



The Prevalence, Mechanism and Evolution of Agrochemical Resistance in Natural Populations

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Abstract

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Agrochemical resistance is a growing problem endangering global food security. The cumulative increase in the number of resistant species since the introduction of the first synthetic pesticides in the 1940s and the projected increase of the global population place more stringent requirements on the yields of agricultural food production. An increased amount of damage is predicted to result from resistance to currently used agrochemicals due to the dearth of new chemicals being developed. Despite this, resistance biology lacks methods to study the phenomenon as a whole, beyond the identification of resistance conferring mutations in particular pest or lab populations.

This thesis tests the utility of using the model organism *C. elegans* to study the prevalence of naturally occurring resistance alleles, the dynamics of resistance emergence in controlled conditions, and the genetic basis of resistance from multiple sources. Assessing the presence of natural variation in chemical susceptibility utilising a genetically diverse panel of *C. elegans* indicated that resistance-conferring variation is widespread, as suggested by the identification of wild isolates naturally resistant to carbendazim and aldicarb. The observation of rapid adaptation to carbendazim exposure suggested a link between a high prevalence of natural resistance with fast emergence of resistance for benzimidazoles, while compounds of other modes of action, with high and low variation, saw no adaptation in experimental populations under the conditions tested. Identification of the genetic basis of

carbendazim-resistance in strains of three different biological origins linked a *C. elegans* β -tubulin to the trait in all cases, indicating a high level of convergence and lack of stochasticity in the evolution of benzimidazole resistance. Furthermore, no fitness cost of benzimidazole resistance in the compound-free environment was identified under laboratory conditions, which is troubling as the presence of a fitness cost underlies many resistance management strategies.

Overall, the findings presented in this thesis provide evidence that *C. elegans* is a useful model system to study resistance biology in a holistic manner, particularly in an evolutionary context. An increased understanding of the natural prevalence, evolutionary biology and molecular basis of the phenomenon would better inform field strategies of resistance mitigation as well as computational models that study the interplay of complex parameters in emergence of resistance. Ultimately, this improved understanding could assist in minimising the danger of rapidly emerging resistant pest populations on global food security.

Thesis supervisors: Prof. Alison Woollard and Dr. Anthony Flemming

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List of Abbreviations

ABC	Adenosine triphosphate-binding cassette
ACh	Acetylcholine
AChE	Acetylcholinesterase
APRD	Arthropod Pesticide Resistance Database
BAM	Binary version of a SAM file
BBPS	Body bends per second
BPDB	Bio-Pesticides DataBase
Bt. toxin	<i>Bacillus thuringiensis</i> toxin
CeNDR	<i>Caenorhabditis elegans</i> Natural Diversity Resource
CGC	<i>Caenorhabditis</i> Genetics Centre
chitin	Chitin biosynthesis
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
CYP	Cytochrome P450
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethyl sulfoxide
DNA	Dioxyribonucleic acid
EMS	Ethyl methanesulfonate
EU	European Union
FAO	The Food and Agriculture Organization of the United Nations
FDR	False Discovery Rate
FRAC	Fungicide Resistance Action Committee
GABA	gamma-Aminobutyric acid

GFP	Green fluorescent protein
GST	Glutathione S-Transferase
HRAC	Herbicide Resistance Action Committee
IPCS	International Programme on Chemical Safety
IPM	Integrated pest management
IRAC	Insecticide Resistance Action Committee
KH ₂ PO ₄	Monopotassium phosphate
MOA	Mode of action
Mt	Mitochondria
NA	Not applicable
nAChR	Nicotinic acetylcholine receptor
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NEB	New England Biolabs
NGM	Nematode growth media
OJEU	The Official Journal of the European Union
PAM	Protospacer adjacent motif
PCR	Polymerase chain reaction
PPDB	Pesticide Properties DataBase
ppm	Parts per million
PRM	Pesticide resistance management
R	Resistant
R4P	Research and Reflection Ring on Pesticide Resistance
RMP	Resistance management plan
RNA	Ribonucleic acid

S	Sensitive
SAM	Sequence Alignment Map
SD	Standard deviation
SNP	Single nucleotide polymorphism
ssODN	Single stranded oligonucleotide
Tub	Tubulin
VGSC	Voltage-gated sodium channel
VSDB	Veterinary Substances DataBase

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Chapter 1: Introduction

1.1. Introduction to agrochemical resistance

Over 105 years ago, in 1914, A.L. Melander presented the first paper detailing the emergence of resistance to sulphur-lime spray in orchard aphids in Clarkston, Washington, USA. His manuscript argued, that without suitable management approaches, emergence of resistant populations would become a problem in pest control. While the comments included with the publication suggest that this report ought to have provoked considerable discussion (Melander, 1914), publications suggesting suitable resistance management strategies remained scarce for the next 50-60 years.

The introduction of the first synthetic pesticides (e.g. dichlorodiphenyltrichloroethane (DDT), cyclodienes and organophosphorous insecticides) brought with them increased large-scale intensive use and an accompanying rapid increase in the number of reports of resistance cases (Sparks and Nauen, 2015). Further discovery of active compound with pesticidal properties meant that a steady introduction of novel compounds through the 20th century was ensured. Use of pesticides, alongside additional advances in agriculture (new plant varieties, mechanisation, irrigation, crop nutrition), resulted in a 145% increase in the global food production between the 1960s and 2000s, with the yields in USA doubling and production in Western Europe growing by 68% (Pretty, 2008). However, more intensive use of pesticides brought rising reports of resistance, which reached the high point in their frequency in the 1970s or 1980s, with numerous publications outlining the general mechanisms of these resistance cases (Whalon, Mota-Sanchez and Hollingworth, 2008). A severe exemplar of the prevalence of insecticide resistance in

the latter part of the 20th century was the Colorado potato beetle, *Leptinotarsa decemlineata*, which by 1983 was resistant to all modern synthetic pesticides and could only be eradicated in the field by using rotenone (Forgash, 1984; National Research Council, 1986). The rise in the cumulative number of species resistant to different agrochemicals is depicted in Figure 1.1.

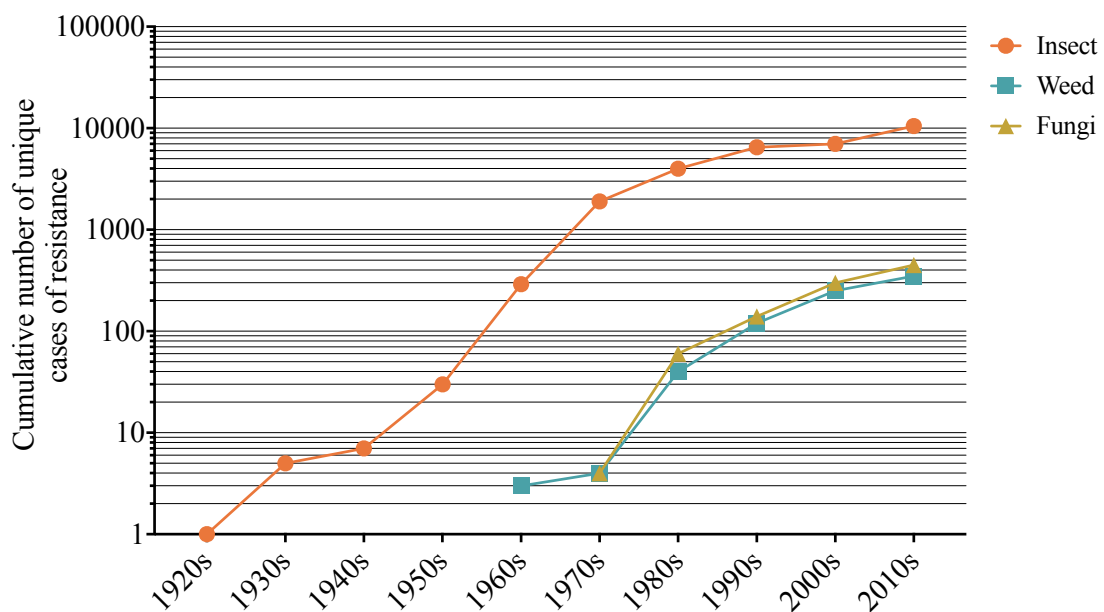


Figure 1.1: Cumulative increase in the number of resistance cases in insects (orange), weeds (blue), and fungi (yellow). Figure modified from (Sparks and Nauen, 2015) who acquired insecticide data from Drs. David Mota-Sanchez, and Mark Whalon, Michigan State University, and fungicide and herbicide data from (FRAC, no date) and (HRAC, no date).

Pesticide resistance is defined as a genetic and heritable (Zalucki and Furlong, 2017) decrease in pesticide susceptibility in a previously sensitive pest population (Whalon, Mota-Sanchez and Hollingworth, 2008; Tabashnik *et al.*, 2014). The emergence of resistance comes about through natural selection where, upon exposure to the pesticidal compound, only the individuals in the pest population that are most adapted to (and least affected by) the new environment will have the ability to, first, survive and, secondly, reproduce. This ultimately results in cumulatively more

resistant populations as the sensitive isotypes disappear due to the pesticide selection (Tabashnik *et al.*, 2014). This means that even though a number of chemical pest control methods are available, as shown in Table 1.1, resistance has been reported across all compound classes (FRAC, 2018; Mota-Sanchez and Wise, 2019).

Just as the effectiveness of antimicrobial agents, which are foremost in protecting global health, is under threat from widespread resistant bacterial strains, some of which carry multi-drug resistance (World Health Organisation, 2016), global food security is threatened by the emergence of agrochemical-resistant species of insects, fungi, and weeds (Tabashnik *et al.*, 2014), as exemplified by Figure 1.1. This pattern suggests that whenever humans design a method to limit the fitness of a pest or a weed, natural selection is likely to result in emergence of resistance (Gould, Brown and Kuzma, 2018). As such, pesticide resistance poses a momentous ecological, global health, and economic problem (Hoy, 1998).

1.1.1. Pesticide resistance endangers global food security

Growth of the global population, which is expected to reach 9.7 billion by 2050 (United Nations, 2019), as well as increases and changes in consumption mean that the demand for food will rise for at least another 30 years until population growth is likely to start to plateau around the middle of this century as suggested by growth modelling projections by the United Nations (Godfray *et al.*, 2010; United Nations, 2019). Limited amounts of agricultural land, water and energy threaten global food security. Further limitations to agricultural food production are expected to be imposed by changing climate conditions (Godfray *et al.*, 2010; Food and Agriculture Organization of the United Nations (FAO) *et al.*, 2019). With these possible changes in suitable agricultural land and global population, it will be more important than ever

to maximise the production capabilities of the land available and minimise the losses in yield to pests, weeds, and plant pathogens by using crop protection strategies and agrochemicals (Fisher *et al.*, 2018; Hawkins *et al.*, 2019).

Table 1.1: General modes of action of pesticides currently used. Examples of particular targets and examples of compound classes used to target the modes of action have been highlighted to provide a sense of the diversity of compounds available for pest control. Source: Fungicide Resistance Action Committee and Insecticide Resistance Action Committee (2019).

<i>General MOA</i>	<i>Example targets</i>	<i>Examples of pesticide classes</i>
Metabolism	Amino acid Nucleic acid Lipid	Anilino-pyrimidines Phenylamides, carboxylic acids Carbamates, aromatic hydrocarbons
Muscle & nerve	ACh pathway Chloride channel Sodium channel	Carbamates, organophosphates Phenylpyrazones, organochlorines Pyrethroids, DDT
Growth & development	Juvenile hormone mimics Growth or biosynthesis inhibitors Cytoskeleton	Juvenile hormone analogues Benzoylureas, diacylhydrazines Benzimidazoles, toluamides
Respiration	Electron transport in mitochondria ATP synthase Complex I	METI insecticides, phosphides Organo fentins, diafenthiuron Quinazoline
Midgut	Microbial disruption of insect midgut membranes	<i>Bacillus thuringiensis</i> <i>Bacillus sphaericus</i> Baculoviruses
Unknown	Not certain	Phtalamic acid, benzotriazines, pyridazinones, guanidines etc.

In this mission it is important to keep in mind the durability of the chemicals and utilise appropriate mitigation strategies to maintain longevity in the field in order to prevent the emergence of resistant species for as long as possible (Brevik *et al.*, 2018). This is particularly important because the rate by which pest species are becoming resistant to existing agrochemicals seems to be outpacing the speed of discovery of new active compounds and their registration for use (Fisher *et al.*, 2018; Gould, Brown and Kuzma, 2018). This is exemplified by resistance to at least one agrochemical recorded in 546 arthropod (Tabashnik *et al.*, 2014) and 190 plant pathogen species (FRAC, 2018).

The slowing down of the rate of discovery of new suitable pesticides is to an extent explained by more stringent health and safety requirements, which were not in place when the first synthetic pesticides were developed (Chandler *et al.*, 2011). The first cases of resistance to synthetic insecticides such as DDT in the 1940s and 50s did little to raise concern about the emergence of resistance due to a rapid rate of discovery of new insecticides, suggesting that humans would be able to stay ahead of the evolution of resistance. However, the early success of pesticide research and intensive use of chemicals resulted in the emergence of species by the 1990s that were also resistant to novel agrochemicals of both the same and different modes of action, due to resistance mechanisms that resulted in cross-resistance (Roush, 1993).

1.1.2. Pesticide resistance causes economic damage worth billions of dollars every year

The proper use of pesticides enables the growth of agricultural produce in a safe, sustainable, and affordable way by maintaining high production yields and maximising the efficiency with which agricultural land and resources such as water are used (Popp, 2011). Based on the prices in 2002, the attainable maximum crop production was worth \$1.5 trillion. The actual crop production was 36.5% less than that at \$950 billion. The production without crop protection would have been \$455 billion, less than half of the actual crop production. The losses prevented by crop protection amount to \$415 billion (Agrios, 2005), highlighting the importance of crop protection chemicals and their continued use in global food production. Unfortunately, inappropriate pest management and the emergence of resistance result in a reduction in production capabilities due to damage by pests that can not be dealt with (Godfray *et al.*, 2010).



Two-spotted spider mites
Tetranychus urticae



Northern root-knot nematode
Meloidogyne hapla



Fungi
Aspergillus nidulans



Colorado potato beetle
Leptinotarsa decemlineata



German cockroach
Blattella germanica



Mosquito
Culex pipiens



Diamondback moth
Plutella xylostella



Whitefly
Bemisia tabaci



Peach-potato aphid
Myzus persicae



Western corn rootworm
Diabrotica virgifera virgifera



Penicillium mould
Penicillium digitatum



Grey mould
Botrytis cinerea

Figure 1.2: Images of major global pest species (such as Western corn rootworm, whitefly) and diverse pests mentioned throughout this chapter.

Currently, the global expenditure on pesticides is expected to be around \$40 billion annually (Popp, 2011). Out of this, based on numbers available from 2001, around \$12 billion is spent in the United States every year (National Research Council, 2000). The cost relating to dealing with pest resistance is estimated to account for about 10% of this expenditure. This cost results from having to spray the areas multiple times, dispose of useless chemicals, or abandon rotations that are no longer usable, with just the additional treatments accounting for a \$40 million increase in the insecticide bill (Carrasco-Tauber and Moffitt, 1992; Pimentel and Lehman, 1993).

Despite the widespread use of chemical pesticides, 10-35% of the United States agricultural production is lost to pest damage (Pimentel and Lehman, 1993). Estimates on the proportion of this production loss that is due to resistant pests vary, but, if it is as low as 10%, it would represent at least a \$2-\$7 billion annual loss for the \$200 billion United States food industry. 10% is often used as a conservative figure while it is widely acknowledged to be an under-estimate (Palumbi, 2001). With the rise in the reliance and use of pesticides (Epstein, 2014) as well as accumulating reports of resistance (Sparks and Nauen, 2015; FRAC, 2018), this proportion can only be expected to increase.

One of the major factors is the dearth of new compounds, despite increasing amounts being spent on research and development, and the high cost of regulatory approval (Thacker, 2002). Since 1995 the amount spent in all three areas has increased across the globe with overall expenditure nearly doubling from \$150 million to \$280 million (Borel, 2017). Considering that the estimate of the amount spent on research, development, and regulatory approval of a single pesticide was around \$80 million in 1999 (*The future role of pesticides in US agriculture*, 2000), it

is rather devastating when resistance to the fresh agricultural product emerges within a matter of years after its introduction such as in the case of spirotetramat, an insecticide of novel mode of action belonging to the chemical class of tetrone and tetramic acid derivatives (Bielza *et al.*, 2019). This agrochemical was introduced for pest control in 2007 (University of Hertfordshire, 2019), however, reports of resistance already existed in citrus red mite in Chinese citrus orchards by 2010. In this particular case, reduced susceptibility was the result of cross-resistance between spirotetramat, introduced in 2000 (University of Hertfordshire, 2019), and spirotetramat (Hu *et al.*, 2010), highlighting the importance of appropriate resistance management strategies, which will be discussed below.

1.1.3. Factors influencing the emergence of field resistance

The emergence of field resistance, both rate and mechanism, is affected by the properties of the agrochemical, which may have nothing to do with their mode of action. For example the mobility of pests within treated areas, and uneven pesticide application that introduce variation to localised compound concentrations can be major factors affecting resistance evolution (Gould, Brown and Kuzma, 2018).

The emergence of resistance is affected by the half-life of a compound, meaning the time taken for the amount of compound present to decrease by half (Taylor and Georgiou, 1982), as a short half-life would result in rapid disappearance of the compound from the environment. Compound concentration and selection pressure on a pest population would then depend on how soon after pesticide treatment the population encountered the treated area. It is in general understood that if pests are exposed to compound at the highest concentration, straight after the treatment was applied, the rate of resistance evolution would be faster than if most

encounters were later on. This is explained by a higher proportion of susceptible individuals being eradicated, which results in decreased competition between the sensitive and the resistant strain in the area and therefore increases the ease by which resistant individuals can spread. At the same time, a low pesticide dose could result in a higher likelihood of emergence of resistance as the size of the pathogen populations remains larger (than in high dose), while the number of resistant mutants produced per unit time remains the same (Taylor and Georgiou, 1982; Hobbelen, Paveley and Van Den Bosch, 2014; Gould, Brown and Kuzma, 2018).

Additionally, in the field, pesticides are applied to complex surfaces where equal coverage of plants or leaves is difficult to achieve resulting in differing selection pressures across the treated areas. Therefore, the effect of compound treatment on the rate of resistance emergence in the field can be difficult to gauge. Possible variation in agrochemical concentration across the resistance evolution period affecting the process in ways that can be difficult to interpret by later analysis of resistant isolates (Sumerford *et al.*, 2013; Gould, Brown and Kuzma, 2018).

1.1.4. Evolutionary origins of pesticide resistance

Pesticide resistance, is an evolutionary process and a prime example of adaptation to a new environment via natural selection (Palumbi, 2001). Four separate evolutionary origins of pesticide resistance may be distinguished, as covered in a recent review by Hawkins *et al.* (2019).

Firstly, resistance can arise from *de novo* mutations that occur once a change in the environment has occurred, where the mutation confers a fitness benefit and therefore is selectively advantageous (Messer and Petrov, 2013). There may be

multiple independent *de novo* mutations or a mutation may arise once and sweep through the population (Hawkins *et al.*, 2019).

Pesticide resistance has also been reported to arise from standing variation where polymorphisms conferring resistance are already present in the population. In this example, the polymorphisms are present at low frequencies but upon application of selective pressure the frequencies rapidly increase (Barrett and Schluter, 2008). Standing variation applies to both monogenic or polygenic traits that under a selective change become advantageous (Pritchard and Di Rienzo, 2010).

Additionally, pest species may have a degree of intrinsic resistance to the pesticide as a result of naturally high frequencies of resistance-conferring polymorphisms in the population. These pre-existing adaptations may not be sufficient to confer high levels of resistance but may have increased activity under selection of the pesticide and provide a slight fitness benefit for example by increase in efflux by overexpression of transporter genes as demonstrated by grey mould fungus *Botrytis cinerea* (Kretschmer *et al.*, 2009).

Finally, interbreeding or horizontal gene transfer may result in the transfer of the resistance allele from a resistant to a susceptible species (Hawkins *et al.*, 2019). While horizontal gene transfer is most common in bacteria and poses a particular danger in the case of antimicrobial resistance, it has also been reported in some fungi. The transfer of a gene encoding a critical virulence factor from one fungal pathogen to another (from *Stagonospora nodorum* to *Pyrenophora tritici-repentis*), evidenced to have occurred just before 1941, resulted in the emergence of a new wheat disease (Friesen *et al.*, 2006). This example of increased virulence of fungal pathogens by interspecific gene transfer thus also jeopardises the long-term durability of fungicides.

1.1.5. Mechanisms of resistance

Mechanisms of pesticide resistance can, most broadly, be divided into monogenic and polygenic (Ffrench-Constant, 2013) with the majority of reported resistance mechanisms being monogenic (Whalon, Mota-Sanchez and Hollingworth, 2008).

Resistance mechanisms can also be characterised by the physiological changes that have taken place (for example the up-regulation of detoxifying pathways) and resulted in heritable resistance in a no longer susceptible population. These common mechanisms encompass changes at the target site, resulting in target-site resistance, and non-target site resistance, which encompasses changes in metabolism, and penetration of the chemical, as well as removal by efflux pumps, active compound binding at a non-target site, resistance via a downstream molecular change, and behavioural resistance (Roush, 1993; Ffrench-Constant, Daborn and Le Goff, 2004; Brent and Hollomon, 2007; Whalon, Mota-Sanchez and Hollingworth, 2008; Ffrench-Constant, 2013; R4P Network, 2016; Fisher *et al.*, 2018). The major mechanisms of resistance are summarised in Figure 1.3 providing an overview of the differences in the molecular changes resulting in resistance between mechanisms.

1.1.5.1. Target-site resistance

Changes resulting in decreased sensitivity at the site of action are among the most important classes of resistance mechanisms (Brent and Hollomon, 2007). As an agrochemical will have one or multiple specific binding sites on a critical macromolecule within the pest, efficacy of pesticides across the full range of modes of action can be affected by this mechanism of resistance (Whalon, Mota-Sanchez and Hollingworth, 2008). As demonstrated below, the molecular sites targeted by

pesticides can tolerate varying degrees of modulation resulting in altered chemical susceptibility phenotypes.

Target-site resistance can take the form of point mutations resulting in a change in the amino acids critical to the binding of the active compound. Mutations of this sort have been reported across a range of plant pathogens resistant to benzimidazoles, strobilurins, succinate dehydrogenase inhibitors and azoles (Fisher *et al.*, 2018) as well as acetylcholinesterase (AChE) inhibitors (Silver, Van Emden and Battersby, 1995) among many other widely used pesticides (Whalon, Mota-Sanchez and Hollingworth, 2008). Among notable examples is a single mutation in cytochrome *b*, Gly143Ala, which has emerged in more than 20 species of plant pathogens under selection by strobilurins (Lucas, Hawkins and Fraaije, 2015).

Target site resistance may also manifest in changes in the level of expression of the compound, resulting in over-expression of the target (Hamamoto *et al.*, 2000; R4P Network, 2016; Fisher *et al.*, 2018) An example of resistance is found in plant pathogen *Penicillium digitatum* where a 100-fold upregulation in the expression of the sterol 14 α -demethylase gene results in resistance to demethylation inhibitors, which are widely used agricultural and medical fungicides (Hamamoto *et al.*, 2000).

Where a null mutant is viable, target site resistance can be conferred by the elimination of the gene product altogether as demonstrated by a *Drosophila melanogaster* mutant that is resistant to a juvenile hormone agonist methoprene. The resistance is conferred by complete lack of *Methoprene-tolerant* gene transcript during the period of development that is insecticide sensitive (Wilson and Ashok, 1998).

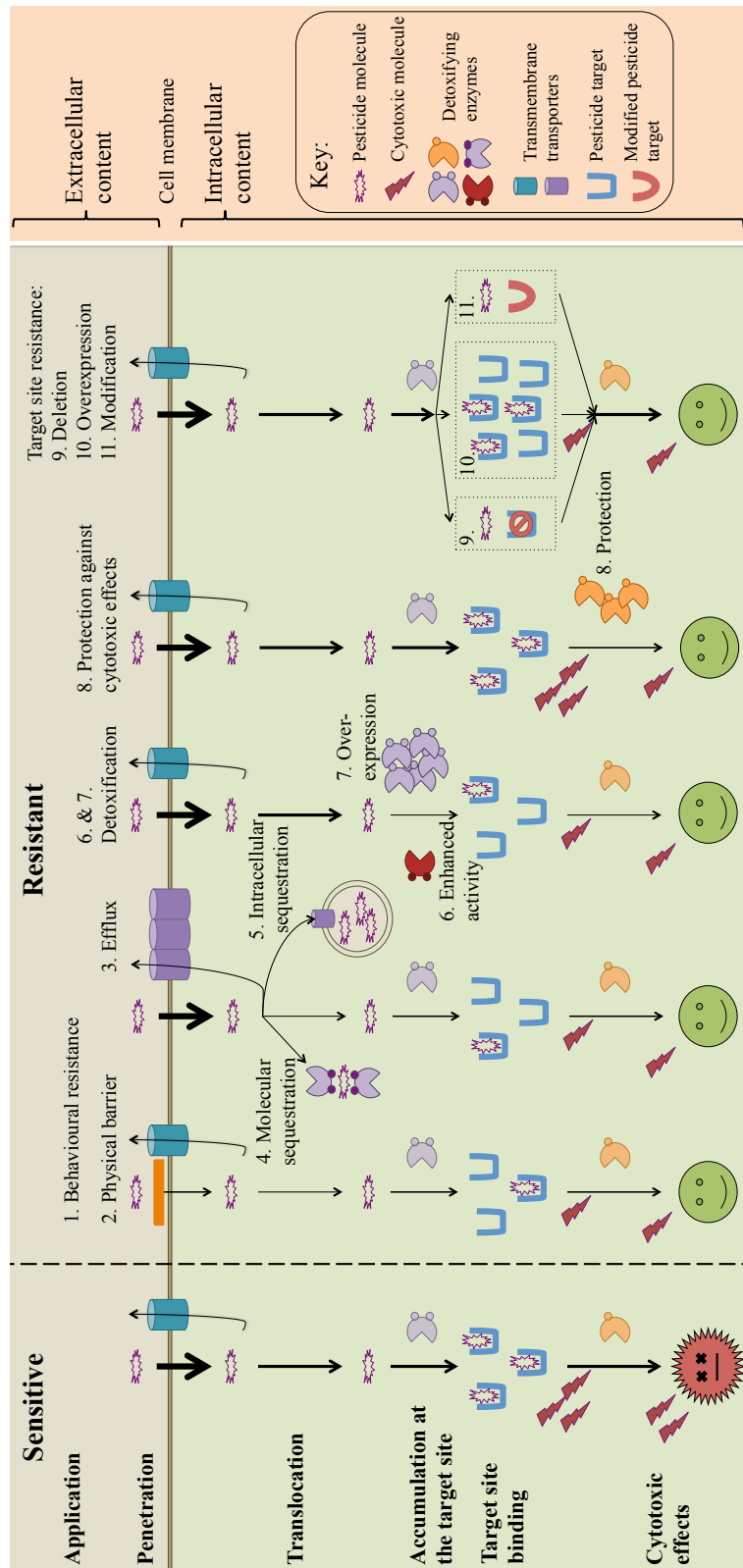


Figure 1.3: Summary of resistance mechanisms recorded in pests against pesticides. Illustrative example of mode of action of pesticides in susceptible pests (left), compared with the variety of mechanisms that can result in resistance (right). Behavioural resistance (1.) reduces the exposure of the pest to the pesticide through avoidance or repulsion. A physical barrier (2.) can reduce agrochemical uptake and result in penetration resistance. The accumulation of compound at the target site can be reduced by efflux by transporter proteins (3.), molecular sequestration (4.), or intracellular sequestration (5.) that all result in the active compound being directed away from the target site. In addition detoxification by enhanced enzymes (6.) or the overexpression of enzymes (7.) can neutralise the pesticide. Upon effective target site binding, resistance can be conferred by protection from the cytotoxic effects of pesticide action (8.). Resistance also results from target site resistance and can be the result of absence of the target molecule (9.), overexpression of the target (10.), or a mutation in the target resulting in modification incompatible with pesticide binding (11.). Figure adapted from (R4P Network, 2016).

1.1.5.2. Metabolic resistance

Another common mechanism of resistance involves the metabolic destruction of the toxic agent (Whalon, Mota-Sanchez and Hollingworth, 2008). The majority of examples of metabolic resistance involve one of three large multigene families: esterases, cytochrome P450 monooxygenases, and glutathione S-transferases (GST). Esterases can confer a considerable reduction or elimination of toxicity of ester pesticides (such as organophosphates and carbamates) by hydrolysis. Cytochrome P450 monooxygenases comprise a versatile system for the bio-transformation of pesticides by facilitating deactivation or excretion of the compound. Glutathione S-transferases enhance intracellular reactions resulting in the conjugation of the active compound and tripeptide glutathione, which ultimately leads to the more active excretion of the pesticide from the cell (Whalon, Mota-Sanchez and Hollingworth, 2008; Panini *et al.*, 2016).

Metabolic resistance can be conferred by the overproduction of pesticide-degrading enzymes, achieved via gene amplification or expression upregulation (Puinean *et al.*, 2010) or by modified and more efficient pesticide degradation (Cui *et al.*, 2015; R4P Network, 2016) that results in enzymatic cleavage and excretion (Panini *et al.*, 2016).

The activity of esterases, with numerous ester insecticides in use, plays an important role in resistance to current pesticides (Whalon, Mota-Sanchez and Hollingworth, 2008). An example of esterase-based resistance involves the peach-potato aphid *Myzus persicae*. In this species, overproduction of insecticide-detoxifying esterases was first implicated in the late 1960s (Devonshire *et al.*, 1998). As it is possible that as much as 1% of the total body protein of an aphid could contain these esterases, it is not surprising that they confer resistance to a range of

agrochemicals and are an important mechanism of resistance (Devonshire and Moores, 1982). Structural mutants of carboxylesterases have been described in the blowfly *Lucilia cuprina*, resistant to diethyl and dimethyl organophosphorous insecticides (Oakeshott *et al.*, 2005) providing an example of improved efficacy in pesticide degradation.

In addition, the toxic compound may become sequestered, via a molecular or vesicle-bound mechanism, preventing an interface between the agrochemical and the physiological processes of the pest (Panini *et al.*, 2016; R4P Network, 2016). Sequestration, the selective uptake, modification, and storage of plant secondary chemicals, is a common phenomenon among herbivorous insects (Petschenka and Agrawal, 2016). The GST system was suggested to offer resistance to pyrethroid insecticides in the coleopteran *Tenebrio molitor* by binding to the compound molecules in a molecular sequestering mechanism (Kostaropoulos *et al.*, 2001). While reports of vacuolar sequestration resulting in pesticide resistance appear rare, an example has been identified in a winter annual weed that by vacuolar separation is able to withstand much higher doses of the herbicide paraquat (Brunharo and Hanson, 2017). Additionally, major adaptations to plant toxins in milkweed butterfly were linked evolutionarily to vacuolar sequestration rather than adaptation to the toxic diet (Petschenka and Agrawal, 2015), suggesting that the mechanism is in place among insects to enable survival in other toxins and therefore could enable survival in pesticides.

Finally, metabolic resistance can be protection against cytotoxicity upon the binding of the agrochemical by neutralization of the molecules generated (R4P Network, 2016). Elevated levels of GSTs were found to reduce mortality and weaken the lipid peroxidation induced by pyrethroids in a laboratory colony of the brown

grasshopper, *Nilaparvata lugens*, upon a selection experiment. This demonstrates the metabolic power of GSTs, already widely known for their role in metabolising organochlorine and organophosphorous insecticides, in insecticide resistance via protection from oxidative stress, effectively extending their range of efficacy to another class of agrochemicals (Vontas, Small and Hemingway, 2001).

1.1.5.3. Penetration resistance

Pests have also been reported to become resistant by changes that result in reduced uptake of the agrochemical. This mechanism is usually associated with light resistance in the range of two- or three-fold (Whalon, Mota-Sanchez and Hollingworth, 2008) in comparison to the far higher effects observed with metabolic or target-site resistance. For example, reports suggest that metabolic changes in the Colorado potato beetle, *L. decemlineata*, result in massive 2,000-fold resistance to carbofuran (Alyokhin *et al.*, 2008). However, even a two- to three-fold decrease in susceptibility can enhance the overall resistance as an additive factor due to a lower penetration being effective across a diverse range of pesticides (Whalon, Mota-Sanchez and Hollingworth, 2008).

The penetration mechanism has been observed in the mosquito *Culex pipiens* to organophosphate insecticides (Raymond, Heckel and Scott, 1989) and in addition in bed bugs (*Cimex* species) where penetration resistance contributes to the decreased susceptibility observed to pyrethroids in multiple examples (Dang *et al.*, 2017). Reports of penetration resistance also exist for the German cockroach, *B. germanica*, to pyrethroids (Wu *et al.*, 1998) and in the house fly, *M. domestica*, to a compound from the benzoylurea class (Pimprikar and Georgiou, 1979). However, in particular the example of bed bugs demonstrates that the resistance is often supplemented by

other mechanisms, such as metabolic or target site resistance, resulting in a higher level of final resistance than possible by just penetration resistance (Dang *et al.*, 2017).

1.1.5.4. Reduction of pesticide accumulation at the target site

Pesticide resistance can also be achieved by reducing pesticide accumulation at the target site by effective removal by efflux pumps. In this mechanism the toxic compound is removed in an energy-dependent manner utilising the ATP-binding cassette (ABC) superfamily of trans-membrane pumps (Whalon, Mota-Sanchez and Hollingworth, 2008). The mechanism is well established in antibiotic resistance in bacteria where it confers reduced susceptibility to multiple drugs (Langton, Henderson and Herbert, 2005). As these transporters are found in both prokaryotic and eukaryotic systems (Dermauw and Van Leeuwen, 2014) it is perhaps unsurprising that the same mechanism underlies resistance to all major classes of fungicides in the membrane transporter mutants of the fungi *Aspergillus nidulans* (Andrade *et al.*, 2000).

In addition, an increasing number of studies now point to the importance of the ABC superfamily of transporters in arthropod resistance, with the recent implication of the proteins in the case of resistance to Bt toxins in *Lepidoptera*. This is of particular interest due to the high use of Bt toxins in pest control (Dermauw and Van Leeuwen, 2014).

Overall, the literature available suggests that limited work has gone into the documentation of resistance conferred by transmembrane efflux (Dermauw and Van Leeuwen, 2014), but current findings imply its rising importance, suggesting that

further research into this mechanism of resistance across pest species will be done in the future, particularly as diagnostic methods improve (R4P Network, 2016).

1.1.5.5. Resistance via insecticide binding at a toxicologically harmless site

Pesticide resistance can be conferred by inhibition of the target site of the pesticide by an alternative pathway or enzyme in a compensation mechanism (R4P Network, 2016). This mechanism has been described in homopterans and mosquitoes where carboxylesterases act as a sink for the organophosphate and carbamate insecticides and, in this example, it is considered an important source of resistance (Whalon, Mota-Sanchez and Hollingworth, 2008).

1.1.5.6. Behavioural resistance

Resistance may also arise through behavioural means, where a change in the behaviour of a pest results in avoidance of the agrochemical (Georghiou, 1972; Roush, 1993; Panini *et al.*, 2016). IRAC defines pesticide resistance as ‘a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species’ (IRAC, no date). The heritability and evolution, however, are rarely investigated in the reports of behavioural resistance (Roush, 1993; Zalucki and Furlong, 2017). Consequently behavioural resistance remains enigmatic. As such it encompasses various possibly polygenic behaviour traits, such as avoidance, as well as receptor based aversion (Zalucki and Furlong, 2017).

One of the best-studied examples of a change in insect behaviour that results in decreased efficacy of an insecticide is the avoidance observed in the German cockroach (*Blattella germanica*). A change in a glucose receptor results in avoidance of sugar baits used to administer the toxicant and as a result the otherwise susceptible pests appear resistant to the insecticide used (Hostetler and Brenner, 1994; Wang, Scharf and Bennett, 2004; Zalucki and Furlong, 2017).

1.1.6. Fitness cost of resistance

Resistance can be conferred by any of the mechanisms outlined above, suggesting that there are a multitude of ways that nature can get around the controls imposed by humans (Palumbi, 2001) that effectively are meant to reduce the fitness of the pests in comparison to the no compound environment. However, as a population adapts to a new environment, a fitness cost in the no compound environment may be introduced (Hughes and Andersson, 2015; Ffrench-Constant and Bass, 2017).

A fitness cost can be understood as ‘a trade-off, in which alleles conferring resistance to a pesticide reduce fitness in environments lacking the pesticide’ (Tabashnik *et al.*, 2014). The principle of a fitness cost of resistance underlies multiple resistance management strategies, discussed below, but it is also worth noting that reports of resistance not conferring a fitness cost and mutations that reduce the fitness cost of pesticide resistance exist.

An example of fitness cost introduced by the mechanism of resistance, which has later been reduced by further mutations that modify the fitness cost, is demonstrated in the resistance to an organophosphate pesticide in the two-spotted spider mites, *Tetranychus urticae*. These mites carry resistance-conferring mutations and duplications of the acetylcholinesterase (AChE) gene. While the resistance

conferring mutations on their own significantly lower the catalytic activity of the enzyme, suggesting an apparent fitness cost, in the presence of the two copies of the AChE gene, the catalytic efficiency is comparable to that of wild type *T. urticae* AChE (Kwon *et al.*, 2012).

The presence of a fitness cost of resistance in no compound ensures that once selection pressure of the pesticide is removed, the frequency of resistance alleles will decrease and over time the population will become sensitive again. However, the absence of a fitness cost altogether or occurrence of further mutations resulting in the loss of the fitness cost, as described above, would mean that resistance alleles would remain in the population in a no compound environment (French-Constant and Bass, 2017). Ultimately this would result in the failure of the pesticide in the area, as it can no longer be used to control the pest population.

Additionally, the different alterations (target site, upregulation of metabolic pathways etc.) that result in resistance to antimicrobials or antimalarial drugs are, usually, associated with a fitness cost in the absence of the compound (Hughes and Andersson, 2015). In the case of artemisinin, a drug used in malaria therapy, resistance is associated with significant fitness cost to *Plasmodium falciparum* in the absence of drug selection. The cost results in the malaria parasites growing less well in animals and being outcompeted by wild type (drug-sensitive) parasites upon the removal of drug pressure in the clinical setting, possibly explaining the slow development of resistance in the field (Rosenthal, 2013).

1.1.7. Current proactive measures to delay the emergence of resistant populations

The emergence of resistant species to agrochemicals has plagued agriculture since the early 20th century, with a particular surge in the problem since the introduction of early synthetic pesticides like DDT in late 1940s due to increased use of chemical crop protection methods. All strategies to manage pest resistance rely on an understanding of biological factors affecting the emergence of resistance as well as the possible influences of involved stakeholders, such as the farmers who will be applying the pesticides (Onstad, 2014). This correlates well with the key factors in resistance development that are in general categorised as biological, genetic, and operational (FAO, 2012).

First, high rates of resistance emergence stem from the inherent goal of pest management to reduce the pest population and maintain it at a very low level. The probability of resistance evolution is much lower when emphasis is on the prevention of damage caused and that is achieved with a modest reduction in pest population. Nevertheless, often a significant pest reduction and a higher likelihood of resistance emergence cannot be avoided. This is where pesticide resistance management (PRM), a term for the collection of strategies designed to mitigate resistance emergence, becomes important as a method to delay the emergence of resistance and minimise the interference of resistance with agricultural production efficiency (Onstad, 2014).

In the past, reactive PRM has been used in the field. This meant that each pesticide was used for several years until the emergence of widespread resistance and effective failure of the control method. In many ways a wasteful and inefficient strategy, it stemmed from the belief that novel pesticides would always be available for use upon treatment failure due to resistance (Onstad, 2014). However, the slower

discovery and regulatory approval of pest control chemicals since the 1990s means that this is no longer a sustainable strategy (Gould, Brown and Kuzma, 2018).

Currently, using integrated pest management (IPM), first conceptualised in the 1950s when the damage done by resistant species became apparent, combined with preventative pesticide resistance management (PRM) is the recommended strategy to create a suitable approach for a given pest and cropping system (FAO, 2012; Onstad, 2014). A successful resistance management plan (RMP) should, therefore, be based on IPM principles, which advise the use of pesticides only when necessary, and would enable the management of resistance (FAO, 2012). The management of resistance is centred, as outlined by Onstad *et al.* (2014), on four factors:

1. diversification of causes of mortality and selection of multiple mechanisms;
2. reduction of selection pressure for each mortality mechanism;
3. maintenance of refuge or immigration to promote mixing of susceptible and resistant individuals;
4. prediction using monitoring and models.

Diversification means that the effect of the pesticide must be well-understood to enable informed choice of compounds with different modes of action for use in the field. Reduction of selection pressure should enable a less rapid increase in the frequency of resistance by one mechanism and thereby reduce the rate by which a *de novo* mutation conferring resistance can spread through a population under compound selection. Measures ensuring maintenance or migration of sensitive individuals to a treated area safeguards that upon the loss of selection pressure, the proportion of resistance in the population will rapidly decrease, in the case of a high fitness cost in no compound, and reduce slightly, in the case of a low or cost-free situation. Use of monitoring to assess the spread of resistance in a field population and models to

understand how this may change based on various factors is important to choose the best strategies to mitigate resistance by effectively slowing or reversing the spread of resistance alleles in a field population of pests.

These factors should take into account the biological peculiarities of the pest, the effect on any adjacent host crops, as well as the availability of alternative non-chemical pest management measures and multiple classes of pesticides. It is also important to consider all treatments performed in the same area in a year, to make sure that only the recommended application rates are utilised, to involve all stakeholders especially where a strategy covers a large area, and to evaluate and refine the RMP where appropriate (FAO, 2012). As quickly becomes apparent, managing pesticide resistance is a complex multivariate problem that is rarely solved well with a stock solution and requires the careful consideration of all specific components.

Table 1.2: Summary of most commonly used resistance management strategies.

Strategy	Explanation	Assumptions
<i>Mixtures</i>	Compounds are applied as mixtures of two or more.	No individuals resistant to both compounds. Cross-resistance to both compounds should not be possible. Compounds matched in environmental persistence.
<i>Alternations</i>	Applications of single compounds are altered.	No individuals resistant to both compounds. Fitness cost of resistance to either compound is high.
<i>Mosaic</i>	Compounds applied in a spatial patchwork.	Migration of susceptible individuals into the treated area. Fitness cost of resistance is high. Compound combinations with negative cross-resistance.

However, some resistance management strategies that are more widely used exist. These include utilising mixtures of pesticides with different mechanisms of resistance or modes of action and rotating the pesticides used (Hoy, 1998; FAO,

2012) and will be covered alongside others below. A summary of these strategies is included in Table 1.2 and further discussed under headings 1.1.7.1, 1.1.7.2, 1.1.7.3.

1.1.7.1. Pesticide mixtures

Firstly, making use of mixtures of pesticides can be effective in controlling resistance emergence. Utilising a pesticide mixture, the pests resistant or partially resistant to one compound will be eradicated due to sensitivity to the other compound and vice versa. This tactic assumes that no or very few individuals resistant to both pesticides are present within the population, as they would survive the treatment. Using this tactic of resistance mitigation requires that components are carefully researched in order to avoid situations where the emergence of resistance to one compound would also result in resistance to the other, or where the persistence of one compound is significantly longer or shorter than the other, resulting in the selection of resistance to only one compound upon degradation of the other compound in the mixture. It is also important that, in the field, the mixture is applied at the label rate to ensure appropriate efficacy (FAO, 2012).

1.1.7.2. Alternations

Secondly, alternating pesticides is a well-used tactic to manage resistance evolution, where two or more pesticides are alternated over time. This results in every individual being exposed to only one material, while the population is treated with more than one product (Hoy, 1998; FAO, 2012). It is a suitable approach where multi-pesticide resistant pests are rare, resulting in eradication by the other compound if the first was ineffective. The interval between applications must be sufficient to enable the return of the initial susceptibility to the compound used (FAO, 2012). This means

that, additionally, the resistance to the pesticides used should carry a high fitness cost so that the proportion of resistant pests would decline in the absence of the pesticide (Ffrench-Constant, 2006).

1.1.7.3. Mosaics

Hybrid tactics, such as the mosaic, where multiple toxins are applied to different locations within treated areas are also used (Roush, 1989). In this strategy, the pesticides are applied as a spatial patchwork so that different sites are treated with different products. This resistance mitigation strategy requires that migration of susceptible individuals occurs into the treated area and that fitness costs of resistance in no compound are high, in order to maintain a low proportion of resistance alleles in the population. Compound combinations with negative cross-resistance, where resistance to one compound results in increased sensitivity to another, are particularly well suited for this strategy (Hoy, 1998).

1.1.7.4. Additional strategies

In addition to the aforementioned tactics, restricting the number of treatments per season, the concentration of treatments, and applying pesticides only when strictly necessary is favourable. This would result in the eradication of fewer susceptible pests and enable faster susceptibility regeneration and reduction in the frequency of resistance alleles if resistance carries a high fitness cost in the absence of the treatment (FAO, 2012; Hobbelen, Paveley and Van Den Bosch, 2014; Onstad, 2014). It is also recommended that poor coverage of the area treated, frequent treatments of organisms with large populations and short generation times, and simultaneous treatment of different life cycles of a pest with single or related compounds are

avoided (FAO, 2012). Finally, combinations with alternative pest management tactics, such as biological or cultural controls, should be implemented (Phillips, Graves and Luttrell, 1989).

1.1.8. Challenges in pesticide resistance management

Challenges of pesticide resistance management are underpinned by the complex setting of the measures to control the emergence of resistance with various events able to contribute to emergence of resistance in the field. Adoption of poor farming practices like no rotation of crop or no cleaning of farm equipment, for example, can lead to the spread of pests and exacerbate the problem of resistance despite management measures (FAO, 2012; Gould, Brown and Kuzma, 2018).

Designing and implementing strategies to control resistance in multiple pest species within the same area is even more difficult as the peculiarities of all pest organisms involved need to be taken into account. Any approach adopted will interact with and affect other species, the differences in pest life cycles, field coverage, and recovery of susceptibility have to be taken into account (Gould, 1994; Wearing and Hokkanen, 1994). Solutions to this problem will most likely arise from computational models as demonstrated by the successful identification of optimal strategies under different insect life histories where heterogeneous timing of selection had to be taken into account (Sudo *et al.*, 2018).

As briefly mentioned earlier, monitoring of field resistance before, during, and after utilising pest treatment and using models to understand resistance development in multivariate conditions is key to sustainable resistance mitigation. Importantly, it provides early warnings that the particular RMP utilised should be adjusted in order to prevent failure of resistance management by an increase in the frequency of resistance

in the population that would result in the compound being no longer effective (Hoy, 1998; FAO, 2012).

Ultimately resistance may evolve to any pesticide and pest management strategy (Onstad, 2014). Mitigation of resistance emergence is, however, key and the tactics and principles outlines above must be kept in mind when developing suitable strategies to give time for innovation in pest control and new compounds (Gould, Brown and Kuzma, 2018). This relies on utilising computational modelling as well as experimental methods to identify the complex contributions of multiple variants in management strategies.

1.1.9. Modelling of resistance emergence

The evolutionary dynamics of pesticide resistance emergence and the contribution of different factors have been widely investigated through the use of computational models. It should be highlighted that any model is only as good as the algorithm and specific parameters included (Hoy, 1998), but the method itself offers considerably larger freedom than experimental methods, meaning that a larger range of starting conditions and management strategies can be tested.

Current models of resistance have been able to investigate the interplay of a range of biological parameters and agrochemical application strategies, but more spatially complex strategies like rotation or mosaic, as well as the effect of migration, remain under-studied despite increased computational capacity (Bourguet *et al.*, 2010). Therefore, modelling of resistance emergence should be fully utilised to gauge the interplay of different parameters. The interpretations of the models, however, must be field appropriate, taking into account space and time as sources of variation in selection pressure, must explore situations and methods of potential strategic

interest, meaning strategies that are not yet used, and ultimately must be supported by experimental findings where possible.

The shortcomings of current mathematical models include the lack of relevant experimental data, for example on the initial frequency of resistance alleles, as well as lack of investigating evolutionary forces other than selection, such as mutation and genetic drift, and working with strategies that have not yet been studied (Bourguet *et al.*, 2010). Models are limited by the assumptions that they are based on and this reduces applicability to real world problems. Reiterating the observations utilising an experimental model would strengthen the model, verifying the results, with the potential to use the two in a synergistic combination to better inform of the contributions of multiple parameters in the realistic evolutionary dynamics of the emergence of pesticide resistance.

1.2. *Caenorhabditis elegans* as a model organism to study multiple aspects of pesticide resistance

1.2.1. Introduction to *C. elegans*

Caenorhabditis elegans is a free-living nematode found naturally, primarily in rotting compost, worldwide. The organism is a major model species and has been used in biological research to answer questions about developmental biology, neurobiology etc. Sydney Brenner first proposed the use of nematodes to answer fundamental questions in these research areas in 1963. *C. elegans* has since been used to study general metazoan biology from gene regulation to metabolism as well as utilised to experimentally investigate evolutionary dynamics.

C. elegans is easy to culture and grow in large quantities under laboratory conditions. They have a rapid life cycle with the worms able to, once hatched from eggs, progress through four larval stages (L1-L4) and develop into adults within 3 days at 20°C. The life cycle of *C. elegans* is illustrated in Figure 1.4.

C. elegans primarily exists as a self-fertilising hermaphrodite, but males arise in populations at frequencies of <0.2% (Altun and Hall, 2009). Therefore, only a single worm is required to maintain stocks, considerably simplifying population upkeep.

The worms themselves are small, with freshly hatched L1 larvae about 0.25mm in length and adults about 1mm, meaning that small spaces are required for maintenance of multiple different populations. Further facilitating ease of use is the ability of the species to undergo an alternative dauer developmental pathway under stressful environmental conditions, meaning that lines survive for long periods of time, as well as the possibility of freezing down strains, meaning that particular populations can be stored for long periods of time at -80°C (Brenner, 1974;

Stiernagle, 2006). In an evolutionary context, this enables the archiving of experimental lines generation by generation.

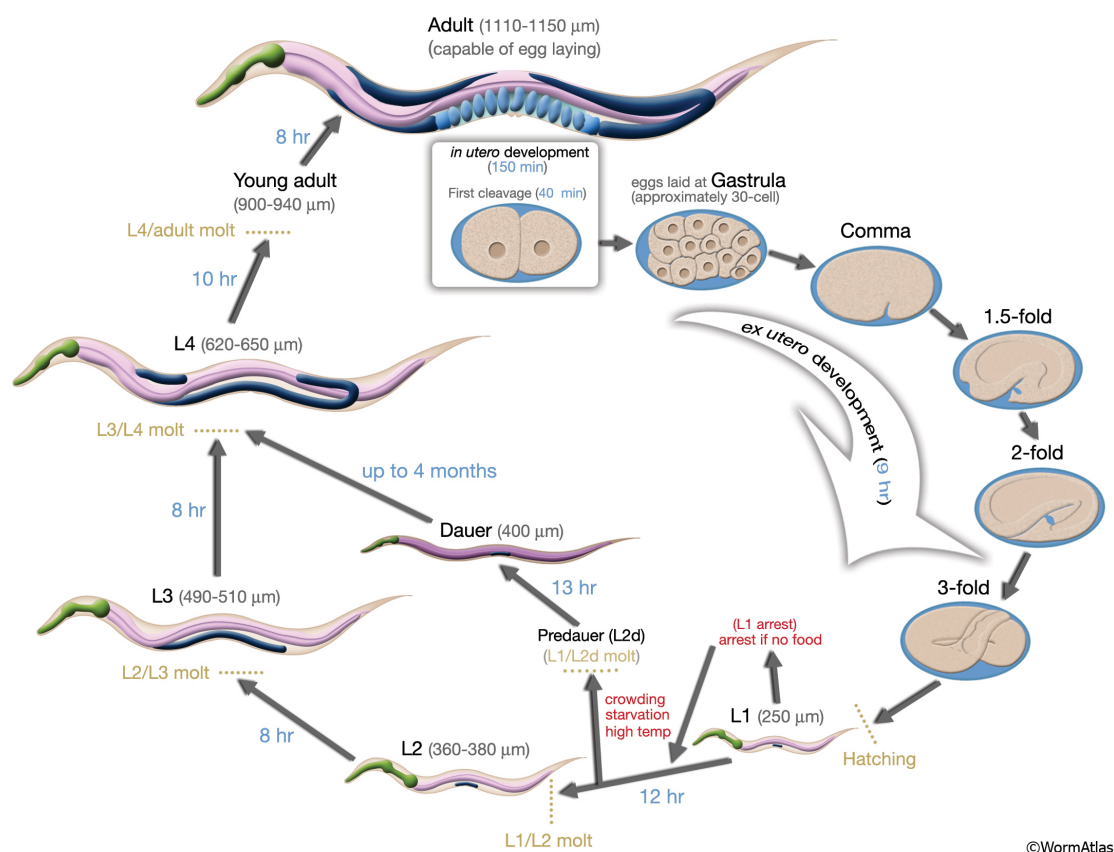


Figure 1.4: The *C. elegans* life cycle at 22°C highlighting the stages that the species progresses through during development and the average time taken by Bristol N2 to progress through said developmental stages. Figure from WormAtlas (www.wormatlas.org).

1.2.2. Benefits of using *C. elegans* to study pesticide resistance

The qualities detailed above provide ample examples of advantages of using *C. elegans* instead of pest species to study pesticide resistance, considering pest species are often very difficult to cultivate under lab conditions. Structural and functional similarities of molecular and cellular pathways (for example in the ACh pathway (Sluder *et al.*, 2012)) in *C. elegans* and a variety of pests means that the effects of a pesticide can be studied in this model organism, while worms benefit from

higher ease of manipulation under lab conditions. This is exemplified by the use of *C. elegans* in the search for new active compounds (Simpkin and Coles, 1981; Leung *et al.*, 2013) as well as their use in mode of action (MOA) and toxicology studies (Burns *et al.*, 2006, 2015). This is due to the simple life cycle that does not rely on different environmental conditions at particular stages, like the northern root-knot nematode, *Meloidogyne hapla* (Madeiras, Wick and Plant, 2014), and the availability of a wide range of experimental tools and resources discussed below.

1.2.3. Availability of a range of resources and established methods

The combination of resources and methods is the strongpoint of utilising *C. elegans* to further the understanding of pesticide resistance. For example, the genome is fully sequenced and well-annotated (available at www.wormbase.org), and a sizeable collection of strains is available through the *Caenorhabditis* Genetics Centre (CGC) at the University of Wisconsin. Additionally, there are well-established protocols to obtain mutant lines using mutagenising agents like ethyl methane sulfonate (EMS) (Kutscher and Shaham, 2014) or to introduce particular modifications by using the CRISPR-Cas9 nucleoprotein complex for genome editing (Paix *et al.*, 2015). These resources already provide a valuable insight to the relationship between phenotype and gene function, which, in the context of pesticide resistance is highly valuable.

In fact, a mutagenesis screen of *C. elegans* for resistance to spirotetramat identified the role of the A1847V mutation of *pod-2* in conferring the reduced susceptibility in worms. A homologous mutation has since been identified in the silverleaf whiteflies (*Bemisia tabaci*) that have evolved resistance to spirotetramat in the field (Anthony Flemming, personal communications), serving as an example of

the insight to be gained from the use of *C. elegans* mutagenesis screens in predicting genetic basis of resistance outside of the lab.

1.2.4. *C. elegans* wild isolates

The CGC house, among strains of most *Caenorhabditis* species, a collection of *C. elegans* wild isolates. The reference strain that has been most widely used across the world in worm labs is N2, originally isolated from Bristol, but well over 150 different wild strain isotypes and even more geographically different isolates are now archived. While *C. elegans* is in general not considered particularly diverse at the molecular level ((Barriere and Felix, 2005; Barrière and Félix, 2005; Rockman and Kruglyak, 2009; Andersen *et al.*, 2012)), many wild isolates show phenotypic variation among them under lab conditions (Barriere and Felix, 2005).

Among the best studied of the phenotypes that vary among *C. elegans* wild isolates are the Clumping and Bordering behaviour (Hodgkin and Doniach, 1997; de Bono and Bargmann, 1998; Barriere and Felix, 2005) as well as the ability of the males to leave a copulatory plug (Hodgkin and Doniach, 1997). The formation of the plug, in nature thought to prevent the female of the species from being mated with again (Mangels *et al.*, 2016; Schneider, Mangels and Dean, 2016), does not fully prevent remating in *C. elegans* isolates, but does make it less likely to happen (Barker, 1994). This phenotypic variation has been mapped to one major locus called *plg-1* (Hodgkin and Doniach, 1997). The Clumping and Bordering behaviour, where aggregates of *C. elegans* adults form on the borders of *E. coli* on standard culture plates, is caused by genetic polymorphism at a single locus, *npr-1*. The exact ecological relevance of the phenotype, which is a striking evolutionary change in *C. elegans* (gain of function of NPR-1), is unclear (Barriere and Felix, 2005). However,

the lower oxygen levels in big clumps of worms or at the borders of the bacterial lawn may drive the behaviour (Gray *et al.*, 2004). Interestingly, the *npr-1* locus is also associated with speed of recovery upon ethanol exposure in *C. elegans* (Davies *et al.*, 2004).

A more methodical sampling of the wild isolates began in 2000 with the move to develop *C. elegans* into a model for evolutionary biology and ecology research (Frézal and Félix, 2015). The sampling of *C. elegans* variants has resulted in the increased use of the species in experimental evolution (Gray and Cutter, 2014), studies on natural variation in phenotypes (Lee *et al.*, 2016), as well as further work into identifying the genomic diversity and history of the species (Andersen *et al.*, 2012) utilising the new wild isolates as well as the bank of knowledge amassed using the Bristol N2 since the 1960s.

The initial report by Andersen *et al.* (2012), which was expanded on by Cook *et al.* (2016), provided a systemic overview on genomic diversity and evolutionary history of *C. elegans*. The initial analysis of 97 distinct *C. elegans* wild isolates, indicated that selective sweeps have reduced the chromosome-wide diversity in the species and that, on average, a wild isolate differed from the reference Bristol N2 by 3,613 single nucleotide polymorphisms (SNPs). As of 2016, 152 *C. elegans* isotypes have been identified (Cook *et al.*, 2016) and the genomic relatedness and geographical distance appears to weakly correlate on a local scale (defined as less than 700km), with some structure to the genetic relatedness of wild isolates and the location of isolation. However, no correlation was present at distances larger than 700km, based on the analysis of 41,000 polymorphisms that were identified in 200 wild strains used (Andersen *et al.*, 2012). It was also reported that functional variants were more likely to be located on the arms of the chromosomes due to the relationship

between polymorphisms and rates of recombination (Rockman, Skrovanek and Kruglyak, 2010). More specifically, *C. elegans* chromosomes are structured in semi-discrete domains with different gene density, recombination rate, evolutionary conservation, and repeat sequence density profiles on chromosome tips, centres, and arms (The *C. elegans* Sequencing Consortium, 1998; Rockman and Kruglyak, 2009). Chromosomal centres have high gene density and low recombination rates relative to chromosome arms for which the measures, respectively, are low and high (while chromosome tips have intermediate gene density and no recombination). Therefore it would be expected to observe more functional variants in chromosomal centres, where there are more functional sites, with an equal rate of mutation acting across all sites. Instead the mapping of functional variants identified that they are mostly localised on chromosome arms (Rockman, Skrovanek and Kruglyak, 2010; Andersen *et al.*, 2012).

In order to further enable the use of *C. elegans* in the study of how natural variation shapes different traits, the *C. elegans* Natural Diversity Resource (CeNDR, available at www.elegansvariation.org) was set up. The resource comprises sequencing data across available wild isolates as well as a detailed online catalogue of wild isolates from across the globe (Cook *et al.*, 2017). Additionally, *C. elegans* wild isolates have already been used to understand natural variation in responses to agrochemicals with reports of naturally occurring resistance in CB4856 to avermectin, a widely used nematicide (Ghosh *et al.*, 2012). Therefore, the study of wild isolates enables to investigate how naturally occurring polymorphisms can contribute to chemical susceptibility phenotypes in relation to pesticide resistance.

1.2.5. Experimental evolution with *C. elegans*

Short generation times and laboratory tractability mean that *C. elegans* is also highly suited to study the evolution of traits and adaptation to new environments over multiple generations with ease (Gray and Cutter, 2014). For example, rapid experimental evolution to the pesticide levamisole has been demonstrated to entail no cost and to result in changes to the mating system (Lopes *et al.*, 2008). As well as utilising *C. elegans* in studies of adaptation to novel environments, natural selection can be measured utilising individuals of different phenotypes in competition experiments. Competition experiments stand out as the effect of the starting frequencies of identifiable genotypes and the environmental conditions on population changes can be identified. This provides information on the fitness of specific genotypes in a defined context as well as enables to compare the effect of different environments (Teotonio *et al.*, 2017).

Utilising *C. elegans* to study pesticide resistance poses a multitude of benefits from ease of use to well-established experimental protocols. However, the caveats of using this species for studying pesticide resistance must also be considered. While, for example, using *C. elegans* in laboratory evolution to understand adaptation to novel environments can provide invaluable insight to resistance emergence and factors underlying it, additional field-realistic studies will be required to supply insights that take into account the peculiarities of pests and pesticide exposure in a complex environment. It is worth noting, at the same time, that making use of *C. elegans* prior to field studies can provide a useful and considerably cheaper primer to understanding pesticide resistance that can be enhanced with further, more costly, field studies as well as mathematical models.

1.3. Aims of the project

Pesticide resistance is a global problem endangering our food security. While resistance is widespread, a limited amount of experimental work has gone into identifying suitable models to study the evolutionary dynamics of pesticide resistance. Improved understanding of the effects of all parameters could be applied in the field to assist in resistance mitigation. The prevalence of naturally occurring resistance alleles is suggested to play an important role in the emergence of resistance in the wild. While reports of naturally occurring resistance exist, for example to *Bacillus thuringiensis* toxins used in transgenic plants, the absence of a suitable laboratory model to investigate the phenomenon has complicated the assessment of how prevalent resistance alleles are in wild populations. Utilising a panel of genetically diverse *C. elegans*, I wanted to characterise the amount of natural variation present in susceptibility to a range of agrochemicals. Additionally, I wanted to identify whether a unifying mechanism (e.g. permeability or developmental rate) could explain the sensitivity profiles observed. In order to investigate the evolutionary dynamics of the emergence of pesticide resistance, I used *C. elegans* in experimental evolution. This multi-generational experiment enabled me to observe the effects of adaptation to pesticide selection, in real time, and allowed the comparison of adaptation to different agrochemical environments to show whether a relationship between natural variation and resistance emergence existed. Finally, I identified resistance to a benzimidazole compound in wild isolates, experimentally evolved lines, and a chemically induced mutant. This presented me with an opportunity to compare the genetic basis, as well as the fitness cost of resistance in a no compound lab environment, using competition experiments, from a diverse range of biological sources that provided insights into the

discussion on whether evolutionary outcomes could be predicted or if that was not possible due to high dependency on specific life history.

In summary, I aimed to:

- characterise the susceptibility of a genetically diverse panel of *C. elegans* wild isolates to a range of agrochemicals and identify isolates more sensitive and resistant to specific agrochemicals;
- identify the developmental time and cuticle integrity of *C. elegans* wild isolates to determine whether variation in chemical susceptibility is the result of a significant difference in either measure;
- perform a 15-generation evolution experiment of *C. elegans* under no compound and three compound different conditions;
- discern whether the multigenerational selection of a sensitive *C. elegans* line on different compounds resulted in increased adaptation to the environment and whether a fitness cost in a no compound environment under lab conditions was introduced by the selection;
- establish the genetic basis of resistance resulting from multi-generational selection on compound;
- characterise the genetic basis of resistance to carbendazim in a *C. elegans* wild isolate and a chemically induced mutant by a SNP linkage experiment utilising whole genome sequencing;
- identify the contribution of a particular polymorphism in the carbendazim-resistance phenotype observed in a *C. elegans* wild isolate by utilising the CRISPR-Cas9 complex in a gene editing experiment;

- utilise competition experiments to characterise the fitness cost of carbendazim resistance in a chemically induced mutant and to experimentally model the spread of resistance through populations under different conditions.

Chapter 2: Materials & Methods

2.1. Worm methods

2.1.1. *C. elegans* maintenance

C. elegans were maintained using standard techniques as described by Brenner (Brenner, 1974). Worms were grown on standard Nematode Growth Media (NGM) at 20°C with *Escherichia coli* OP50 as food source.

Wild isolates including Bristol N2, and myo-1::GFP and myo-3::GFP strains, were obtained either from the Woollard or Hodgkin lab stocks in Oxford (both the Department of Biochemistry, University of Oxford) or Caenorhabditis Genetics Centre (CGC).

The EEV-A₀ strain, described by Teotonio et al. (2012), used as the ancestral population in experimental evolution work was obtained from the King lab in Oxford (Department of Zoology, University of Oxford).

Strains were defrosted for use every 6 months, with a 2-week recovery period before use in experiments.

2.1.1.1. *Strains used*

C. elegans strains used are detailed in Table 2.1.

Table 2.1: The worm strains used in the experiments.

Strain	Genotype	Notes
N2	Wild isolate	Sampled from Bristol, UK
AB1	Wild isolate	Sampled from Adelaide, Australia
CB4851	Wild isolate	Sampled from Bergerac, France
CB4853	Wild isolate	Sampled from Altadena, USA
CB4854	Wild isolate	Sampled from Altadena, USA
CB4856	Wild isolate	Sampled from Hawaii, USA
CB4857	Wild isolate	Sampled from Claremont, USA
CB4858	Wild isolate	Sampled from Pasadena, CA, USA

CX11262	Wild isolate	Sampled from Huntington Gardens, Los Angeles, USA
CX11307	Wild isolate	Sampled from Huntington Gardens, Los Angeles, USA
ED3005	Wild isolate	Sampled from Edinburgh, Scotland
ED3012	Wild isolate	Sampled from Edinburgh, Scotland
ED3017	Wild isolate	Sampled from Edinburgh, Scotland
JU1088	Wild isolate	Sampled from Kakegawa, Japan
JU1400	Wild isolate	Sampled from Sevilla, Spain
JU1491	Wild isolate	Sampled from Le Blanc, France
JU1580	Wild isolate	Sampled from Orsay, France
JU1652	Wild isolate	Sampled from Montevideo, Uruguay
JU258	Wild isolate	Sampled from Ribeiro Frio, Madeira
JU311	Wild isolate	Sampled from Merlet, France
JU363	Wild isolate	Sampled from Franconville, France
JU775	Wild isolate	Sampled from Lisbon, Portugal
JU792	Wild isolate	Sampled from Frechendets, France
LKC34	Wild isolate	Sampled from Madagascar
MY23	Wild isolate	Sampled from Münster, NW Germany
PB306	Wild isolate	Sampled from Connecticut Valley, USA
QX1211	Wild isolate	Sampled from San Francisco, California
EEV-A ₀	Highly polymorphic strain used in multigenerational selection experiments (Chapter 4)	Obtained in a funnel cross of 16 <i>C. elegans</i> wild isolates (AB1, CB4507, CB4852, CB4855, CB4856, CB4858, JU319, JU345, JU400, MY1, MY16, N2, PB306, PX174, PX179, RC301). Highly polymorphic strain with <i>fog-2</i> introgressed. No significant loss of genetic diversity observed after maintenance at standard conditions for 140 generations. Discussed further in 4.1.4.1. Reference: Teotonio <i>et al.</i> , 2012
AW1783	Chemically induced carbendazim-resistant mutant	Obtained from an EMS mutagenesis experiment using Bristol N2 by C. Casey (Woollard lab, not published). Strain not outcrossed.
myo-1::GFP	<i>myo-1::gfp</i>	<i>C. elegans</i> strain with pharyngeal Green fluorescent protein (GFP) marker
myo-3::GFP	<i>myo-3::gfp</i>	<i>C. elegans</i> strain with body wall muscle GFP marker

2.1.2. Bleaching of large worm populations

In order to obtain populations of synchronised L1 worms, large populations were bleached. Gravid hermaphrodites were washed off plates using 3 ml of M9

buffer into 15 ml Falcon tubes. 3 ml of a 1:5 dilution in water of 2x bleach solution (freshly made up to consist of 25% 4M NaOH, 40% sodium hypochlorite, and 35% water) was added to the tubes and the tubes were vortexed. After a 10-minute incubation at room temperature the tubes were centrifuged at 1500 rpm at 4°C for 90 seconds. The supernatant was removed and 10ml of M9 was added to wash the sample. Tubes were again centrifuged at 1500rpm at 4°C for 90 seconds, supernatant removed, and the wash repeated once. Finally the pellet of eggs was carefully pipetted onto a fresh NGM plate with ample *E. coli* OP50, dried, and left to incubate at 20°C until required for further use.

2.1.3. Chemical susceptibility screen

The chemical susceptibility screen was performed using 24-well plates with 0.5 ml NGM per well. 20 µl of OP50 was added to each well and the plates dried in a flow hood for up to 60 minutes until the bacterial lawn was completely dry. 30 µl of compound in solvent (50% iso-2-propanol, 40% water, 10% dimethyl sulfoxide (DMSO)) was added to each well to create a final concentration of 5ppm (parts per million) or 50ppm. Upon addition of the compound solution plates were dried in a flow hood for 45 minutes. 10 synchronised L1 stage worms were transferred to each well. The plates were sealed with Parafilm, to prevent drying out, and placed in 20°C for 4 days.

On day 4 the wells were phenotyped and placed into one of 4 severity groups: 1, 2, 3, 4. The groups are described in Table 2.2. '4' was assigned where the worms were unaffected by the compound present. The L1s transferred had developed into healthy adults with no movement or reproduction defects, there were plenty of next generation (F1) worms present on the plates, and at least 8 out of 10 worms initially

transferred were present on the plate. ‘3’ was assigned where there were some movement and fecundity changes with perturbed development observations. In this category 5-7 of the initial worms transferred had survived. ‘2’ was assigned where fewer than half of the initial worms had survived and severe developmental, movement and reproduction defects were observed. ‘1’ was assigned where no worms were alive by day 4.

Table 2.2: Descriptions of the categories of the wells in the chemical susceptibility screen after four days of incubation.

Group	No of survived F ₀ worms	Defects observed		
		Development	Movement	Reproduction
4	8-10	None	None	None
3	5-7	Yes	Yes	Some
2	1-5	Severe	Severe	Severe
1	0	No survivors	No survivors	No survivors

2.1.3.1. Compounds used in chemical susceptibility screen

The compounds below were kept dissolved in solvent (50% iso-2-propanol, 40% water, 10% DMSO) at room temperature, wrapped in Parafilm, and used in the assay at concentrations outlined below in ppm with the corresponding μM concentration. The chosen concentrations were above the highest allowed residue amounts on agricultural produce in European Union (EU), which for compounds used were between 0.02 and 5ppm (‘COMMISSION REGULATION (EC) No 149’, 2008). The recommended concentrations of the compound solutions applied in field range from 20 to 500ppm (determined by compound formulations on sale in 2019). It would be difficult to accurately estimate compound concentration in the area treated, as that would depend on environmental conditions and availability of water. However, it is reasonable to assume that the highest concentration of compound in the treated area will be upon the treatment, with the concentration from then readily decreasing

through wash-off and degradation. This means that the concentrations used (outline in Table 2.3) fall within this field-relevant range of compound concentration.

Table 2.3: Final concentrations of compounds used in the screen of chemical susceptibility in ppm and μM alongside highest allowed residue on agricultural produce and concentration of applied solution in the field. NA is noted where the compound is not widely in agricultural use or applied as a solid, or no relevant information was available.

	Concentration used		Highest allowed residue (EU)	Concentration of applied solution in the field
Compound	ppm	μM	ppm	ppm
Abamectin	50	55	2	20
Aldicarb	50	253	0.05	NA
Amitraz	50	164	0.1	200
Carbendazim	50	252	2	500
Cartap	50	203	0.1	500
Cyanotropane	50	697	NA	NA
Cyprodinil	50	214	NA	500
DDT	50	136	1	NA
Diafenthiuron	5	13	NA	500
Dieldrin	50	126	0.2	NA
Diflubenzuron	50	155	5	65
Fenoxycarb	50	160	2	50
Fenvalerate	50	115	1	200
Fluazinam	5	10	3	500
Flufenoxuron	50	99	2	50
Fluopyram	5	12	NA	415
Indoxacarb	50	91	3	150
Levamisole	50	236	NA	150
Lufenuron	50	94	1	100
Metaflumizone	50	95	NA	NA
Monepantel	5	10	NA	25
Oxamyl	50	220	0.03	500
Pirimicarb	50	202	5	500
Pyriproxyfen	50	150	0.6	100
Rotenone	5	12	0.02	50
Ryanodine	50	98	NA	NA
Spinosad	50	33	10	115
Spiroindoline	50	167	NA	NA
Spirotetramat	50	129	NA	500

2.1.4. Clustering of chemical susceptibility screen results

Clustering of chemical susceptibility screen results was performed to identify groups of wild isolates that responded similarly across the panel of compounds used. Cluster 3.0 (de Hoon *et al.*, 2004) was used for clustering. No data filtering or data adjustment on the chemical susceptibility data used was required. Non-parametric distance measures with Kendall's T were used to calculate distance measures based on which hierarchical complete linkage clustering was performed. Treeview (Page, 1996) was used to visualise results. An average of all responses recorded in wild isolates to every compound was included as the 'mean' response in order to establish the location of strains that overall behaved the closest to the mean of the group across the panel of agrochemicals assayed.

2.1.5. Food consumption race

To assay fitness and fecundity of *C. elegans* strains in different compound environments I performed food consumption races in a protocol modified from Hodgkin and Barnes, 1991 and Xiong, Pears and Woollard, 2017. For this 24-well plates were prepared as they were for the chemical susceptibility screen (0.5 ml NGM, 20 µl OP50). Compounds were added as 30 µl solutions in solvent to create wells with final concentrations from 5 to 300ppm as required.

10 synchronised L1 worms were picked into each well. Plates were sealed with Parafilm and incubated at 20°C. After 4 days, the wells were looked at each day until the first day of the full consumption of the entire bacterial lawn.

2.1.6. Thrashing and body size measurements after chronic compound exposure

A thrashing assay was used to identify quantifiable differences in movement between strains upon compound exposure.

L1s were transferred onto 50ppm aldicarb plates (5cm) for 4 days and incubated at 20°C. After 4 days, adults on all plates were picked onto food-free plates prior to use in the thrashing assay. 10 adults were picked into a 10 µl drop of M9, left to acclimatise for 15 seconds and then their movement was recorded in a 30 second video using a Leica MZFLIII fluorescence microscope fitted with a Hamamatsu digital camera (ORCA-05G) to take videos and HC ImageLive to view the video.

The videos were analysed using Fiji (Schindelin *et al.*, 2012) and wrMTrck (Nussbaum-Krammer *et al.*, 2015) and values of body bends per second (BBPS) and body size (pixels) obtained.

2.1.7. Single worm bleaching assay

In order to quantify differences in cuticle integrity between *C. elegans* wild isolates a single worm bleaching assay was performed. This protocol was modified from Wormbook Protocol 5: ‘Egg prep in a drop: Small scale method for removing bacterial or yeast contaminants’ (Stiernagle, 2006) and also described by Xiong, Pears and Woollard, 2017. For this assay, single gravid hermaphrodites were placed in a drop (10 µl) of freshly made up 2x bleach (25% 4M NaOH, 40% sodium hypochlorite, and 35% water) on parafilm and the time taken until the visible rupture (a break in the cuticle) of the body of the worm was measured.

2.1.8. Developmental time

In order to identify differences in developmental time between *C. elegans* wild isolates the developmental time of single worms was quantified as a measure of hours required from L1 to the first egg laid. The 24-well plate format was used with 0.5ml NGM and 15 μ l of OP50 per well. Single L1s were picked into wells. The plates were incubated at 20°C for 60 hours and then checked every hour for the first egg laid by the gravid hermaphrodite.

2.1.9. Multi-generational selection experiment in agrochemicals

A 15-generation selection experiment was performed in four conditions: no compound, 30ppm carbendazim, 90ppm aldicarb, 90ppm dieldrin. Six replicate populations were created per condition. The dioecious strain EEV-A₀ ('Portuguese strain', further discussed in 4.1.4.1 and Teotonio *et al.* (2012)) was utilised in this work and had been frozen down according to established protocols (Stiernagle, 2006) prior to use.

A diagram of the selection experiment is included in Chapter 4 as Figure 4.2. The populations were synchronised using the large-scale population bleaching protocol outlined above. Worm eggs were then hatched on no compound 9cm OP50 plates at 20°C. The next day, worms were washed off plates using 2ml M9. Numbers of worms present in 1 μ l were counted ten times in order to calculate how many microlitres were required for the transfer of 1400 worms. At the same time 9cm OP50 plates were prepared with compound or no compound. Compound solution was added in 300 μ l solvent to reach the required final concentration and dried for 45-60 minutes in a flow hood until completely dry. Once the compound plates were dry, 1400 synchronised L1 worms were added based on the counts per 1 μ l. Unused worms

were placed back onto the no compound plates, left for 3-5 days until starved and were then frozen down in 50% M9 and 50% freezing solution (2.93g NaCl, 3.4g KH₂PO₄, 150g Glycerol, 2.8ml NaOH (1M), 500 ml distilled water) at -80°C. Worms were grown on compound plates for 48 hours at 20°C, after which they were washed off with 2 ml M9. The washed worms were then transferred onto no compound plates ('recovery period') for another 72 hours. The recovery period was added in order to enable the worms to lay a sufficient number of eggs to be taken to the next generation of the experiment. The populations were then individually bleached using the large-scale population bleaching protocol outlined above and another generation started as described above in the method. The method was repeated for 15 generations and populations from all generations frozen down for later characterisation.

2.1.10. Effect of compound selection on EEV-A₀ brood size

Brood size counts for strain EEV-A₀ were performed in the 24-well plate format with compound or no compound exposure. Early larval stage worms were grown in compound or no compound environment for 2 days until the distinction between males and females was possible and then transferred as a male-female pair to a single well. The plates were incubated at 20°C. At 24-hour intervals for 3 days the worms were transferred to the next well. The offspring present in the well were counted by visual inspection after the parental worms had been removed.

2.1.11. Genetic cross for SNP linkage mapping of carbendazim resistance

In order to identify carbendazim-resistance causing genes in ED3005 and AW1783 SNP linkage mapping was performed. Both resistant strains were independently crossed with CB4856 males. F1 cross-progeny was allowed to self on

30ppm carbendazim plates for 24-48 hours and confirmed by single worm polymerase chain reaction (PCR) using M117 primers (details under heading 2.2.2) followed by EcoRI (enzyme from NEB) restriction digest (45 minutes at 37°C with a further 20 minute inactivation period at 65°C). An F1 genotyping step was required to confirm cross-progeny due to the semi-dominance of the resistance observed. Cross-progeny in F1 were not fully sensitive to the compound (like the CB4856 parental strain) or fully resistant (like the parental resistant strains) and exhibited an intermediate phenotype in carbendazim that phenotypically resembled the resistant parent more. Therefore, it was essential to make sure that cross-progeny had been acquired by a genotyping step. Healthiest F2s were transferred onto 30ppm carbendazim plates and allowed to self for 24 hours. F3 progeny were checked for any carbendazim-sensitive worms by visual inspection and, where none were detected, single F3 worms were picked to self on 9cm plates to create a novel recombinant carbendazim resistant cross-progeny line. This approach was also performed with no carbendazim selection on no compound plates.

The lines obtained from this cross were subjected to a genomic deoxyribonucleic acid (DNA) preparation protocol (detailed under the heading 2.2.4), which was utilised to obtain the whole genome sequence and identify single nucleotide polymorphism (SNP) ratios (details covered under the heading 2.2.5) to establish locations linked to the carbendazim-resistance by the absence of unique CB4856 SNPs present.

2.1.12. Competition experiments between resistant and sensitive strains

Competition experiments were performed to compare the fitness of the carbendazim-sensitive strain myo-1::GFP and chemically induced carbendazim-

resistant strain AW1783 in ancestral no compound and different carbendazim environments over 5 generations and in 6 replicate populations per generation. The myo-1::GFP strain was chosen as a visual comparison of myo-1::GFP (pharyngeal GFP marker) and myo-3::GFP (body wall muscle GFP marker) determined that the pharyngeal GFP was an easier marker to utilise for the counting of worms.

4 different overall conditions were tested, with a no compound control for all, described in Table 2.4.

Table 2.4: Description of the conditions tested in the competition experiment using AW1783 and myo-1::GFP.

	Starting proportion %resistant out of 100%	Compound treatment
1	50	30ppm
2	50	10ppm
3	50	G1, 3, 5: 30ppm; G2, 4: no compound
4	1	30ppm

To begin, the worm populations were synchronised using the large scale bleaching protocol described above. After 1 day on no compound plates post bleaching, the number of L1 worms in 1 μ l was determined. Compound plates, as well as plates with no compound (both with ample OP50 (100 μ l)) were prepared, prior to transferring the worms, by adding the required amount of compound in 300 μ l of solvent. The worm counts were then used to transfer a total of 1000 worms in the 50% or 1% resistant proportions outlined above. The plates were then incubated at 20°C for 5 days upon which the worms were washed off using 2 ml M9. Worm counts per 1 μ l were established again for each population and 1000 worms transferred onto compound or no compound plates for another generation. This was repeated for 5 generations. Similar competition experiments have previously been described, among others, by Theologidis *et al.*, 2014 and Hahnel *et al.*, 2018.

The number of GFP-tagged myo-1::GFP worms was recorded in a 60 μ l drop of washed off worms in triplicate per population using a Leica MZFLIII microscope with Prior Lumen 200 fluorescence illumination system.

2.2. DNA methods

2.2.1. Worm lysis

Worm lysis buffer was prepared immediately prior to use by addition of 10 μ l of Proteinase K to 50 μ l of worm lysis buffer. Single worm lysis was performed using PCR tubes and 5 μ l of Proteinase K and lysis buffer mixture with single gravid hermaphrodites picked into the liquid. Worm lysis of 50 worms was performed using PCR tubes and 10 μ l of Proteinase K and lysis buffer mixture with 50 gravid hermaphrodites picked into the liquid.

The protocol used for all worm lysis (hot lid on):

Temperature ($^{\circ}$ C)	Time (minutes)
60	60
80	20
10	∞

2.2.2. PCR protocols and primers used

PCR reactions were done using worm lysis samples as genomic DNA templates. The total volume of each PCR reaction was 25 μ l and contained: 2 μ l of template, 5 μ l of Onetaq buffer (NEB), 0.125 μ l of TaqOne polymerase (NEB), 0.5 μ l of dNTP mix (final concentration of 200 μ M for each of DATP, dTTP, dCTP, and dGTP), 0.5 μ l of forward and reverse primer (200 μ M final concentration), and 17 μ l of PCR-grade water. Each PCR reaction mix underwent the following temperature cycles (hot lid on):

Cycle	Temperature (°C)	Time	x30
Initial denaturing	95	30 sec	
Denaturing	95	30 sec	
Annealing (Tm-5°C)	54	15 sec	
Extension	68	90 sec	
Final extension	68	5 min	

Primers were designed to have as little secondary structure as possible and to have compatible primer annealing temperatures. Primers used are listed below in Table 2.5.

Table 2.5. List of PCR primers used.

Primer name	Sequence	Tm (°C)	Notes
M117_for	AGTAATCGAAGCGTCGTCG	56.7	identification of cross-progeny SNP linkage cross
M117_rev	TACGCCTGCCTATTCTCATG	57.3	
ben-1 Ex234 fwd	TGGAGCACGTTTCCAGCAAC	59.4	genotyping for <i>ben-1</i>
ben-1 Ex234 rev	ATTCGCGCTCTATAGCAACCA	57.9	
ben-1_Ex234_2_fwd	AAGTTTGCGGAGATCAGCATTG AG	61	sequencing primer for <i>ben-1</i>
N2_crispr_fwd	CATATTTGCCGCCTGAAA	62.2	identification of edited targets in CRISPR, paired with ben-1 Ex234 rev
ED3005_crispr_fwd	GACATATTCCGCCTGAAA	58.6	identification of not edited targets in CRISPR, paired with ben-1 Ex234 rev

2.2.2.1. *PCR cleanup for sequencing of ben-1 fragment to identify polymorphisms in carbendazim-resistant strains*

PCR cleanup was performed using the NucleoSpin Gel and PCR Clean-up kit and the following protocol.

The volume of the sample was made up to 50 µl with water and 100 µl of Buffer NT1 was added. The sample was then loaded into a gel tube in a collection tube and centrifuged at 11,000xg for 30 seconds. The membrane was washed with 700 µl of Buffer NT3 and spun at 11,000xg for 30 seconds. The wash was repeated once. The membrane was then dried by centrifugation at 11,000xg for 1 minute. The drying step was repeated once. To elute the DNA Buffer NE was heated up and the membrane column was placed in a new 1.5 ml microcentrifuge tube. Then 30 µl of Buffer NE was added to the membrane and incubated at room temperature for 5 minutes. The column inside the microcentrifuge tube were then centrifuged at 11,000xg for 1 minute to elute the PCR product.

2.2.2.2. *Gel electrophoresis*

Gel electrophoresis was used to resolve the product after a PCR reaction and after PCR cleanup. For this 1% agarose gel was prepared in TBE Buffer (45 mM Tris-base, 45 mM boric acid, 1 mM EDTA) with added Ethidium Bromide (final concentration 0.5 µg/ml). Samples mixed with loading dye buffers (10 mM Tris.Hcl pH 7.4, 1 mM EDTA, 10% glycerol, 0.1% w/v xylene cyanol or bromophenol blue) and molecular markers (100bp or 1kb from NEB) were loaded into the set gels and electrophoresed at 80V for 20-45 minutes. DNA was visualised on a UV transilluminator and captured using a camera with AlphaImager software.

2.2.2.3. Sanger sequencing of *ben-1* fragments to identify polymorphisms in carbendazim-resistant strains

DNA fragments of interest were amplified and purified as described above. Samples were then diluted for the accepted concentration for PCR fragments (10 ng/μl) and submitted to Source Bioscience Sanger Sequencing Services (Source Biosciences Limited, Nottingham, NG8 6PX, United Kingdom). Sequencing primers were submitted at a concentration of 4 μM.

2.2.3. Gene editing using CRISPR-Cas9 in wild isolate ED3005

In order to investigate the contribution of a *ben-1* polymorphism, resulting in a Lys58fs mutation, in carbendazim resistance of the *C. elegans* wild isolate ED3005; I decided to use the CRISPR-Cas9 system. A co-CRISPR strategy targeting the locus of interest *ben-1* as well as a screening locus *dpy-10* was utilised as described by Paix, *et al.* (2015). Constructs were designed according to recommendations outlined in Supporting Information of Paix, *et al.* (2015) and are detailed in Table 2.6. The activity of the nuclease Cas9 relies on the presence of two small RNAs: CRISPR RNA (crRNA) and trans-activating RNA (tracrRNA). The sequence of interest is targeted by the crRNA, which binds to the complementary target sequence. TracrRNA refers to the trans-activating RNA, which binds to the crRNA and to Cas9 forming the ribonucleoprotein complex. The formation of the complex leads to the cleavage of the complementary sequence. This introduction of a double strand break allows for the incorporation of a repair template, which includes the edit of interest (Paix *et al.*, 2015).

In the co-CRISPR strategy the successful incorporation of the *dpy-10* repair template results in the presence of short fat (dumpy) worms in the F1, which can be utilised as a visual marker that the gene editing has been successful.

Table 2.6: Constructs used in the gene editing experiment using CRISPR-Cas9. Constructs designed according to recommendations by Paix et al. (2015).

Construct	Sequence
tracrRNA	AACAGCAUAGCAAGUUA AAAUAGGCUAGUCCGUUAUCAACUUGAAA AAGUGGCACCGAGUCGGUGCUUUUUUU
<i>ben-1</i> crRNA	uaauuguuaccuuuuucagG+GUUUUAGAGCUAUGCUGUUUUG
<i>ben-1</i> repair template	ttaattctctcaatgtgaattacaaaattcactaattgttaccattttcagGCGGCAAATATGTCCCACGC GCTGTTCTCGTCGATCTTGAGCCAGGAACC
<i>dpy-10</i> crRNA	GCUACCAUAGGCACCACGAG+GUUUUAGAGCUAUGCUGUUUUG
<i>dpy-10</i> repair template	CACTTGAACTTCAATACGGCAAGATGAGAATGACTGGAAACCGTACCG CATGCGGTGCCTATGGTAGCGGAGCTTCACATGGCTTCAGACCAACAGC CTAT

For storage at -80°C, crRNA constructs were reconstituted in Tris pH 7.5 at 8 µg/µl. Single stranded DNA repair templates were stored at -80°C reconstituted in H₂O at 500 ng/µl. TracrRNA was stored in -80°C at a concentration of 4 µg/µl in Tris pH 7.5.

2.2.3.1. *Worm injections*

Worms were injected in one gonad one day post-L4 at 20°C. Injection mix was made up fresh for the day of injections and stored on ice.

The injecting protocol detailed by Paix et al. (2015) was used to create the following injection mixture:

	Concentration ($\mu\text{g}/\mu\text{l}$)	Volume (μl)
Cas 9	10	5
tracrRNA	4	5
<i>dpy-10</i> crRNA	8	0.4
<i>dpy-10</i> ssODN	0.5	0.55
<i>ben-1</i> crRNA	8	1
<i>ben-1</i> ssODN	1	2.2
H ₂ O		5.85
Total		20

Worms were stabilised on agar pads (75 μl of 2% agar dried at 60°C for 15 minutes) in Halocarbon oil 700 prior to injection. Worms were recovered from injection pads with M9 and placed on fresh NGM plates with *E. coli* OP50 food source.

2.2.3.2. Screening for the edit of interest

Injected P0s (60-80) were singled out on NGM plates. F1s were examined for Rollers 4-5 days post-injection and plates with 30-70% Roller worms were identified. These served as the ‘jackpot broods’ where edits in the target gene were most likely. Tens of rol/*dpy* and also normal worms from the plates with ‘jackpot broods’ were picked and selfed on NGM plates. F1 worms were genotyped by single worm PCR to confirm the presence of the edit. F1s with confirmed presence of the edit were singled out again on NGM plates to obtain worms homozygous at the locus of the edit. This was again confirmed by single worm PCR, outlined above, and confirmed homozygous edits left to self on NGM plates for later use.

2.2.4. DNA preparation for whole genome sequencing to perform SNP

linkage mapping of carbendazim resistance in ED3005 and AW1783

In order to identify the genetic basis of carbendazim-resistance in ED3005 and AW1783 a SNP-linkage based mapping experiment was designed (further details in Chapter 5, under heading 5.3.2). DNA samples were prepared of N2, ED3005, AW1783, CB4856, to obtain reference sequences that could be used to establish genomic locations that were important in the resistance phenotype, as well as the F3 cross-progeny of CB4856 and ED3005 or AW1783 selected from the SNP-linkage experiment described above (heading 2.1.11). QIAGEN DNeasy Blood & Tissue Kit was used to purify whole genome DNA for sequencing and SNP capture in the SNP linkage experiment according to 'Purification of Total DNA from Animal Tissues (Spin-Column Protocol)' (QIAGEN, 2006).

For this all worms on 9cm plates were washed off using M9 and pelleted down in a 1.5ml microcentrifuge tube. The supernatant was removed and the pellet washed once using M9. As much of the supernatant as possible was removed before the addition of 180 µl of Buffer ATL (tissue lysis buffer for the purification of nucleic acids) and 20 µl of Proteinase K. The sample was then frozen down at -80°C for 30 minutes before incubation at 56°C for 1 hour. 4 µl of RNase A (100mg/ml) was added, mixed by vortexing and incubated for 2 minutes at room temperature before continuing. The sample was vortexed for 15 seconds. Then 200 µl of Buffer AL (lysis buffer, which aids in keeping digested proteins in solution to avoid precipitation) was added to the sample and mixed thoroughly by vortexing. 200 µl of ethanol (96-100%) was added to the sample and mixed again thoroughly by vortexing. The mixture was then pipetted into the DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at 8000rpm for 1 minute. The flow-through and collection tube were

discarded. The DNeasy Mini spin column was placed in a new 2 ml collection tube and 500 µl of Buffer AW1 was added. The sample was centrifuged for 1 minute at 8000rpm and the flow-through and collection tube were discarded. The DNeasy Mini spin column was placed in a new 2 ml collection tube and 500 µl of Buffer AW2 added. The sample was centrifuged for 3 minutes at 14,000 rpm to dry the DNeasy membrane and the collection tube was emptied. The sample was then centrifuged for another 3 minutes at 14,000 rpm and flow-through and collection tube discarded. The DNeasy Mini spin column was then placed in a clean 1.5 ml microcentrifuge tube and 50 µl of water was pipetted onto the DNeasy membrane. The sample was incubated at room temperature for 1 minute and then centrifuged for 1 minute at 8000rpm to elute. The elution step was repeated with 50 µl of water. The concentration was then recorded on NanoDrop 1000 (Thermo Fischer Scientific), a spectrophotometer, which uses absorbance of the sample at 260 nm to identify the concentration of the sample according to the analysis constant selected. The sample was stored at 4°C until sent off to sequencing.

2.2.5. Sequencing and SNP calling

The samples prepared in ‘Whole genome DNA preparation for sequencing’ (2.2.4) were sequenced using Illumina technology with paired end sequencing run. Syngenta Jeallott’s Hill site bioinformatics team performed data analysis and SNP calling to obtain SNP frequencies according to the protocol outlined below in Figure 2.1. I prepared the samples for sequencing and performed the further filtering to identify linkage.

The quality of the samples was checked using a program called FastQC (created by Babraham Bioinformatics, available at

(<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). The quality of the sequencing reads is assessed by returning a pass, fail or warning response on 11 different basic parameters: basic statistics, per base sequence quality, per tile sequence quality, per sequence quality scores, per base sequence content, per sequence GC content, per base N content, sequence length distribution, sequence duplication levels, overrepresented sequences, adapter content, Kmer content. An alignment of the reads for all samples to the *C. elegans* genome (C_elegans_Bristol-N2 v2.) was completed and the percentage of aligned reads was obtained. The alignment rate was used to obtain a predicted coverage, and analysis of samples with at least 10x coverage was continued.

Removal of contaminating adaptors was performed by using Trimmomatic (Bolger, Lohse and Usadel, 2014). This removes residual adapters, the first 13 bases, leading and trailing low quality or N bases. By scanning the read with a 4-base sliding window Trimmomatic cut when the average quality dropped below an established threshold. Sequences below 20 bp in length were also excluded.

Once the quality had been confirmed and contaminating adaptors removed, in order to call single nucleotide polymorphisms the data set was aligned to a sequenced and well-annotated genome. The *C. elegans* genome used was C_elegans_Bristol-N2 v2., which was obtained annotated from ENSEMBL that had imported information from Wormbase WS250. The trimmed and properly paired reads for each sample were aligned to the genome to create a sequence alignment map file using BWA which was then converted into a binary format (BAM) to enable use downstream in the pipeline. In order to enable faster and easier usage of the alignment file, the BAM file was sorted by the leftmost coordinate and indexed in order to enable faster usage of the large alignment file.

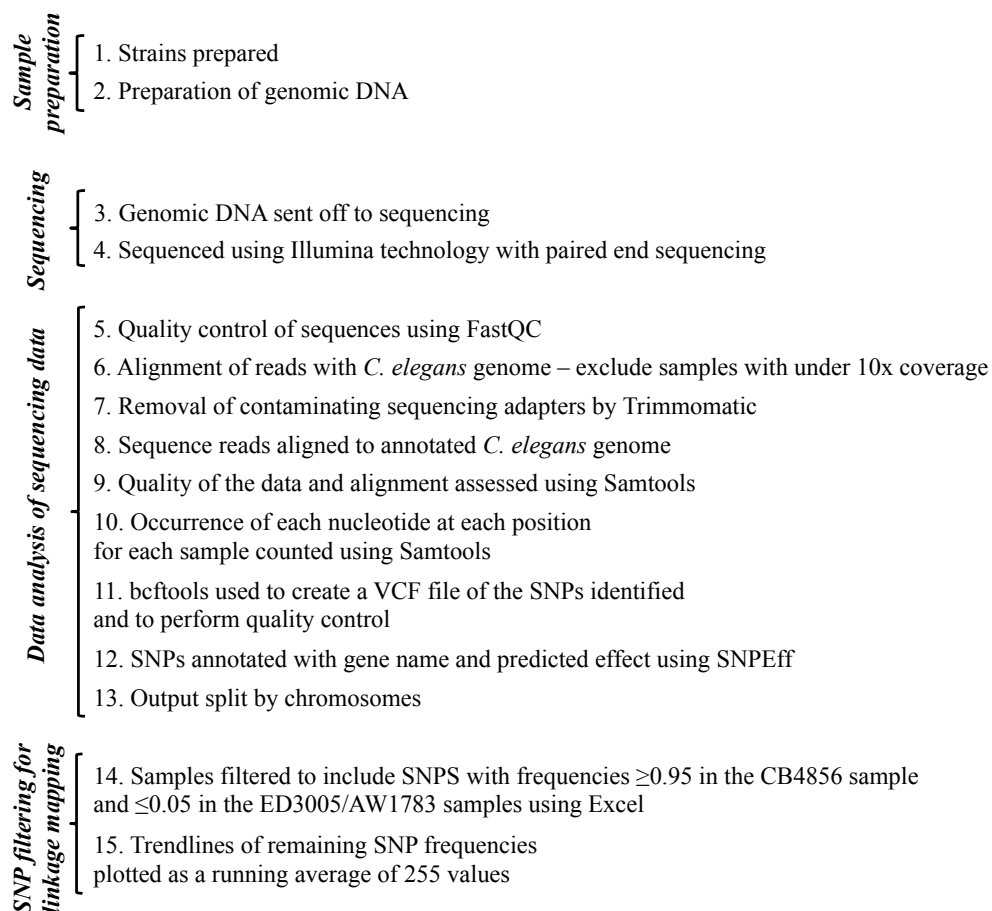


Figure 2.1: Outline of the stages of the SNP linkage mapping of carbendazim resistance from sample preparation to the SNP filtering for linkage mapping. The steps labelled ‘Data analysis of sequencing data’ (5.-13.) were completed by Syngenta in-house bioinformatics team.

Samtools (Li *et al.*, 2009) was then used to get an understanding of the quality of the data and the alignment. Picard (Broad Institute, 2019) was also used to locate and tag duplicate reads in the SAM file.

Next, Samtools mpileup was used to collect summary information from the input BAMs and to compute and store the occurrence of each nucleotide at each position for each sample. The binary bcf file outlining the called SNPs was then converted into a VCF file using bcftools. This outlined the alleles that were at a high enough count and sufficiently different from the reference to be considered SNPs.

Filtering of SNPs was done with bcftools to ensure good quality across the sample and these were then annotated using SNPEff to include the names of genes

containing SNPs, together with the predicted effect of the particular SNP on the gene. The output was split by chromosome for every sample.

The sample was further filtered for SNP linkage plots using Excel. All samples across all chromosomes were filtered to include SNPs for which frequencies were ≥ 0.95 in the CB4856 sample and ≤ 0.05 in the ED3005/AW1783 samples in order to plot the SNP ratios of unique CB4856 SNPs present in all of the samples. The remaining positions were used to create a scatter plot showing the trendlines of SNP ratios that remained after filtering in all relevant samples. A moving average across 255 data points (the largest option in Excel) was plotted to visualise the genomic regions linked with carbendazim resistance in the resistant ED3005 and AW1783. This would be seen as regions of no unique CB4856 SNPs incorporated in the cross-progeny of resistant and CB4856 that were selected for on carbendazim (2.1.11). The cross-progeny selected on no compound is expected to have an equal distribution of CB4856 and resistant strain SNPs across all chromosomes. This approach is further illustrated in Figure 5.3.

In order to obtain a list of potential targets in the linked region, the genes that fell into the linked region were identified. Then the SNPs in the region were filtered to only include those of moderate and high effect and would result in either a stop codon or a missense mutation.

2.3. Bacterial methods

2.3.1. OP50

A uracil-deficient strain of *Escherichia coli* was used as a *C. elegans* food source, as used by Brenner (Brenner, 1974), to prevent overgrowth of the bacterial lawn when seeded onto NGM plates.

It was grown up by stabbing the OP50 glycerol stock with a pipette tip, without selecting for single colonies, and inoculating the tip in 200 ml 2xTY liquid media (16g tryptone, 10g yeast extract, 5g NaCl in 1l water) shaking overnight at 37°C. It was then aliquoted into 15 ml centrifuge tubes and stored at 4°C until used for seeding standard NGM plates.

2.4. Microscopy methods

A low power dissection microscope fitted with a 10x objective (Nikon SMZ800) was used for everyday worm maintenance.

Images of wells in the chemical susceptibility assay and counts of fluorescent worms in the competition experiment were obtained with Leica MZFLIII scope. This is fitted with a Prior Lumen 200 for fluorescence and a Hamamatsu digital camera (ORCA-05G) that uses the HC ImageLive programme to view and take images.

2.5. Statistical methods

Unless otherwise specified, statistical analysis was performed using GraphPad Prism Version 7.0e for Mac OS X created by GraphPad Software in La Jolla California USA (available at www.graphpad.com).

2.5.1. Developmental time and bleaching sensitivity

Statistical significance was assessed using GraphPad Prism with p-values obtained from one-way ANOVA with Tukey's multiple comparisons test and corrected for multiple comparisons. The response for each wild isolate was compared to every other wild isolate, as, in this context, there was no appropriate 'control' group.

2.5.2. Chemical susceptibility screen

Statistical significance was assessed using GraphPad Prism with p-values obtained from Kruskal-Wallis 1-way analysis of variance, which is a non-parametric ANOVA based on ranks and suitable for the comparison of more than two groups, on responses to every environment. By ranking all of the values from and creating sums of ranks, without regard to which group the values belonged, it was possible to identify compounds that caused significant variation in the susceptibilities observed in the panel of wild isolates, where a small p value (<0.05) signified the presence of populations with different distributions when performing the chemical susceptibility screen and was not caused by random sampling.

This analysis was then followed by Dunn's multiple comparison tests for group pair comparisons where the Kruskal-Wallis test showed significant differences, and corrected for multiple comparisons. This approach enabled me to identify significant differences in susceptibility to a single chemical from the responses of the panel of wild isolates used without an obvious control group and by comparing all variants to each other.

2.5.3. Food consumption of wild isolates in 50ppm carbendazim

Statistical significance of food consumption of wild isolates JU1580, CX11307, N2, CB4856, QX1211, ED3005, ED3012, and JU363 in 50ppm carbendazim was determined using a permutation test run in Matlab (The Math Works Inc.). The code used is shown in Appendix I.

2.5.4. Thrashing and body size

Statistical significance between no compound and four-day aldicarb exposed wild isolates was determined by multiple t tests using GraphPad Prism, one for each wild isolate for both BBPS and body size measurements separately. Bonferroni-Dunn method, with $\alpha=0.05$, was used to correct for multiple comparisons.

2.5.5. Food consumption race data of evolved lines

Where visual observation was not sufficient, statistical significance was assessed using GraphPad Prism with p-values obtained from one-way ANOVA across time, where generations 5, 10, 15 were compared to generation 0, with Dunnett's multiple comparisons test and significance between selection treatments tested in multiple t-tests, one for each generation, and corrected for multiple comparisons.

2.5.6. Brood size data

Statistical significance was calculated using GraphPad Prism with p-values obtained from one-way ANOVA with Dunnett's multiple comparisons, where generations 5, 10, and 15 were compared to generation 0, and corrected for multiple

comparisons. Changes between no compound and compound selection treatment were determined by multiple t tests.

2.5.7. Comparison of ED3005 and AW1783 in a food consumption race in a range of carbendazim concentrations

The responses of ED3005 and AW1783 in a food consumption race in a range of carbendazim concentrations (5ppm-300ppm) were compared using multiple t tests with corrections for multiple comparisons.

Chapter 3: Natural Variation in Chemical Susceptibility in Wild Populations

3.1. Introduction

3.1.1. The role of naturally occurring resistance alleles in the emergence of pesticide resistance

Reasons for resistance emergence are manifold and are often underpinned by not following appropriate resistance management methods. However, the efficiency and long-term suitability of these strategies depends heavily on the initial proportions of naturally-occurring resistance alleles (Génissell *et al.*, 2003). Understanding this parameter of pesticide resistance relies on both experimental and computational approaches.

The parameter of natural resistance, defined by the presence of resistant animals in a population not previously exposed to pesticides, is regularly incorporated in models of resistance emergence with around 88% of relevant studies including it (Bourguet *et al.*, 2010). On the other hand, experimental determination of the prevalence of naturally-occurring resistance has so far been limited and focused on specific cases of pesticide resistance, such as resistance in specific pest species to the genetically modified plants that fight pests using *Bacillus thuringiensis* (Génissell *et al.*, 2003).

However, what appears to be lacking is a more holistic experimental approach to determine the presence of resistance-conferring alleles in an easy to use model organism without limitations to the type of pesticide that can be assessed. This would be an important experimental tool that could be used, in an approach akin to that of *in silico* resistance modelling, to estimate the prevalence of naturally-occurring

resistance via a non-target organism to common agrochemicals, to understand how the observed responses relate to reports of resistance in pest populations, and in the future, to new agrochemicals to predict their performance in the field prior to market release. Importantly, this could also aid in the adoption of appropriate resistance management techniques by modifying the assumptions set with current methods. These strategies are based on the assumptions that, if the use of a compound concludes, the resistance will disappear as the resistance alleles carry a high fitness cost in an environment lacking the selection pressure of the chemical, and that the presence of resistance to one compound results in low or non-existent resistance to other compounds. However, the presence of high frequencies of resistance-conferring alleles in wild populations prior to the use of a pesticide would imply that they do not impose a large fitness cost in a no compound environment, meaning that with equal selection for and against the polymorphism it will be under balancing selection and, therefore, be able to remain in the population (Ffrench-Constant, 2006). Thus, a method to assess and quantify the presence of resistance-conferring polymorphisms to a range of compounds as well as their effect on the susceptibility of related and unrelated compounds is required to gain knowledge about the natural prevalence of resistance in order to ensure the longest possible lifespans for current and future agrochemicals.

3.1.2. *C. elegans* as a model for wild populations

The work in this chapter proposes the use of *C. elegans* wild isolates as a model to assess the prevalence of resistance-conferring polymorphisms in wild populations.

C. elegans is a widely used model organism, and due to the benefits of well-established methods, high quality genome assembly, and fully traced cell lineage is commonly used in research to study development, ageing, neuroscience, and behaviour. In general, for this work, the Bristol N2, a highly lab-adapted *C. elegans* variant (Sterken *et al.*, 2015), is utilised but, importantly for this chapter, a bank of wild isolates with richer genetic diversity is also available for research.

These wild isolates cover the natural diversity present within the species and represent an invaluable experimental toolkit for those interested in understanding the real-world implications of naturally occurring polymorphisms (Félix and Braendle, 2010; Frézal and Félix, 2015; Sterken *et al.*, 2015; Cook *et al.*, 2017). Additionally, due to the species mostly reproducing by self-fertilisation, heterozygosity is decreased and linkage disequilibrium is increased between polymorphisms, meaning that different collections of polymorphisms are maintained in more stable collections in every wild isolate line (Teotonio *et al.*, 2012).

The presence of pesticide resistance conferring natural variation has already been reported by Ghosh *et al.* (2012) who describe moderate resistance to avermectin in the wild isolate CB4856 (Ghosh *et al.*, 2012). This resistance-conferring polymorphism highlights that while avermectin has been used as an agrochemical for a considerable time, *C. elegans*, being a non-parasitic nematode in the wild (Frézal and Félix, 2015), has not been a target organism in its use and thus the organism is suitable to characterise the levels of naturally occurring resistance, from target site resistance to penetration or metabolic resistance, to compounds that have been used in agriculture since the introduction of the first synthetic pesticides in the early part of 20th century.

At the same time, recent reports of laboratory and field resistance to spirotetramat have connected *C. elegans* and whiteflies through homologous mutations that result in the resistance observed. Interestingly, the spirotetramat resistant *C. elegans* mutant was isolated in the lab prior to the identification of field isolates with a homologous resistance conferring gene (A. Flemming, personal communications). This indicates that, despite the species not being a target for pesticides by virtue of not being a pest, *C. elegans* can serve as an invaluable tool for identifying the genetic basis of resistance and even predicting it.

C. elegans is also widely used as a model in the discovery of new anthelmintic compounds (Choy and Thomas, 1999; Jones, Buckingham and Sattelle, 2005; Lackner *et al.*, 2005; Kokel *et al.*, 2006; Kwok *et al.*, 2006; Burns *et al.*, 2015; Mathew *et al.*, 2016) and is used to assess the environmental toxicity of a number of agents including more broad-spectrum pesticides (Leung *et al.*, 2008). This indicates the biological activity of a range of agrochemicals in this model organism and renders it suitable for use in a screen characterising the natural variation in chemical susceptibility to a range of pesticides.

3.2. Chapter aims

This chapter aims to characterise natural variation in chemical susceptibility in genetically diverse wild populations using the resources of a model organism.

Specifically, to:

- establish *C. elegans* wild isolates as a suitable experimental model to study the effects of naturally occurring variation on pesticide susceptibility in lieu of wild pest populations;
- investigate the effect of a range of agrochemicals on genetically diverse *C. elegans* wild isolates;
- understand how the responses of wild isolates relate to each other and whether similarities observed are explained by genetic relatedness or geography;
- determine the widest ranges of chemical susceptibility observed in the wild isolates.

3.3. Results

3.3.1. Choosing a suitable genetic range of *C. elegans* wild isolates

C. elegans is traditionally used as a model organism to study cell, developmental, and molecular biology by utilising the lab-adapted Bristol N2. I was interested in making use of the *C. elegans* wild isolate collection to establish its usefulness as a model for variation in wild populations and to study the effects of naturally occurring polymorphisms on chemical susceptibility in an effort to provide a tool to assess the presence of naturally occurring resistance to any pesticide.

Recent work had characterised genetic variation within *C. elegans* identifying 152 independent isotypes (Andersen *et al.*, 2012; Cook *et al.*, 2016). I used this work to select a panel of 27 wild isolates that would cover the genetic variation of the species as currently known. The strains selected were: N2, AB1, CB4851, CB4853, CB4854, CB4856, CB4857, CB4858, CX11262, CX11307, ED3005, ED3012, ED3017, JU1088, JU1400, JU1491, JU1580, JU1652, JU258, JU311, JU363, JU775, JU792, LKC34, MY23, PB306, QX1211. The genetic relatedness of the strains used is shown in Figure 3.1, which highlights the wild isolates used with red dots on a similarity tree. Table 3.1 highlights the geographic locations of the wild isolates selected. In line with current understanding of intra-species variation of *C. elegans*, this selection of isogenic strains is expected to cover the full genetic variation of the species as currently available (Barriere and Felix, 2005).

The choice of wild isolates was also guided by the Convention on Biological Diversity (United Nations, no date), which limits the use of strains from particular countries, as Syngenta, our industrial partner, has agreed to follow the rules of the convention.

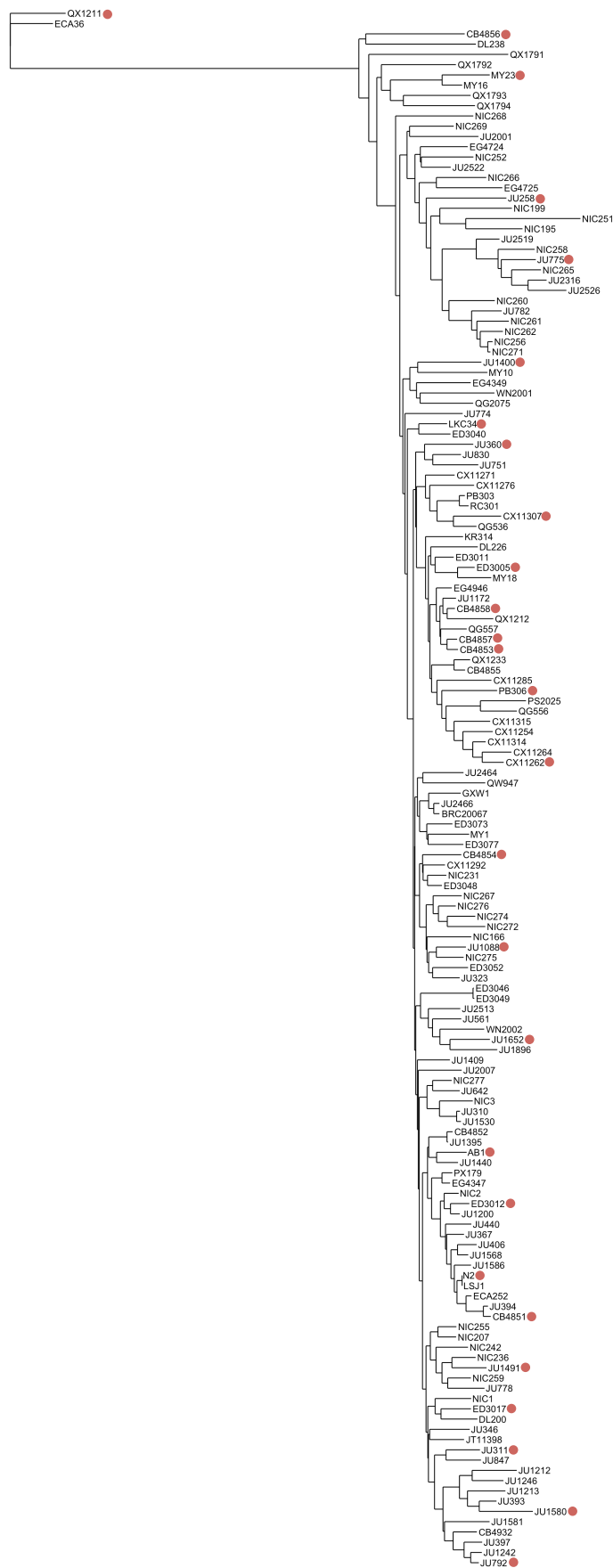


Figure 3.1: Neighbour-joining tree of 152 *C. elegans* wild isolates modified from Cook *et al.* (2016) to include red dots next to the wild isolates chosen for this study.

Table 3.1: Sampling locations of *C. elegans* wild isolates selected with landscape and substrate from which the strain was isolated from indicated. Data collected from CeNDR (Cook *et al.*, 2017).

Strain	Location	Landscape	Substrate
<i>N2</i>	Bristol, UK	urban garden	compost
<i>AB1</i>	Adelaide, Australia	urban garden	compost
<i>CB4851</i>	Bergerac, France	urban garden	soil
<i>CB4853</i>	Altadena, USA	urban garden	vegetable
<i>CB4854</i>	Altadena, USA	urban garden	vegetable
<i>CB4856</i>	Hawaii, USA	rural garden	fruit
<i>CB4857</i>	Claremont, USA	not known	rotting fungus
<i>CB4858</i>	Pasadena, CA, USA	urban garden	flower
<i>CX11262</i>	Huntington Gardens, Los Angeles, USA	botanical garden zoo	soil
<i>CX11307</i>	Huntington Gardens, Los Angeles, USA	urban garden	soil
<i>ED3005</i>	Edinburgh, Scotland	urban garden	compost
<i>ED3012</i>	Edinburgh, Scotland	urban garden	compost
<i>ED3017</i>	Edinburgh, Scotland	urban garden	compost
<i>JU1088</i>	Kakegawa, Japan	botanical garden zoo	soil
<i>JU1400</i>	Sevilla, Spain	rural garden	rotting fruit
<i>JU1491</i>	Le Blanc, France	forest	rotting stem
<i>JU1580</i>	Orsay, France	rural garden	rotting fruit
<i>JU1652</i>	Montevideo, Uruguay	urban garden	compost
<i>JU258</i>	Ribeiro Frio, Madeira	agricultural land	soil
<i>JU311</i>	Merlet, France	rural garden	snail
<i>JU363</i>	Franconville, France	urban garden	compost
<i>JU775</i>	Lisbon, Portugal	botanical garden zoo	rotting fruit
<i>JU792</i>	Frechendets, France	rural garden	compost
<i>LKC34</i>	Madagascar	not known	rotting nut
<i>MY23</i>	Roxel, Germany	urban garden	compost
<i>PB306</i>	Connecticut Valley, USA	not known	isopod
<i>QX1211</i>	San Francisco, California	urban garden	rotting fruit

3.3.2. Design of the screen to assess agrochemical susceptibility

In the design of the chemical susceptibility screen it was important that it would provide information about the multigenerational effects of chronic compound exposure on a strain, would be suitable for a range of agrochemicals, and be manually high throughput (in the absence of equipment, such as a worm sorter, required for automated high throughput assays). As suggested by previous screening work using *C. elegans* (Burns *et al.*, 2006) I opted for a 24-well plate format with solid media and food in every well as well as the platform to add compounds, and placed 10 synchronised early larval stage worms into each well as illustrated in Figure 3.2. In this setup the worms were then grown at 20°C for four days at which point the worms in the wells were phenotyped, as outlined in Figure 3.3, and the well was photographed.

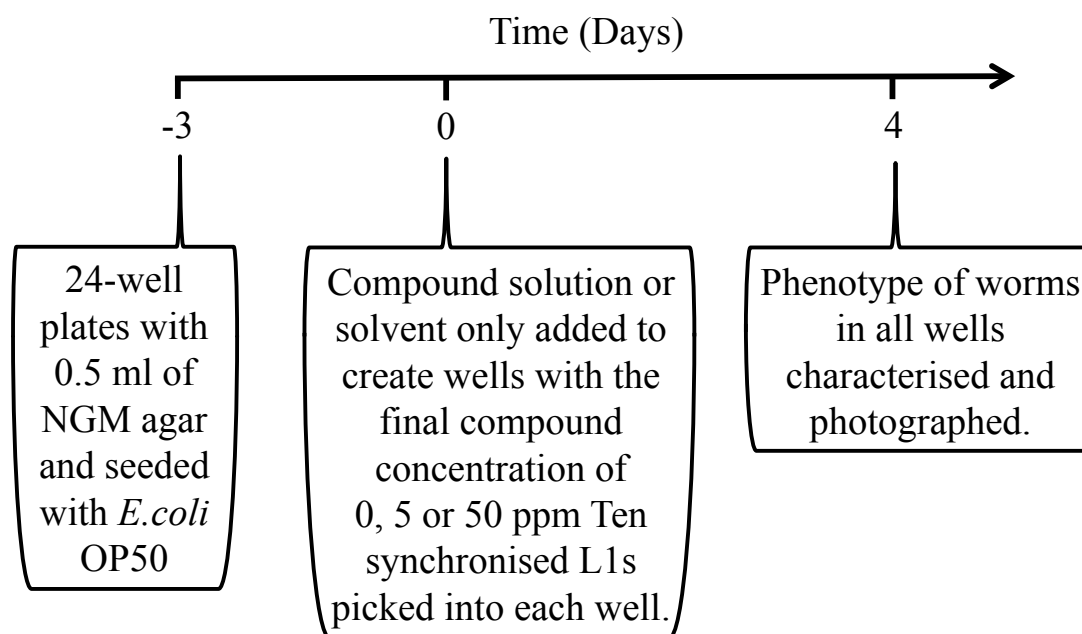


Figure 3.2: Timeline of the chemical susceptibility screen outlining the preparation of plates with NGM agar and *E. coli* OP50 food source three days prior to the start of the screen. Compound solution was added to the relevant wells in the morning before picking ten synchronised early larval stage worms into each well. The plates were then incubated at 20°C for four days at which point the phenotype of the worms in the wells was characterised (as outlined in Figure 3.3 below) and the wells were photographed.

3.3.2.1. *Scoring the chemical susceptibility of wild isolates*

In order to characterise the response of a strain to a compound after the four-day exposure I scored it as one of four different categories as illustrated in Figure 3.3.

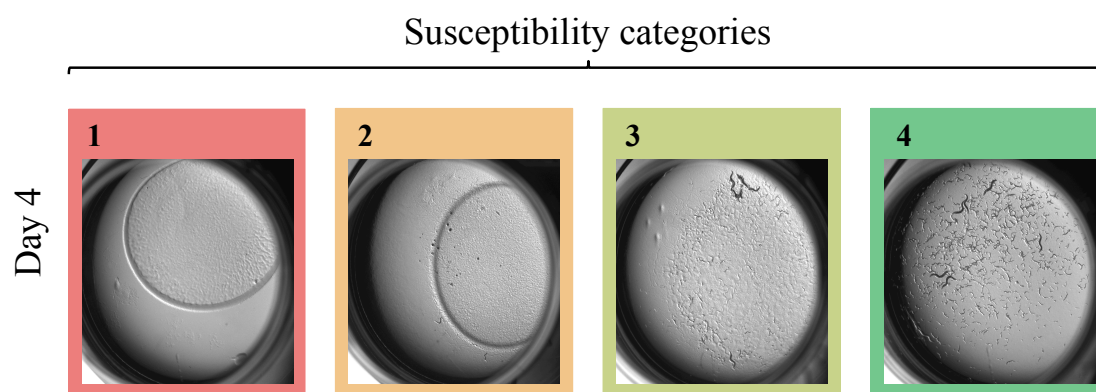


Figure 3.3: The four categories of the responses of the worms in wells after a four-day exposure to the compound. Groups were given numerical values from 1-4 from left to right on the figure while the colours correspond to the responses reported in the chemical susceptibility screen Table 3.5.

The groups coloured red, orange, light green, and dark green were assigned a numerical whole number value from 1-4 according to the severity of phenotype observed in compound. These values run from left to right on Figure 3.3. Number '4', indicated by dark green, was assigned where no obvious difference was observed in comparison to no compound control. The worms were unaffected in terms of movement, development, and fecundity. '3', indicated by light green, was assigned to wells where small effects were observed. This meant some lethality in transferred L1s or their progeny, movement defects, slightly perturbed development, and/or reduced fecundity. I assigned '2', indicated by orange, to wells where high lethality of transferred L1s and their progeny, highly reduced or missing egg-laying, severe movement defects and developmental delay were observed. Finally, '1', indicated by

red, was assigned where the transferred worms had died in the environment by the fourth day of chronic exposure and no progeny were observed.

3.3.2.2. Observing the multigenerational effects of chronic agrochemical exposure

The outlined design fulfilled the first criteria of the chemical susceptibility screen as it enabled us to observe multigenerational effects of chronic pesticide exposure. Within the four-day duration of the assay the transferred early larval stage worms will have the time to develop into adults, within the first two days, and begin to lay eggs, in the third and fourth day, that would then be able to begin progressing through the *C. elegans* life cycle.

3.3.3. Characterising the developmental time and cuticle integrity of the *C. elegans* wild isolates

C. elegans development has been extensively studied, utilising the Bristol N2 variant. While a number of the wild isolates have been utilised in various studies in the past (e.g. Hodgkin and Doniach, 1997; Teotónio, Manoel and Phillips, 2006; Ghosh *et al.*, 2012; Lee *et al.*, 2016), only limited and non-comprehensive data was available on the set of 25 wild isolates chosen for this study. Therefore, I set out to characterise the developmental time and cuticle thickness of the wild isolates. These were parameters that I considered to be important to establish as uniform in the wild variants in order to enable the legitimate comparison of differences in chemical susceptibility of the isolates as caused by variation in the molecular targets of the chemicals. In theory, as a faster developmental time or thicker cuticle could carry a benefit for the strain in the chemical susceptibility screen and make it less susceptible

to compounds or the effect of the compound on fecundity less noticeable (Johnstone, 2000; Xiong, Pears and Woollard, 2017), it was important to identify whether there were wild isolates with significantly differences in cuticle or developmental time.

3.3.3.1. *Results in the panel of wild isolates suggest cuticle integrity will not confer resistance to chemicals*

In order to characterise cuticle integrity of 27 wild isolates, I tested the time taken to rupture the cuticle of gravid hermaphrodites. My results, shown in Figure 3.4, suggest that there is variation in cuticle thickness between wild isolates with significant differences in cuticle rupture times. Results of the statistical analysis of bleaching results are shown in Table 3.2.

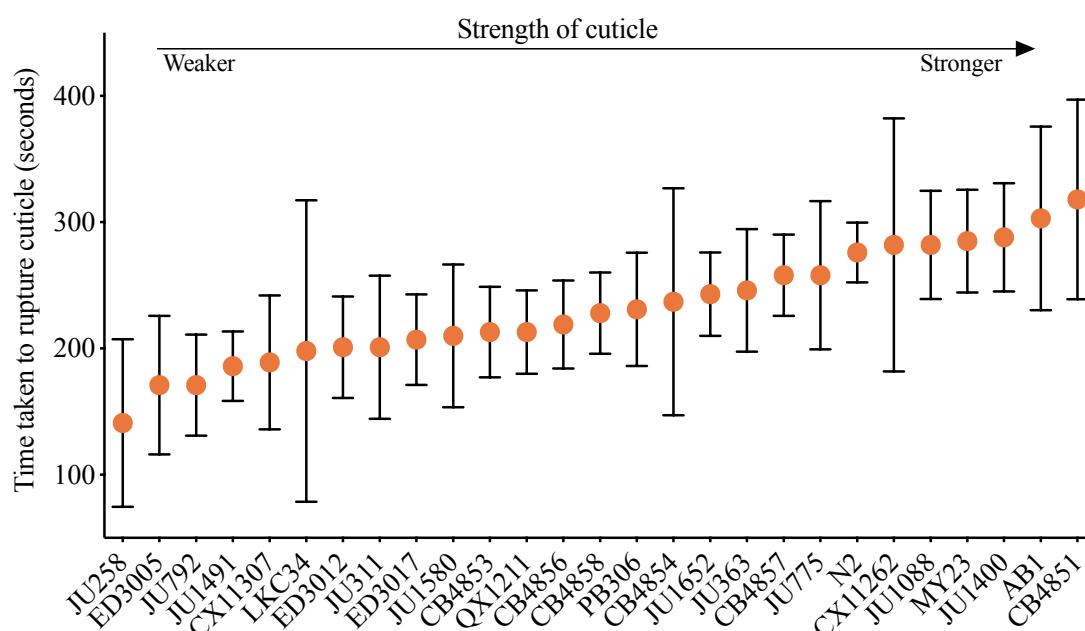


Figure 3.4: Time taken to rupture the cuticle of 27 *C. elegans* wild isolates in seconds (n=10). Values are plotted showing the mean $\pm$ standard deviation (SD). Five wild isolates with weak cuticle and 7 wild isolates with strong cuticle are highlighted. The identification of cuticle differences is based on statistical analysis and the identification of significant differences in pairwise comparisons upon one-way ANOVA and Tukey's multiple comparisons test. Results of statistical analysis are outlined in Table 3.2.

In particular there were seven more durable strains: CB4851, AB1, JU1400, MY23, JU1088, CX11262, N2. By contrast, there were five strains that exhibited weaker cuticles in the test: JU258, ED3005, JU792, JU1491, CX11307.

I wanted to compare the weak cuticle wild isolates to published data available on cuticle mutants, such as *bus-5* (*e2985*) and *agmo-1* (*e3016*) that both hold mutations in genes important for cuticle integrity (Loer *et al.*, 2015; Feng, Shou and Butcher, 2016). Cuticles of both *bus-5* (*e2985*) and *agmo-1* (*e3016*) rupture in under 50 seconds if subjected to the same bleach treatment as used with wild isolates (Xiong, Pears and Woollard, 2017). This suggests that weaker cuticle wild isolates, that on average rupture at around 150 seconds, have considerable thicker cuticle than weaker cuticle mutants of *C. elegans*. Therefore, while variation in cuticle integrity is present, the strains were deemed suitable for use in a chemical susceptibility screen and expected to not result in sensitivity in slightly weaker cuticle wild isolates or resistance in stronger cuticle wild isolates. While not expecting to see differences resulting from cuticle permeability, I intended to keep the variation in this trait in mind in analysis of the results of chemical susceptibility of wild isolates to a range of agrochemicals.

3.3.3.2. Genetically diverse *C. elegans* wild isolates exhibit mostly uniform speed of development

In order to identify whether there were differences in the developmental time of the 27 wild isolates chosen for the chemical susceptibility assay, I identified the number of hours taken by first larval stage worms to lay first egg. The results of this assay are shown in Figure 3.5. The results of the statistical analysis of developmental speed results can be found in Table 3.3.

I observed less variation in the analysis of developmental time of wild isolates than for cuticle integrity. Overall two strains with slower developmental times were identified: QX1211, MY23. I was concerned that these strains would be incorrectly identified as compound-sensitive in a chemical susceptibility screen where the multigenerational effects of compounds were assayed because they would start to lay eggs significantly later. However, as QX1211 is considered the most divergent wild isolate, with a notable proportion of SNPs present only in that strain (Andersen *et al.*, 2012), I was reluctant to exclude the strain from our wild isolates panel.

MY23 as well as JU1088 were, however, excluded due to concerns regarding slow development, in the case of MY23, and due to difficulties with maintaining the strains at 20°C and on NGM for assays, in the case of JU1088 (phenotype determined qualitatively and not shown).

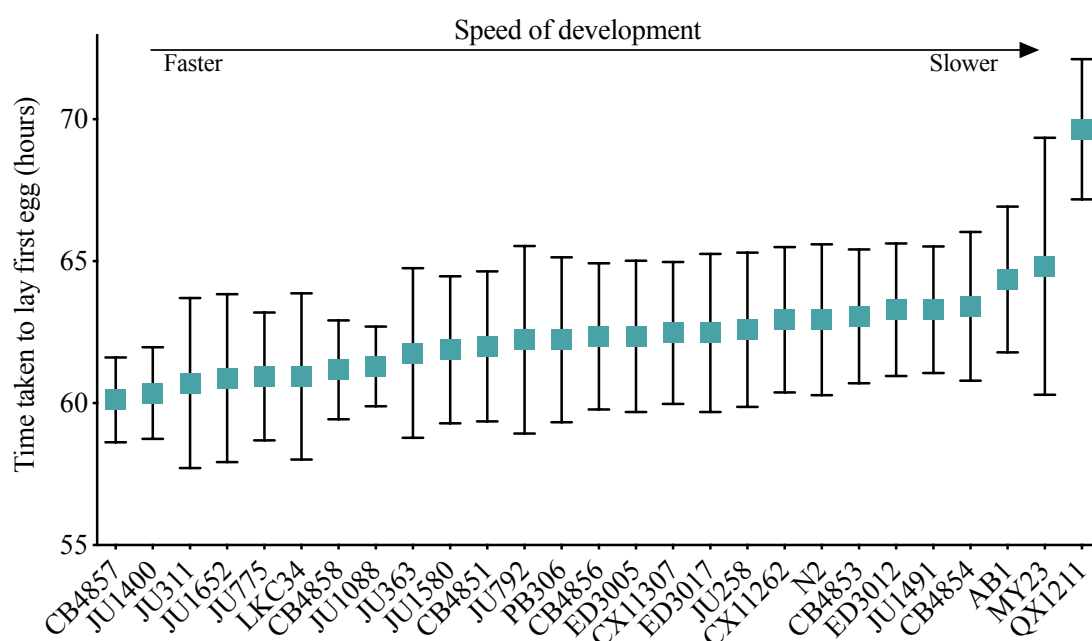


Figure 3.5: Time taken for 27 wild isolates to lay first egg from L1 stage in hours at 20°C (n=17). Values are plotted showing the mean $\pm$ SD. Presence of two wild isolates (MY23, QX1211) with slower development (indicated by grey background) identified by finding statistical differences in multiple comparisons using one-way ANOVA together with Tukey's multiple comparisons test.

3.3.4. Choosing a range of agrochemicals for the chemical susceptibility screen

The aim of the chemical susceptibility screen was to assess the effect of a wide variety of agrochemicals. As such, the agrochemicals were chosen to encompass insecticides, nematicides, and fungicides, with four of the compounds being bio-pesticides (crop protection products that are derived from living microorganisms or natural products (Zhu *et al.*, 2016)): abamectin, rotenone, ryanodine, spinosad. They were also chosen to cover different modes of action, targeting nerve and muscle physiology, development, respiration, amino acid and protein synthesis. Finally, the list of compounds was chosen to include pesticides that were recently discovered, widely used, or no longer used in agriculture due to environmental safety issues. The compounds chosen alongside their specific MOA, target pest populations, and the year of introduction as a pesticide, as outlined by Pesticide Properties (PPDB), Bio-Pesticide Properties (BPPD), and Veterinary Substances Database (VSDB) (Lewis *et al.*, 2016), are indicated in Table 3.4.

Table 3.4: 28 compounds selected for chemical susceptibility screen. Mode of action outlines the general molecular target the compound is thought to act upon to exhibit pesticidal properties. General MOA indicates the physiology that the compound targets based on IRAC, and FRAC MOA classifications. ‘Used’ indicates which pest populations the compound is used to target or has been used to target in the past. The ‘Introduced’ column indicates the year the pesticide was introduced to control pest populations and ‘Current use’ refers to whether the compound is still in use or whether it has been withdrawn or banned by regulatory bodies as indicated by the Pesticide and Bio-Pesticide Properties and Veterinary Substances Database (Lewis *et al.*, 2016) in September 2019.

Compound	Mode of action	General MOA	Used	Introduced	Current use
Abamectin	Agonist of glutamate-gated chloride channel	Nerve & muscle	Anthelmintic, insecticide	1985	In use
Aldicarb	Acetylcholinesterase inhibitor	Nerve & muscle	Anthelmintic, insecticide	1970	In use
Amitraz	Octopamine receptor agonist	Nerve & muscle	Acaricide, insecticide	1972	In use
Carbendazim	Tubulin polymerisation disruptor	Growth & development	Anthelmintic, fungicide	1974	In use
Cartap	Nicotinic acetylcholine receptor blocker	Nerve & muscle	Insecticide	1967	Withdrawn. 2009
Cyanotropane	Nicotinic acetylcholine receptor agonist	Nerve & muscle	Insecticide	2002	In use
Cyprodinil	Inhibitor of methionine synthesis and secretion of hydrolytic enzymes by an unknown mechanism	Amino acid and protein synthesis	Fungicide	1994	In use
DDT	Voltage gated sodium channel agonist	Nerve & muscle	Insecticide	1944	Banned, 1972
Diafenthiuron	Mitochondrial inhibitor	Respiration	Acaricide, insecticide	1988	In use
Dieldrin	GABA receptor antagonists	Nerve & muscle	Insecticide	1949	Banned, 1987
Diflubenzuron	Chitin biosynthesis inhibitor	Growth & development	Insecticide	1975	In use
Fenoxycarb	Juvenile hormone mimic	Growth & development	Insecticide	1985	In use
Fenvalerate	Voltage gated sodium channel agonist	Nerve & muscle	Insecticide	1976	In use
Fluazinam	Mitochondrial inhibitor	Respiration	Fungicide	1990	In use

Flufenoxuron	Chitin biosynthesis inhibitor	Growth & development	Insecticide	1986	In use
Fluopyram	Mitochondrial inhibitor	Respiration	Fungicide	2008	In use
Indoxacarb	Voltage gated sodium channel blocker	Nerve & muscle	Insecticide	2000	In use
Levamisole	Nicotinic acetylcholine receptor agonist	Nerve & muscle	Anthelmintic	1966	Withdrawn, 2000
Lufenuron	Chitin biosynthesis inhibitor	Growth & development	Anthelmintic, fungicide	1990	In use
Metaflumizone	Voltage gated sodium channel blocker	Nerve & muscle	Insecticide	2008	In use
Monepantel	Nicotinic acetylcholine receptor antagonist	Nerve & muscle	Anthelmintic	2009	In use
Oxamyl	Acetylcholinesterase inhibitor	Nerve & muscle	Pesticide	1974	In use
Pirimicarb	Acetylcholinesterase inhibitor	Nerve & muscle	Insecticide	1970	In use
Rotenone	Mitochondrial inhibitor	Respiration	Insecticide, pesticide	1935	In use
Ryanodine	Ryanodine receptor	Nerve & muscle	Insecticide	1968	Banned, 1999
Spinosad	Nicotinic acetylcholine receptor agonist	Nerve & muscle	Insecticide	1997	In use
Spiroindoline	Vesicular acetylcholine transporter inhibitor	Nerve & muscle	Insecticide	2012	In use
Spirotetramat	Acetyl CoA carboxylase inhibitor	Growth & development	Insecticide	2008	In use

3.3.5. Results of the chemical susceptibility screen

The results of the chemical susceptibility screen are outlined in Table 3.5 with the colours in responses indicating the response observed as outlined in Figure 3.3. The results of the Kruskal-Wallis test, also shown in Table 3.5, indicated that there was significant variability, explained by the presence of populations with different distributions in susceptibilities, to 16 out of the 28 chemicals tested. The results of the Dunn's multiple comparisons test to identify the strains that were significantly more resistant or sensitive, shown in Table 3.6, indicated that strains with significantly differing susceptibilities were identified in 8 chemical backgrounds.

This further analysis identified eight out of the sixteen compounds with resistant or sensitive strains, defined by group pair comparisons that were significantly different: aldicarb, carbendazim, diafenthiuron, fluazinam, levamisole, lufenuron, metaflumisol, oxamyl. Therefore these were the compounds where, within the populations of wild isolates, resistance-conferring or sensitivity-causing variation was present to be spotted in a single generation of compound selection.

The compounds with significant variation in responses among wild isolates but no significantly susceptible or resistant strains were: abamectin, cyanotropane, diflubenzuron, fenvalerate, rotenone, ryanodine, spinosad, spiroindoline. This means that differences were found between more complex comparisons of sums of ranks than those considered in pairwise comparisons. Therefore with no identification of significant differences between any two populations in Dunn's multiple comparison test and despite the identification of significantly different populations in the Kruskal-Wallis test, these 8 compounds were considered to have little naturally-occurring variation in chemical susceptibility.

The other compounds screened had no significant differences in responses across the genetically diverse panel of wild isolates used. Uniform responses were recorded for: amitraz, cartap, cyprodinil, DDT, dieldrin, fenoxycarb, flufenoxuron, fluopyram, indoxacarb, monepantel, pirimicarb, spirotetramat. Thus, resistance-conferring polymorphisms were not present in the panel of genetically diverse wild isolates tested and as a result varying susceptibility could not be detected.

Mode of action	Compound																										
		Kruskal-Wallis test																									
		P Value																									
	No compound	0.0855																									
Acetyl CoA carboxylase inhibitor	Spirotetramat	0.0827																									
	Aldicarb	<0.0001																									
	Oxamyl	0.0115																									
Acetylcholinesterase inhibitor	Pirimicarb	0.052																									
	Diflubenzuron	0.0061																									
	Flufenoxuron	0.1342																									
	Lufenuron	0.0001																									
Chitin biosynthesis inhibitor	Dieldrin	0.1971																									
	Abamectin	0.0005																									
	Juvenile hormone mimic	0.2282																									
Mitochondrial inhibitor	Fenoxycarb	0.0005																									
	Diafenthuiuron*	0.0002																									
	Fluzinam*	NA																									
	Fluopyram*	NA																									
Rotenone*	Rotenone*	<0.0001																									
	Cyanotropane	0.0016																									
	Levamisole	<0.0001																									
Nicotinic acetylcholine receptor agonist	Spinosad	0.0029																									
	Cartap	0.0729																									
Nicotinic acetylcholine receptor blocker	Monepantel*	0.5013																									
	Amitraz	0.5335																									
	Ryanodine	0.0044																									
Tubulin disruptor	Carbendazim	<0.0001																									
	Cyprodinil	0.4446																									
	Spiroindoline*	0.0327																									
Vesicular acetylcholine transporter	DDT	0.3808																									
	Fenvalerate	0.0014																									
	Indoxacarb	0.0595																									
	Metaflumisol	0.0006																									

Table 3.5: Results of the chemical susceptibility screen. The responses of 25 *C. elegans* wild isolates, outlined on the top, were recorded upon chronic exposure (4 days) to 28 agrochemicals (indicated on the left with corresponding general mode of action) as well as a no compound environment. Most compounds were tested at a concentration of 50ppm, but compounds marked with an asterisk (*) were tested at a 5ppm concentration. The colours of the response correspond to colours outlined in Figure 3.3: green colours show no sensitivity to compound; orange and yellow show severe and mild effects on development, fecundity, movement respectively; red colours indicate complete lethality and extreme sensitivity. The mean response of n=6 across two biological replicates of three is shown. Far right shows p values and significance for compound datasets obtained in GraphPad by performing a Kruskal-Wallis test. Both p value and significance for fluopyram are marked NA as all values obtained from the screen were the same.

Table 3.6: Significance and P values of group pair comparisons from Dunn's multiple comparison tests. Compound to which the comparisons refer to is indicated on the left.

Compound	Comparison	Sig.	P Value
<i>Aldicarb</i>	CB4857 vs. CX11307	**	0.0027
	CB4858 vs. CX11307	**	0.0027
	CX11307 vs. JU1652	**	0.0027
	CX11307 vs. JU363	*	0.029
<i>Carbendazim</i>	N2 vs. ED3005	***	0.0002
	N2 vs. ED3012	**	0.0012
	N2 vs. JU363	***	0.0002
	AB1 vs. ED3005	*	0.0184
	AB1 vs. JU363	*	0.0184
	CB4851 vs. ED3005	*	0.0184
	CB4851 vs. JU363	*	0.0184
	CB4856 vs. ED3005	**	0.0019
	CB4856 vs. ED3012	*	0.0122
	CB4856 vs. JU363	**	0.0019
	CX11307 vs. ED3005	****	<0.0001
	CX11307 vs. ED3012	****	<0.0001
	CX11307 vs. JU363	****	<0.0001
	ED3005 vs. JU1580	****	<0.0001
	ED3005 vs. QX1211	*	0.0184
	ED3012 vs. JU1580	****	<0.0001
	JU1580 vs. JU363	****	<0.0001
	JU363 vs. QX1211	*	0.0184
<i>Diafenthiuron</i>	CB4851 vs. JU311	*	0.0406
	CB4856 vs. JU311	*	0.0406
	JU311 vs. LKC34	*	0.0406
<i>Fluazinam</i>	CB4851 vs. JU1491	*	0.0362
	CB4851 vs. JU258	*	0.0362
	CB4851 vs. JU311	*	0.0362
	CB4851 vs. JU363	*	0.0362
<i>Levamisole</i>	CB4853 vs. CB4856	***	0.0002
	CB4853 vs. CX11307	****	<0.0001
	CB4853 vs. JU311	**	0.0042
	CX11307 vs. ED3017	**	0.0056
	CX11307 vs. JU1491	**	0.0056
	CX11307 vs. PB306	**	0.0056
<i>Lufenuron</i>	N2 vs. CB4856	*	0.0366
	AB1 vs. CB4856	*	0.0366
	CB4856 vs. JU1652	*	0.0366
	CB4856 vs. JU363	*	0.0366
	CB4856 vs. JU792	*	0.0366
	CB4856 vs. LKC34	*	0.0366

<i>Metaflumisol</i>	N2 vs. CB4856	***	0.0003
	AB1 vs. CB4856	***	0.0003
	CB4851 vs. CB4856	***	0.0003
	CB4853 vs. CB4856	***	0.0003
	CB4854 vs. CB4856	***	0.0003
	CB4856 vs. CB4857	***	0.0003
	CB4856 vs. CB4858	***	0.0003
	CB4856 vs. CX11262	***	0.0003
	CB4856 vs. CX11307	***	0.0003
	CB4856 vs. ED3005	***	0.0003
	CB4856 vs. ED3012	***	0.0003
	CB4856 vs. ED3017	***	0.0003
	CB4856 vs. JU1491	***	0.0003
	CB4856 vs. JU1580	***	0.0003
	CB4856 vs. JU1652	***	0.0003
	CB4856 vs. JU258	***	0.0003
	CB4856 vs. JU311	***	0.0003
	CB4856 vs. JU363	***	0.0003
	CB4856 vs. JU792	***	0.0003
	CB4856 vs. LKC34	***	0.0003
	CB4856 vs. PB306	***	0.0003
	CB4856 vs. QX1211	***	0.0003
<i>Oxamyl</i>	CB4858 vs. CX11307	**	0.0068

3.3.6. Detection of groups of similarly-responding wild isolates

Following the characterisation of variation in chemical susceptibility I wanted to establish that there were no strains or groups of strains that emerged as overall more resistant or sensitive to a range of compounds. The identification of an overall resistant strain might indicate the presence of a more general mechanism of resistance in a *C. elegans* wild isolate, for example a strain with a general up-regulation of detox pathways.

I decided that by clustering similarly responding wild isolates together I might be able to establish whether any particular groups of strains were emerging as more resistant or sensitive. This analysis, shown in Figure 3.6, established that the wild isolates could be placed into three groups based on their responses to the 29 conditions tested. These were colour coded blue, yellow, and green (Figure 3.6).

This overall average susceptibility grouped into the yellow cluster alongside the responses of the susceptibility ratings of ten wild isolates. While this meant that these ten wild isolates were in general like the group average response observed, it did not exclude the possibility that in the case of particular compounds they had significantly lower or higher susceptibility.

The blue group represents strains that fared better in aldicarb and oxamyl (both AChE inhibitors), dieldrin (gamma-aminobutyric acid (GABA) receptor agonist), carbendazim (tubulin disruptor), and fenvalerate (VGSC agonist) while the green group encompasses strains that on average coped better in spirotetramat (Acetyl CoA carboxylase inhibitor), fenoxycarb (juvenile hormone mimic), fluazinam (mitochondrial inhibitor), cyanotropane (nAChR agonist), cartap (nAChR blocker), and ryanodine (ryanodine receptor modulator). Therefore it appears that neither the blue or green cluster is overall only resistant or sensitive and that both represent a

mixture of opposing sensitive and resistant traits. Additionally, neither group seems to have increased or decreased susceptibility to compounds of a particular MOA.

As this analysis was done to identify whether there were overall more sensitive or resistant strains, there are limited interpretations to the results of the clustering of responses to all compounds used in the chemical susceptibility screen.

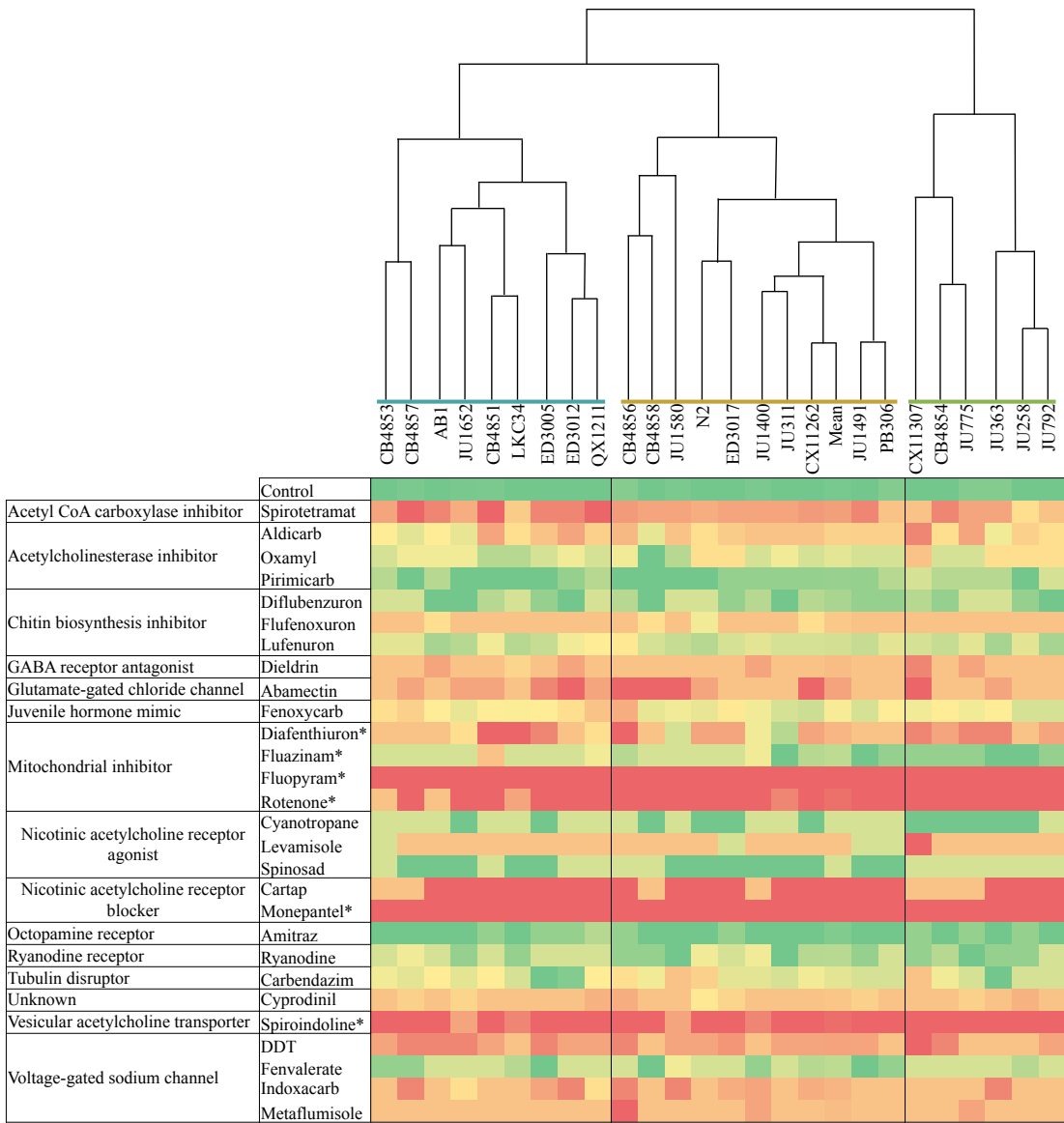


Figure 3.6: Clustering of wild isolates according to the chemical susceptibility screen results showing the wild isolates with most similar susceptibility responses across the panel of agrochemicals used. Isolates were allocated into three groups indicated by blue, yellow, and green lines above the wild isolate names. Results visualised in Tree View and figure modified further using Powerpoint to include chemical susceptibility screen results with compounds used.

3.3.6.1. *Are the clusters based on genetic relatedness of the wild isolates?*

In order to establish whether the formation of the chemical susceptibility clusters had a connection to the genetic relatedness of the wild isolates, I decided to identify the locations of the members of the blue, yellow, and green clusters on the neighbour joining tree referenced in 3.3.1 and shown in Figure 3.1, where it was used to choose the genetically diverse panel of *C. elegans* wild isolates used. The neighbour-joining tree, shown in Figure 3.7 below, does not highlight the co-localisation of any chemical susceptibility clusters. This indicates that the clustering of particular wild isolates and therefore their responses to a panel of agrochemicals cannot be predicted from total genetic similarity to another wild isolate and that the responses are more likely to be the result of smaller polymorphisms that alter particular susceptibility. However, CB4853 and CB4857 are genetically closely related and both belong to the blue cluster, indicating that high genetic similarity has, in this case, resulted in similar chemical susceptibilities.

3.3.6.2. *Geographical scattering of wild isolate clusters reveals the blue group as the most widely spread*

Relatedness of *C. elegans* wild isolates has been found to follow rules where the correlation between genetic relatedness and geographic distance can only be found within 700 kilometre sampling distance with correlations breaking down across distances beyond that (Andersen *et al.*, 2012). I was intrigued to see how the chemical susceptibility clusters and the agrochemical sensitivity patterns they represent would be placed on a world map. A corresponding map of cluster locations can be found on Figure 3.8.

According to the map, the blue cluster spans the most continents being present in Europe, North and South America, Africa, and Australia. Overall, the blue cluster displayed lower susceptibility (than the yellow and green clusters) to pesticides that span a diverse range of modes of action affecting muscle and nerve physiology (aldicarb, oxamyl, dieldrin, and fenvalerate) and a single growth and development perturbing agent (carbendazim), thus indicating that resistance to these agents may be most widespread in this sample of wild populations.

The wild isolates belonging to the yellow cluster can be found in both Europe and North America. The yellow cluster was highlighted as the collection of strains that co-clustered with the overall average response values as the ‘mean’ dataset, thus highlighting the group of wild isolates that across the panel of agrochemicals tested behaved closest to the average of all responses observed in wild isolates.

The strains belonging to the green cluster are found predominantly in Europe with only a single strain in that group isolated from California, USA. The isolates grouping together here were found to be less susceptible to agrochemicals that target

muscle and nerve physiology (cyanotropane, cartap, ryanodine), perturb development (spirotetramat, fenoxycarb), and inhibit mitochondrial activity (fluazinam).



Figure 3.8: Geographical locations of three wild isolate clusters green, yellow, and blue do not identify that isolates from any particular cluster originate from the same geographical location. Map modified from http://johomaps.com/world/worldblank_bw.html.

A limitation that should be highlighted here is the lack of strains used in this work that have been isolated from outside of Europe and North America which raises concerns for missed variation due to sampling bias (Barriere and Felix, 2005). However, the limited number of isolates from outside of Europe and North America as well as the limitations set by the Convention on Biological Diversity, means that it was impossible to achieve uniform coverage of the globe. It is possible that additional isolates are now available from a number of these locations and these should be included in future work.

Additionally, isolates geographical locations with high sampling density could be utilised to capture more of the genetic variation of *C. elegans*. Deep sampling of strains from Germany (Haber *et al.*, 2005) and, more recently, Hawaii (Crombie *et al.*,

2019) have indicated that a high level of the intra-specific genetic diversity is captured using isolates from a small geographic region. In particular, the Hawaiian isolates have been demonstrated to harbour greater genetic diversity than the rest of the worldwide population (Crombie *et al.*, 2019).

3.3.7. Identifying wild isolates displaying overall sensitivity or resistance to agrochemicals

As mentioned above, eight compounds (aldicarb, carbendazim, diafenthiuron, fluazinam, levamisole, lufenuron, metaflumisolet, oxamyl) were identified with significantly differing wild isolate susceptibilities. While the clustering of wild isolate chemical susceptibility did not reveal any groups of strains that were sensitive or resistant to all compounds tested, I was interested in identifying whether the responses to the eight compounds highlighted strains resistant or sensitive to multiple compounds.

By identifying pairs that were significantly different I assigned strains relative labels ‘resistant’ (R) and ‘sensitive’ (S). The ‘resistant’ label was assigned where, in the pair, the strain scored higher in the initial chemical susceptibility screen (out of the two strains compared), and vice versa for the ‘sensitive’ label. By doing this I identified the strains with extreme responses to compounds by only taking into account significant differences observed in pair wise comparisons within the group. As no suitable ‘control’ response was available to be used as an average response to any compound of interest, this was the most appropriate course of action. Results of this analysis are shown in Table 3.7 and are further broken down under headings 3.3.8 and 3.3.9.

In regards to responses observed in general, all eight compounds apart from metaflumisolet had at least a single wild isolate highlighted as ‘sensitive’ or ‘resistant’.

A single sensitive wild isolate was identified in the case of metaflumisolet with most other isolates being only slightly less susceptible.

Table 3.7: Identification of sensitive (S) and resistant (R) wild isolates upon chronic exposure to compound. Compounds used at a 50ppm concentration and those marked with an asterisk (*) used at a 5ppm concentration. Leftmost column highlights the general mode of action of the compound immediately next to it. Modes of action are abbreviated to stand for: acetylcholinesterase (AChE), tubulin (Tub), mitochondria (Mt), nicotinic acetylcholine receptor (nAChR), chitin biosynthesis (chitin), voltage-gated sodium channel (VGSC).

MOA	Compound	N2	AB1	CB4851	CB4853	CB4856	CB4857	CB4858	CX11307	ED3005	ED3012	ED3017	JU1491	JU1580	JU1652	JU258	JU311	JU363	JU792	LKC34	PB306	QX1211
AChE	Aldicarb						R	R	S						R			R				
	Oxamyl							R	S													
Chitin	Lufenuron	R	R			S									R			R	R	R		
Mt	Diafenthiuron*			S		S											R			S		
	Fluazinam*			S									R			R	R	R				
nAChR	Levamisole				R	S			S			R	R				S				R	
Tub	Carbendazim	S	S	S		S			S	R	R			S				R				S
VGSC	Metaflumisolet					S																

3.3.8. Three wild isolates sensitive to multiple compounds

Table 3.7 shows three strains that are sensitive to three or more compounds: CB4851, CB4856, CX11307.

3.3.8.1. Genetic and geographical relatedness do not explain increased susceptibility to multiple compounds

The strains are not closely related based on the neighbour-joining tree of wild isolates (Figure 3.1) and the isolates have been collected from three separate geographical locations with CB4851 sampled from Bergerac (France), CB4856

collected from Hawaii (USA), and CX11307 sampled from Los Angeles (USA). This suggest that that the sensitivity is not the result of a single evolutionarily conserved mechanism despite overlap in the compounds where higher susceptibility is detected.

3.3.8.2. *Developmental time and cuticle integrity of the three susceptible strains*

Results of the developmental time measurements (Figure 3.5) for the three isolates offer no explanation as all three strains laid first eggs at the same time. Cuticle integrity, Figure 3.4, offers a potential explanation to the sensitivity observed with CX11307 as the strain was found to have a weaker cuticle, which could in turn make it more permeable to compounds resulting in more adverse effects on movement, development, and fecundity. At the same time CB4851 was found to have a strong cuticle and CB4856 had average cuticle integrity in the panel of wild isolates tested. These results also indicate that the sensitivity in these three cases is most likely not due to a common cause (a weaker cuticle in all three strains).

3.3.8.3. *Modes of action of the compounds*

Looking at modes of action that the strains are sensitive to offers a different picture. CB4851 is sensitive to two inhibitors of oxidative phosphorylation diafenthion and fluazinam indicating the possibility of a mechanism conferring sensitivity to mitochondrial inhibitors, relative to the other wild isolates screened. The strain was also sensitive to carbendazim, a tubulin disruptor.

CB4856 is sensitive to compounds that encompass five different modes of action: tubulin disruptor (carbendazim), mitochondrial inhibitor (diafenthion), nAChR agonist (levamisole), chitin biosynthesis inhibitor (lufenuron), VGSC blocker

(metaflumisole). It is likely that CB4856 is more susceptible to the five unconnected targets due to a cause other than cuticle integrity and developmental time, given my data. It may be the result of different natural history leading to the exposure of fewer chemical agents prior to isolation from the wild or predisposition to lower scoring in chemical susceptibility screen due to an unidentified parameter.

Sensitivity to two AChE inhibitors (aldicarb, oxamyl), nAChR agonist (levamisole), and tubulin disruptor (carbendazim) was identified in the wild isolate CX11307. In this case the appearance of sensitivity to pesticides affecting two different acetylcholine-related modes of action is notable and potentially indicates a naturally occurring variation in the acetylcholine (ACh) pathway that results in increased susceptibility in comparison to the other wild isolates as no other wild isolates sensitive to multiple agents that affect ACh transmission and signalling were identified.

3.3.9. Identification of wild isolates resistant to multiple agrochemicals

Results shown in Table 3.7 reveal one strain that scores more highly than the group in four different pesticide environments according to the chemical susceptibility screen – JU363 – and two strains that score more highly than the panel of wild isolates in the case of particular modes of action: CB4858, JU311. There were also strains that were resistant to two agrochemicals with unrelated modes of action: JU1491, JU1652.

3.3.9.1. The resistant strains are not connected by genetic or geographical relatedness

Any combination of the five strains do not appear to be closely related based on Figure 3.1 and were isolated from Pasadena, USA (CB4858), Le Blanc, France (JU1491), Montevideo, Uruguay (JU1652), Merlet, France (JU311), and Franconville, France (JU363). Despite three out of the five strains being isolated from the same country it remains unlikely that the strains share a similar genetic cause of resistance via a generalist mode that is effective across a range of compound modes of action.

3.3.9.2. Resistance is not explained by faster developmental time or decreased cuticle permeability

The results of developmental time and cuticle integrity offer no simple explanation as to the cause of resistance as JU1491 was identified as a weaker cuticle outlier and the other four wild isolates were not significantly different from the group in either of the datasets.

3.3.9.3. Resistance to multiple compounds with unrelated modes of action in the wild isolates JU363, JU1491, and JU1652

JU363 was found to be resistant to four different pesticide environments: an AChE inhibitor (aldicarb), a chitin biosynthesis inhibitor (lufenuron), a mitochondrial inhibitor (fluazinam), and a tubulin disruptor (carbendazim). This is interesting as the four compounds affect very different physiology. It is unlikely, while not impossible, that the strain has a naturally occurring mechanism that confers resistance across very different mechanisms of action (such as a up-regulation of detoxifying enzymes), as resistance to other pesticides of similar modes of action, at least, would then also be

expected. However, no resistance to the other AChE inhibitor oxamyl or mitochondrial inhibitor diafenthiuron was observed. More likely the four cases of resistance-conferring alleles (or sets of alleles) have arisen separately. It is possible that alleles for resistance to all four compounds arose from naturally occurring mutation events. It may also be the case that before being isolated from compost in an urban garden in the suburb of Paris in 2005 the strain was exposed to the pesticides leading to selection favouring the worms with the resistance-conferring polymorphisms within the natural population. While it is unlikely that all four compounds would have been used in the area, as they are more widely used in agricultural settings, it is worth noting that aldicarb and carbendazim were both introduced as pest control agents in the 1970s and fluazinam and diafenthiuron in the 1990s leaving time for these selection events prior to the isolation of the wild variant. Finally, as the polymorphisms presumably carried little cost in the environment, they were maintained in the population at high proportions until detected in the chemical susceptibility screen.

JU1491 was found to be resistant to a mitochondrial inhibitor (fluazinam) and nAChR agonist (levamisole) and JU1652 to AChE inhibitor (aldicarb) and chitin biosynthesis inhibitor (lufenuron). The explanation of these two cases of naturally occurring resistance to two pesticides is most likely similar to that of JU363. Both variants were isolated from urban compost in France and Uruguay, respectively; therefore, it is possible that prior to their isolation (in 2005 and 2009 respectively) they went through selection that favoured the animals fittest in the compound environment.

3.3.9.4. *Naturally occurring resistance to acetylcholinesterase inhibitors in CB4858*

Resistance to both AChE inhibitors (aldicarb, oxamyl) was identified in CB4858. This implicates a polymorphism in a gene encoding for AChE itself or, another, encoding for an associated protein that is required for the inhibitor activity. In *C. elegans* there are four AChE genes: *ace-1*, *ace-2*, *ace-3*, *ace-4* (Combes *et al.*, 2001, 2003). Using the CeNDR variant browser (Cook *et al.*, 2017) I identified a polymorphism in CB4858 *ace-3* that was not present in N2 – a missense variant resulting in a Pro27Gln substitution and four missense variants in *ace-4* resulting in altered protein coding: Asp19Asn, Pro145Thr, Thr584Ala, and Gln600Lys amino acid substitutions. Neither *ace-1* nor *ace-2* sequences had any polymorphisms identified in CB4858. Reports in the literature link *ace-3* with resistance to AChE inhibitors while *ace-4* appears to be transcribed but not present at detectable levels *in vivo* (Nguyen *et al.*, 1995; Combes *et al.*, 2001). This makes it an unlikely candidate to cause resistance. It is possible that the observed resistance to AChE inhibitors is therefore mediated by the substitution in *ace-3* in the wild isolate CB4858, but contradictory evidence arises from looking at *ace-3* in wild isolates resistant (CB4857, JU363, JU1652) and sensitive to aldicarb (CX11307). While the same polymorphism is present in the other three resistant strains, it is also present in the aldicarb-sensitive CX11307 instead suggesting that another target in the ACh pathway is responsible. No resistance is present to the postsynaptic ACh receptor agonist levamisole in CB4858. Thus it can be suggested that, instead, presynaptic ACh pathway is implicated, leaving numerous potential candidates (Nguyen *et al.*, 1995; Rand, 2007). Follow-up experiments are required to elucidate the mechanism of this naturally occurring resistance.

3.3.9.5. Resistance to two mitochondrial inhibitors in wild isolate JU311

The wild isolate JU311 was highlighted as resistant to both inhibitors of oxidative phosphorylation (diafenthiuron, fluazinam). This resistance was established at a low concentration of 5ppm for both compounds and disappeared at a ten-fold higher concentration of 50ppm (data not shown) where the dose was lethal to all strains in the chemical susceptibility assay. Fluazinam is reported to be an extremely potent uncoupler, effectively disassociating reactions of ATP synthesis from the electron transport chain (Guo *et al.*, 1991). Diafenthiuron is rapidly desulphurated in cells to a pesticidal carbodiimide, which then acts as a potent inhibitor of electron transfer by blocking the coupling site in a time dependent matter. It has been suggested that the carbodiimide, due to structural similarities to another well-known mitochondrial inhibitor dicyclohexylcarbodiimide, inhibits mitochondrial ATPase (Ruder *et al.*, 1991; Zhang *et al.*, 2017). The wild isolate was not resistant to two other mitochondrial inhibitors tested at 5ppm – fluopyram and rotenone. It is known that fluopyram is a succinate dehydrogenase inhibitor (Faske and Hurd, 2015) and rotenone interferes with electron transport chain by inhibiting the transfer of electrons in iron-sulphur centres in Complex I to ubiquinone (Heinz *et al.*, 2017). It is therefore possible, that if the mechanism of resistance to these two mitochondrial inhibitors is mitochondria-specific, that Complex I and II are not implicated. It is more likely that a difference in another component of the mitochondrial energy production system explains the resistance observed and follow up work is required to identify the potential targets and their role in this example of naturally occurring resistance.

3.3.10. Quantifying the differences in chemical susceptibility

The chemical susceptibility screen, results of which are highlighted in Table 3.5, assessed the sensitivity to agrochemicals in a fast, high-throughput, yet qualitative manner. From this I was able to identify many interesting wild isolates, for example the naturally occurring AChE inhibitor resistant CB4858 that is discussed in 3.3.9.4, and look at the responses of the wild isolates to the entire bank of tested agrochemicals as a whole. This highlights the usefulness of this model in understanding the implications of natural variation in wild populations on pesticide susceptibility.

Attempting to identify the genetic basis of AChE inhibitor resistance in CB4858 by using solely online resources (CeNDR) and clues from the literature showed that this approach was much akin to attempting to find a needle in a haystack – in theory possible but time consuming, inconclusive, and not necessarily practical.

Therefore, quantifying the differences in chemical susceptibility in the agrochemicals where significant differences were observed was important both to verify the responses of the screen more quantitatively and to identify robust resistance suitable for a mapping approach to identify its genetic basis.

Out of the eight compounds highlighted as having wild isolates with different susceptibilities, I decided to quantify the range of responses observed for two of the agents that perturb growth and development (carbendazim, lufenuron) and another two that affect muscle and nerve physiology (aldicarb, levamisole). Mitochondrial inhibitors were not chosen as the susceptibility differences were only present at a lower concentration and the phenotype was thus considered less robust.

3.3.10.1. *Quantifying variation in susceptibility to compounds that perturb growth and development*

Food consumption races were used to quantify differences in the responses of wild isolates to agrochemicals perturbing growth and development. This work aimed to quantify the differences seen between wild isolates in the initial 50ppm concentration tested as well as to identify responses to lower and higher compound concentrations.

3.3.10.2. *Variation in susceptibility to carbendazim reveals multiple resistant wild isolates*

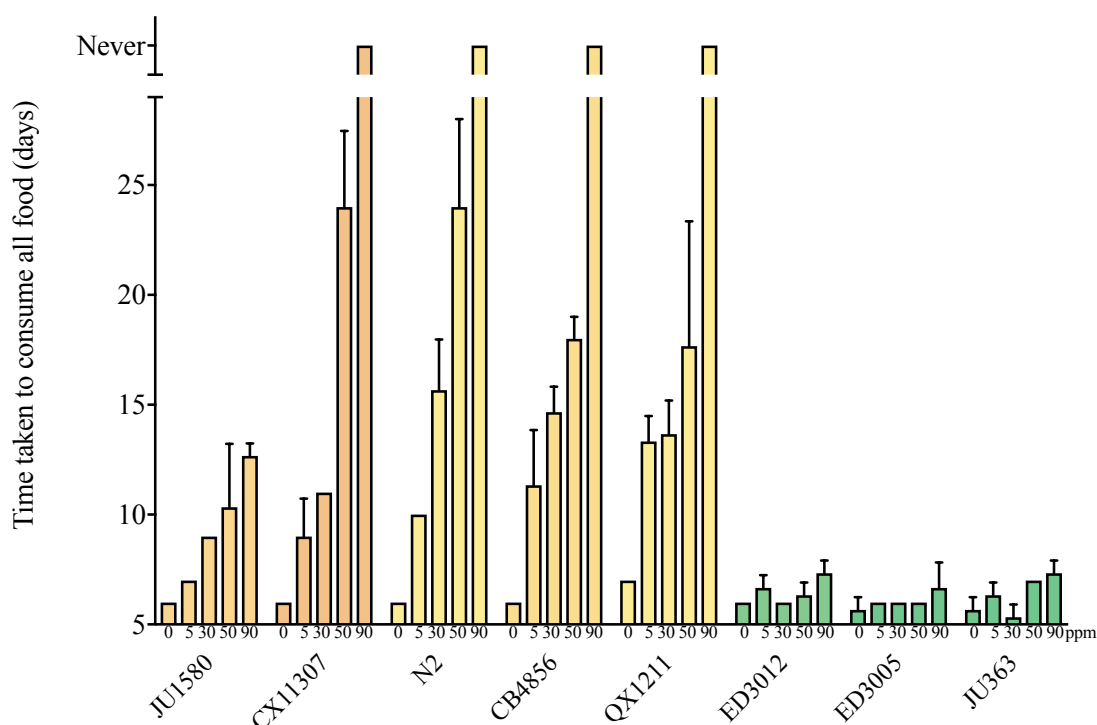


Figure 3.9: Food consumption race of wild isolates JU1580, CX1307, N2, CB4856, QX1211, ED3012, ED3005, JU363, ordered by their respective responses in the chemical susceptibility assay and coloured with their responses in said assay, in 0, 5, 30, 50, and 90ppm carbendazim (n=3). Values are plotted showing the mean $\pm$ SD.

The results of the food consumption race, shown in Figure 3.9, indicated a clear difference at 90ppm between the sensitive strains (CX11307, N2, CB4856, QX1211) and resistant strains (ED3012, ED3005, JU363). Resistant strains were able to finish food in a similar number of days as if no compound was present and sensitive strains did not survive for long enough to consume all food. Across the concentrations used in the assay, the sensitive strains presented a clear dose response curve with the strains requiring longer finishing food in higher concentrations.

I used a permutation test run in Matlab (The MathWorks Inc.) to examine whether statistically significant differences were present when comparing the mean number of days it took to finish food between the N2 strain and the other strains at 50ppm concentration of carbendazim as this was the dose also used in the chemical susceptibility screen. The code used is shown in Appendix I. Non-parametric testing was required in this instance as the very small number of observation ($n = 3$) for each strain did not meet the sample size guidelines for the parametric tests, and I could not make a clear determination that I had normally distributed data. As a non-parametric test, however, permutation testing does not rely upon these assumptions. The p-values for each comparison can be found in Table 3.8. The p-values for comparisons each survived the false-discovery rate (FDR) correction for multiple comparisons (Benjamini and Hochberg, 1995).

Table 3.8: P values for each comparison of food clearing of wild isolates in 50ppm carbendazim performed in permutation test and corrected for multiple comparisons using FDR with all values $p < 0.05$ significant.

Comparison	P value	Sig.
<i>N2 vs CB4856</i>	0.062	ns
<i>N2 vs CX11307</i>	0.557	ns
<i>N2 vs ED3005</i>	0.019	Sig.
<i>N2 vs ED3012</i>	0.0178	Sig.
<i>N2 vs JU1580</i>	0.024	Sig.
<i>N2 vs JU363</i>	0.020	Sig.
<i>N2 vs QX1211</i>	0.157	ns

The results of the food consumption race confirm that wild isolates ED3012, ED3005, and JU363 outperform all sensitive strains in 50ppm, and 90ppm carbendazim. This is in agreement with the results of the chemical susceptibility screen, which identified the strains as resistant to 50ppm carbendazim.

3.3.10.3. Food consumption assay reveals no differences in susceptibility to lufenuron among selected wild isolates

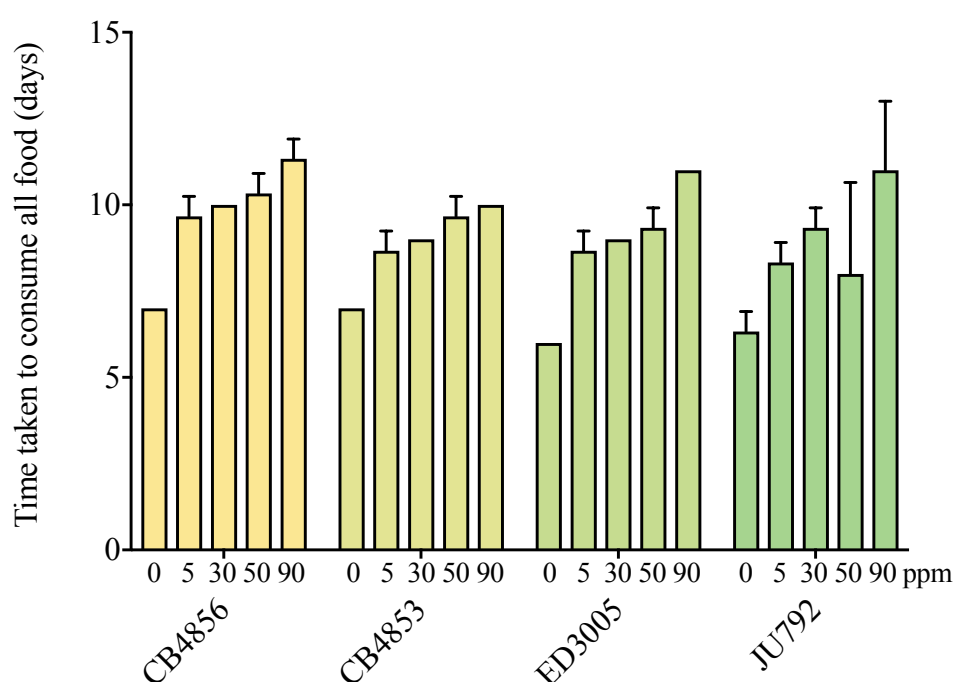


Figure 3.10: Food consumption rate of wild isolates CB4856, CB4853, ED3005, and JU792 in 0, 5, 30, 50, and 90ppm lufenuron (n=3). Values are plotted showing the mean $\pm$ SD and coloured according to the response in the chemical susceptibility screen.

The chemical susceptibility screen identified wild isolates sensitive and resistant to lufenuron. The sensitive strain CB4856 and resistant strain JU792 were assayed together with two intermediate strains CB4853 and ED3005 in order to quantify their responses to lufenuron as shown in Figure 3.10. A dose-response can be identified upon visual inspection but the results of the food consumption race in this

compound did not confirm the presence of naturally lufenuron-resistant or -sensitive wild isolates. These results suggest that differences observed in the chemical susceptibility screen did not confer differences in the food consumption race set-up. The exact cause of this is unidentified, but the uniform food consumption rates indicate that observed lethality in transferred L1s, as well as developmental delay and movement effects did not translate into differences in the time taken to consume all food. This may be explained if the compound had no lethality in the progeny of transferred L1s, meaning that over time the number of worms in the well would have continued to increase until food was consumed and assay completed.

Alternatively, the compound may be broken down over the assay resulting in reduced concentrations over time and therefore explaining differences seen in chemical susceptibility screen, where effect of the compound is noted four days after application, and food consumption race, where the effect is measured over a long time frame (up to 13 days in this case). The half-life of lufenuron is reported to be 9-24 days in aerobic environmental conditions (Sieke, no date), however, suggesting that the concentration would not have dropped sufficiently to support this explanation.

3.3.10.4. *Quantifying susceptibility in variation to compounds affecting muscle and nerve physiology*

A combination of food consumption races and thrashing assays upon chronic exposure to the compound were used to quantify differences in the responses of wild isolates to compounds affecting muscle and nerve physiology.

3.3.10.5. *Results of food consumption assay and thrashing confirm the presence of aldicarb-resistant wild isolates*

Four wild isolates were initially picked to quantify differences seen in responses to 50ppm aldicarb in the chemical susceptibility screen by performing a food consumption race. JU1652 had been identified as a resistant variant in the screen while N2, CB4854, and CB4853 were neither highlighted as sensitive or resistant strains. The results of the food race showed no observable difference between the isolates at concentrations 0, 5, and 30ppm. An obvious difference, as seen in Figure 3.11, arose by the 50ppm concentration where JU1652 was able to finish the food at around 18 days but the other three strains were never able to consume all bacterial food in the well.

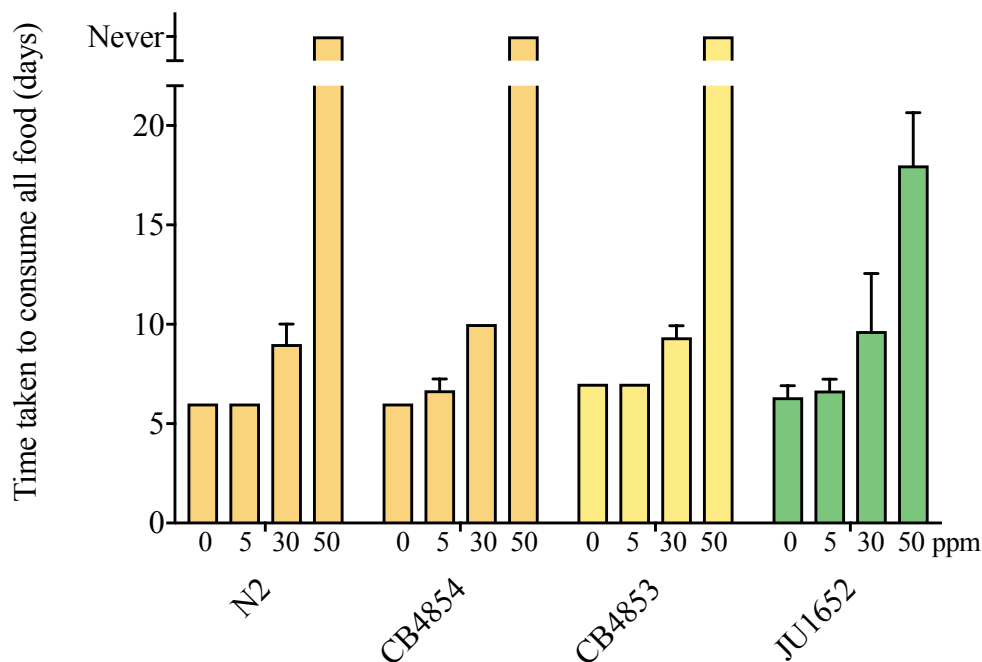


Figure 3.11: Food consumption race of wild isolates N2, CB4854, CB4853, and JU1652 in 0, 5, 30, and 50ppm aldicarb (n=3). Values are plotted showing the mean $\pm$ SD and coloured according to the response in the chemical susceptibility screen.

While the results of the food consumption race showed a difference at 50ppm, the effect disappeared at higher doses and considering the length of the experiment it did not appear to be the most practical approach to quantify the responses of other wild isolates of interest from the chemical susceptibility screen. I decided that exposing the animals to 50ppm aldicarb for four days and then measuring BBPS (thrashing) and body size was an appropriate alternative instead (Nguyen *et al.*, 1995; Rand, 2007).

Data detailing body bends per second and body size for wild isolates after four-day no compound or 50ppm aldicarb exposure were analysed using GraphPad. Multiple t tests (one for every wild isolate) were performed to discover overlapping measurements after the compound and no compound exposure i.e. wild isolates where measurements of BBPS or body size after no compound and 50ppm aldicarb exposure were not significantly different. Results of the statistical analysis are shown in Table 3.9.

This analysis of effect of four-day aldicarb and no compound exposure on BBPS of wild isolates, shown in Figure 3.12, indicated the presence of no aldicarb-resistant wild isolates with the BBPS of all wild isolates significantly different after compound and no compound exposure as shown in Table 3.9. The wild isolates ED3005 and CB4857, with adjusted p values 0.0024 and 0.0011 respectively, were closest to having overlapping (not significantly different) BBPS measurements upon aldicarb and no compound exposure.

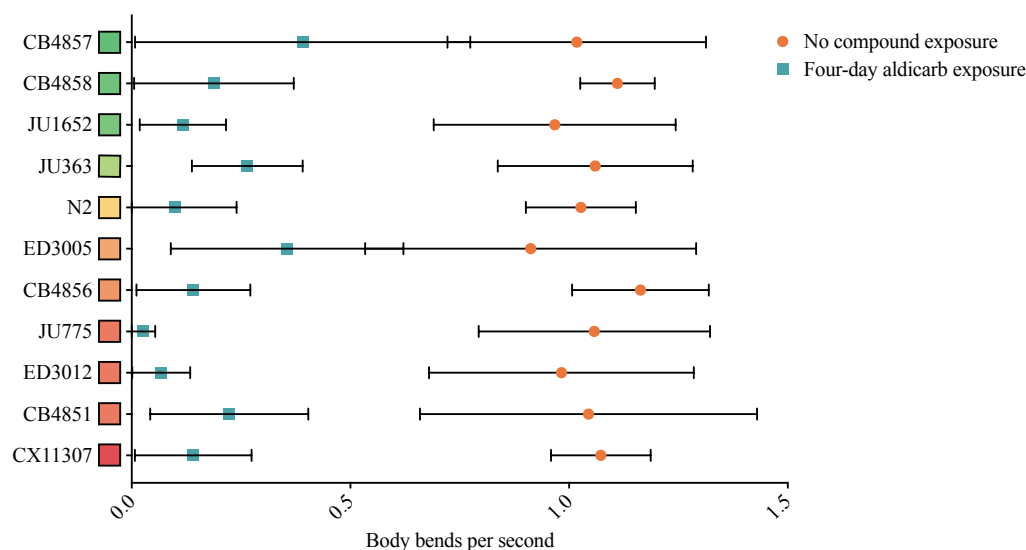


Figure 3.12: Comparison of body bends per minute of wild isolates that were exposed to 50ppm aldicarb and no compound for four days (n=13). The susceptibility of wild isolate to 50ppm aldicarb in the chemical susceptibility screen (Table 3.5) is shown in colour next to isolate, with green indicating resistance, yellow indicating moderate response, and red indicating high sensitivity (explained in Figure 3.3). Values are plotted showing the mean $\pm$ SD.

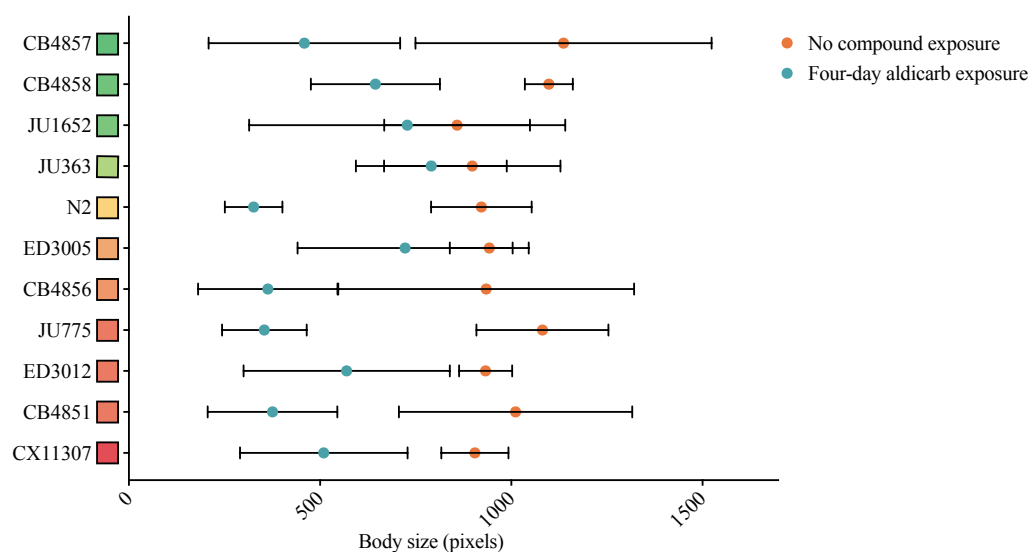


Figure 3.13: Comparison of body size in pixels of wild isolates that were exposed to 50ppm aldicarb and no compound for four days (n=13). The susceptibility of wild isolate to 50ppm aldicarb in the chemical susceptibility screen (Table 3.5) is shown in colour next to isolate, with green indicating resistance, yellow indicating moderate response, and red indicating high sensitivity (explained in Figure 3.3). Values are plotted showing the mean $\pm$ SD.

The same analysis of the body size results, visible in Figure 3.13, showed that the body size of ED3005, JU1652, and JU363 was not significantly different upon four-day exposure to no compound and 50ppm aldicarb. The results of BBPS and body size together suggest a form of aldicarb resistance in these three wild isolates where the strains do not escape the paralysing effect of the compound, as suggested by BBPS results, but the body size of the isolates is not affected by hyper-constriction of body wall muscles as usual, as suggested by body size results.

The wild isolate JU1652 is of particular interest as it showed, firstly, the ability to finish food in 50ppm aldicarb albeit taking just under 20 days to do so (Figure 3.11), secondly, the paralysing effect of 50ppm aldicarb based on BBPS measurements (Figure 3.12), thirdly, no hyperconstriction upon aldicarb exposure based on body size results (Figure 3.13). These results suggest that JU1652 is resistant to aldicarb by a mechanism that does not overcome the paralysing effect of the compound but does perturb usual hyper-constriction seen in the compound.

Table 3.9: Results of multiple t tests, one on each wild isolate, performed to compare results of BBPS and body size, separately, after exposure to no compound and aldicarb for 4 days. Bonferroni-Dunn method with $\alpha=0.05$ was used to correct for multiple comparisons.

Strain	BBPS		Body size	
	Significant	Adjusted P value	Significant	Adjusted P value
CB4851	Yes	<0.0001	Yes	<0.0001
CB4856	Yes	<0.0001	Yes	0.0007
CB4857	Yes	0.0011	Yes	0.0002
CB4858	Yes	<0.0001	Yes	<0.0001
CX11307	Yes	<0.0001	Yes	<0.0001
ED3005	Yes	0.0024	No	0.1552
ED3012	Yes	<0.0001	Yes	0.001
JU363	Yes	<0.0001	No	>0.9999
JU775	Yes	<0.0001	Yes	<0.0001
JU1652	Yes	<0.0001	No	>0.9999
N2	Yes	<0.0001	Yes	<0.0001

3.3.10.6. *Quantification of levamisole-susceptibility indicates levels of variation not identified by a food consumption race*

Four strains were picked for a food consumption race in 0, 5, 30, 50, and 90ppm levamisole. CB4853 and JU1491 were identified as levamisole resistant in the initial chemical susceptibility screen, while ED3012 represented a not significantly resistant or sensitive response in a wild isolate, and CX11307 was identified as a levamisole-sensitive wild isolate.

The food consumption race, Figure 3.14, performed showed contradictory results to those of the screen as at the highest concentration, 90ppm, tested, the two resistant strains were unable to finish the food while the more sensitive strains, CX11307 and ED3012, fared better. Additionally, no differences were observed at the 50ppm compound concentration.

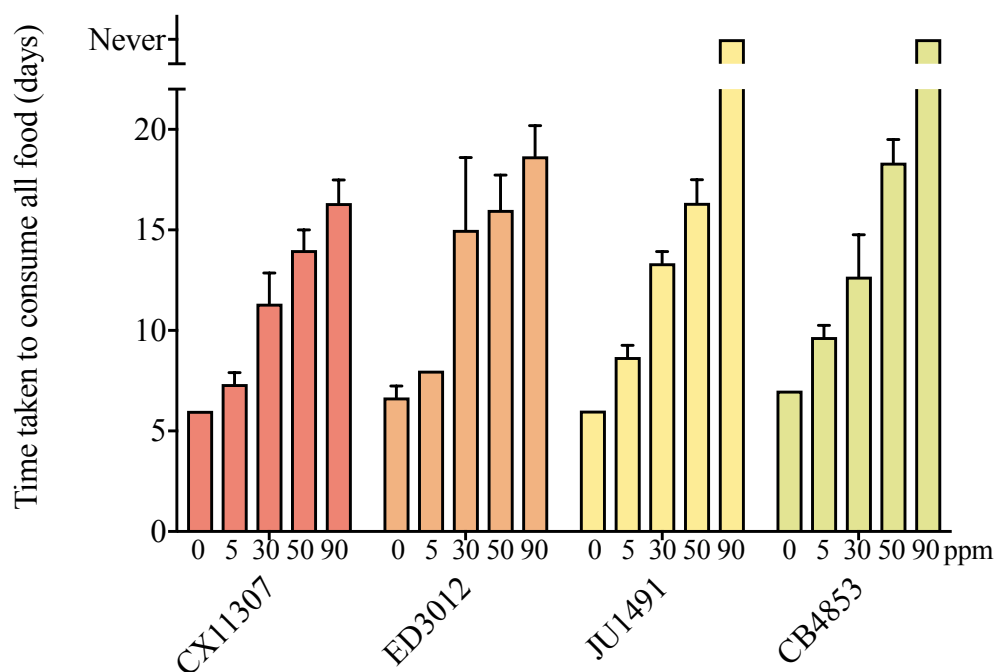


Figure 3.14: Food consumption race of wild isolates CX11307, ED3012, JU1491, and CB4853 in 0, 5, 30, 50, and 90ppm levamisole (n=3). Values are plotted showing the mean $\pm$ SD and coloured according to the response in the chemical susceptibility screen.

The reason for these contradictory results may have been the difference in length of chronic exposure to the compound. Worms were exposed for only 4 days in the screen and over 20 days in the food race. Levamisole is rapidly absorbed in the gastrointestinal tract upon which it is broken down in a matter of hours (Lewis *et al.*, 1980). Therefore, a mechanism of resistance that would slow down the adsorption or speed up the metabolism of the anthelmintic would carry an advantage in the transferred L1s in this environment. Pictures taken on day four of the chemical susceptibility screen, shown in Figure 3.15, illustrate that CB4853 was considerably healthier with adults, eggs, and young adults present upon exposure to 50ppm levamisole. Therefore it is possible that the strains JU1491 and CB4853 are resistant to four-day chronic exposure to levamisole but not to exposures longer than that.

3.3.10.7. *Summary of quantification of natural variation in chemical susceptibility*

Overall I was able to identify quantifiable differences to carbendazim and aldicarb confirming the presence of naturally occurring resistant isolates for these two agrochemicals. I was not able to confirm differences in responses to lufenuron or levamisole with the adopted approaches. As the food race was an indirect measure of fecundity, movement, development, and pharyngeal pumping over a relatively long time period the stand-alone measurements of each of those parameters may have revealed clearer measurements of the amount of variability naturally present in susceptibility to these compounds.

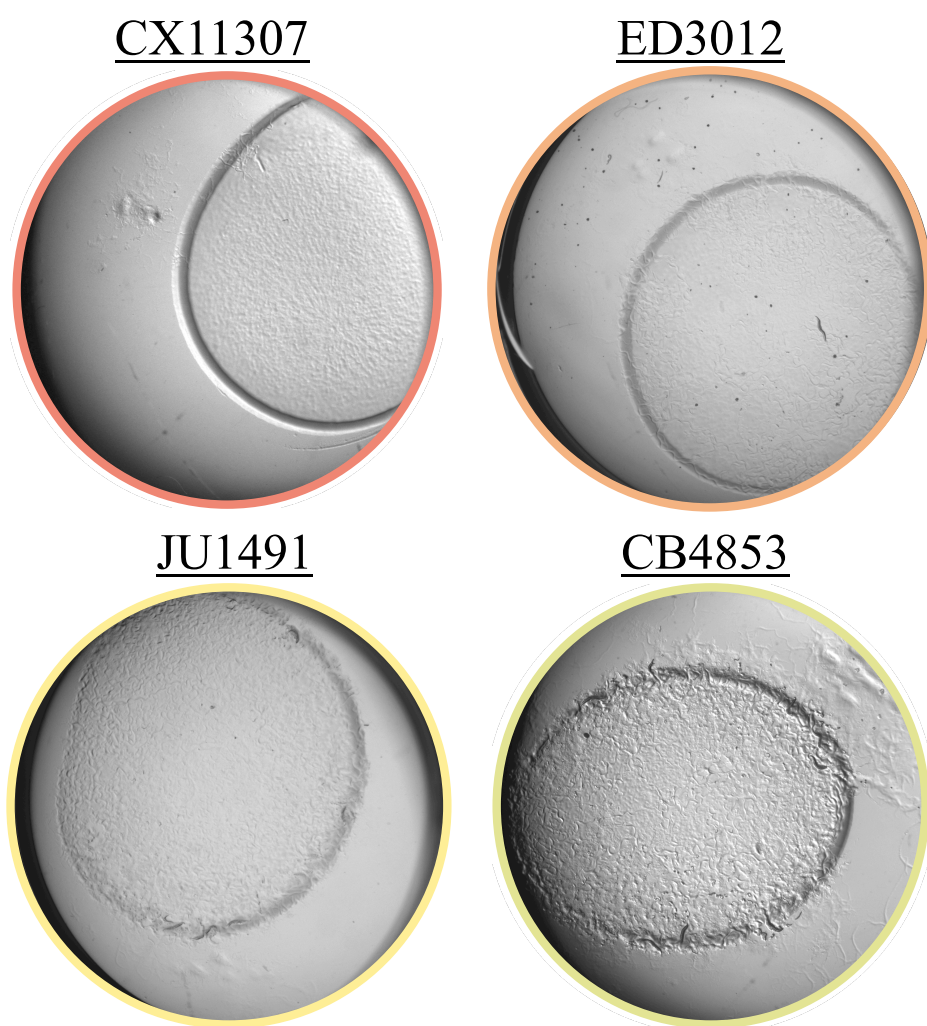


Figure 3.15: Pictures of wells in chemical susceptibility screen of CX11307, ED3012, JU1491, CB4853 after four-day exposure to 50ppm levamisole. Colours correspond to average responses recorded in the chemical susceptibility screen Table 3.5 and are based on the categories outlined in Figure 3.3.

3.4. Discussion and future work

3.4.1. Insights from utilising *C. elegans* wild isolates in a chemical susceptibility screen

The chemical susceptibility screen, shown in Table 3.5, enabled us to observe the responses of a genetically diverse panel of *C. elegans* wild isolates to a range of agrochemicals. While no particular *C. elegans* wild variant was identified as overall more sensitive or resistant to the agrochemicals as per the clustering of the findings of the chemical susceptibility screen, the results indicated that within this panel of *C. elegans* a considerable level of variation in chemical susceptibility responses is present with 8 out of 28 compound environments indicating significant differences between responses of populations. This highlights pesticides and modes of action that may be predisposed to see faster emergence of resistance when introduced to control pest populations in the field due to the spread of naturally occurring resistance-causing polymorphisms. At the same time 20 out of 28 agrochemicals showed a uniform approach across the wild isolates, highlighting pesticides that should have more robust responses and align with slower emergence of resistance in the wild. However, this is a first attempt to experimentally model the emergence of resistance from standing natural variation with reports of resistance existing for all of the pesticides used as indicated by Table 3.10. Further insight to using the method in order to predict emergence of resistance from naturally occurring mutations would be gained by additional testing of novel agrochemicals using this model of natural variation.

3.4.2. Effect of a resistance-allele in no compound is of high importance in the preservation of the allele in a population

It is worth noting that numerous pesticides tested have been in use for decades and for reasonable lengths of time prior to the isolation of the wild isolates. If we suppose that there is a chance the resistance observed in the cases of aldicarb and carbendazim is due to some level of prior selection, we should also assume that at least some exposure to any of the pesticides must have taken place before the sampling of the isolates. Yet there are a number of pesticides that have been used for decades with uniform pesticide susceptibility observed in the panel of wild isolates. Therefore it appears that these are the chemicals that are targeting processes with minimal naturally occurring variation for selection to act upon. In itself this is a strong finding as it indicates that genomic locations in which naturally occurring polymorphisms are least likely to arise and prevail, due to lack of any deleterious effect, and confer resistance are good targets for agrochemical development. As naturally occurring resistance is also often associated with low cost that can have devastating effects in the wild (Ffrench-Constant, 2006) it would be logical, where possible, to take this rule into account. Based on this, my findings would suggest that agrochemicals affecting respiration may be less likely, while tubulin disruptors are more likely, to see rapid emergence of resistance in field as a result of resistance alleles that have prevailed in the gene pool of the pest population.

3.4.3. General physiological mode of action is a poor indicator to predict variation in chemical susceptibility

In general, compounds used in the susceptibility screen have physiological effects that can be divided into three. There are compounds that:

1. affect muscle and nerve by altering normal neurotransmitter activity in neurons;
2. perturb growth and development by inhibiting synthesis of important cell building elements;
3. alter respiration.

I observed variation in the responses of the *C. elegans* wild isolates and the presence of sensitive and resistant variants to compounds in all three classes. Similarly, compounds with uniform responses fell into all three categories. Resistance was observed at a concentration of 50ppm to compounds affecting muscle and nerve physiology and perturb growth and development. Concentrations of 5ppm were used to identify resistant and sensitive isolates to mitochondrial inhibitors that alter respiration.

It is reasonable to expect that the degree of variation observed in wild populations of *C. elegans* natural isolates would at least to an extent reflect the levels of variation in wild populations of relevant pest species across the globe. As such it appears that no particular physiological target as a whole is a particularly advantageous agrochemical development opportunity by having the lowest frequency of naturally occurring resistance-conferring alleles and therefore would have the greatest chance of slow emergence of resistance. The only advantage is detected by mitochondrial inhibitors as naturally-occurring resistance was established at a ten-fold lower concentration suggesting that at higher concentrations selection pressures are sufficient to avoid the survival of any animals even if some of the strains possess polymorphisms that confer a fitness benefit at a lower concentration. The mitochondrial inhibitors used in the screen belong to the high class of toxicological concern according to Pesticides and BioPesticides Properties Database (University of

Hertfordshire, 2019), meaning that this potential advantage is coupled with increasing human safety concerns.

The results of natural variation in chemical susceptibility can also be compared to the emergence of resistance reported by the Arthropod Pesticide Resistance Database (APRD) and Fungicide Resistance Action Committee and this data is outlined in Table 3.10. These reports suggest widespread pesticide resistance in terms of cumulative number of resistance cases recorded but also the number of resistant species present for most of the agrochemicals included. These findings suggest that resistance can emerge in response to treatment with any agrochemicals, regardless of their particular MOA. In conclusion this means that the general physiological effect of a pesticide on its own is a poor indicator of the likely abundance of resistance-causing natural variation in field populations, as supported by reports of resistance to pesticides.

Table 3.10: Current resistance data available on agrochemicals used in the chemical susceptibility screen. ‘Number of total cases’ represents the number of unique cases and ‘Number of species’ the total number of unique species resistant as reported by APRD (Mota-Sanchez and Wise, 2019) as of 17.09.19. or by FRAC (FRAC, 2018). NA is used to mark compounds for which no data was available in either of the collated resistance databases. Monepantel is marked with an asterisk (*) as databases do not report resistance, but literature on resistant pest populations exists (Bartley *et al.*, 2015).

Mode of Action	Compound	Number of total cases	Number of species
Acetyl CoA carboxylase inhibitor	Spirotetramat	16	7
Acetylcholinesterase inhibitor	Aldicarb	5	5
	Oxamyl	11	6
	Pirimicarb	52	6
Chitin biosynthesis inhibitor	Diflubenzuron	15	7
	Flufenoxuron	10	2
	Lufenuron	33	7
GABA receptor antagonist	Dieldrin	221	105
Glutamate-gated chloride channel	Abamectin	279	22
Juvenile hormone mimic	Fenoxycarb	1	1
Mitochondrial inhibitor	Diafenthiuron	6	2
	Fluazinam	1	1
	Fluopyram	32	22
	Rotenone	4	2
Nicotinic acetylcholine receptor agonist	Cyanotropane	NA	NA
	Levamisole	NA	NA
	Spinosad	278	30
Nicotinic acetylcholine receptor blocker	Cartap	16	4
	Monepantel	NA*	NA*
Octopamine receptor	Amitraz	103	7
Ryanodine receptor	Ryanodine	124	15
Tubulin disruptor	Carbendazim	141	131
Unknown	Cyprodinil	12	5
Vesicular acetylcholine transporter	Spiroindoline	NA	NA
Voltage-gated sodium channel	DDT	986	262
	Fenvalerate	172	37
	Indoxacarb	220	15
	Metaflumisole	12	5

3.4.4. Recently introduced pesticides suggest limited natural variation in susceptibility of *C. elegans* despite widespread reports of resistance

The agrochemicals used in the chemical susceptibility screen, as well as encompassing a range of general physiological modes of action, ranged from the very first synthetic pesticides introduced in agriculture, such as DDT, to seven compounds that were only discovered in 2000 or later as shown in Table 3.4. This latter list includes indoxacarb introduced in 2000, cyanotropane discovered in 2002, fluopyram, metaflumisolet, monepantel, and spirotetramat all introduced in 2008, and spiromodoline discovered in 2012. It is encouraging, for those concerned about the prevalence of natural resistance-causing polymorphisms, to note that in this *C. elegans* model of wild populations all of the compounds that were introduced later were highly effective and exhibited a uniform response across the wild isolates panel at the dose utilised, which should have enabled the observation of significant variation in chemical susceptibility. In the case of metaflumisolet a single variant was identified as more sensitive than the rest of the strains meaning that naturally occurring variation in susceptibility to the compound is possible.

Out of the seven recently introduced agrochemicals used in the screen five did not have novel modes of action. This means they share the general mechanism of action with other already established pesticides. However, even in instances where resistant wild isolates were identified for the other compounds working by the same operating principle, the more recently introduced pesticide showed no signs of resistance in *C. elegans* wild isolates. An example of this was observed with cyanotropane and levamisole in the chemical susceptibility screen. This indicates that the differing precise mechanism within the established MOA is sufficient for new compounds to remain effective in the field. In the instance of cyanotropane and

levamisole this means that while both confer a pesticidal effect by being agonists of the nicotinic ACh receptor, the specific mechanisms differ sufficiently for cross-resistance to not be an issue. This highlights the different likelihood of naturally occurring balanced polymorphisms to occur within a particular target mechanism with viable resistant variants present for one and not the other.

Ultimately it means that the general physiological target as a whole is not a good predictor of naturally occurring resistance. Interesting insights may be gained from the analysis of the specific molecular targets of pesticides, some of which may be more likely to collect mutations not affecting the functioning of the animal in a natural no compound environment but providing them with an advantage in the presence of a chemical.

Out of the seven recently introduced compounds used two have novel modes of action. Both spirotetramat and spiroidoline were highly effective with no significant variation observed when comparing the susceptibility of wild isolates. With the exception of cyanotropane, and spiroidoline, reports of resistance exist for all more recently introduced compounds as shown in Table 3.10.

Overall, the results of the chemical susceptibility screen infer that more recently introduced compounds, both those of novel and already established MOA, have no naturally occurring resistance to report in this model of natural variation. This suggests that in agrochemical development targets with low likelihood of naturally occurring and resistance conferring polymorphisms have been chosen.

3.4.5. *C. elegans* wild isolates exhibit uniform sensitivity to bio-pesticides

Responses of *C. elegans* wild isolates were assessed to four bio-pesticides: abamectin, rotenone, ryanodine, spinosad. No naturally occurring resistance to

chronic exposure of these pesticides was found despite the possibility that these compounds may be present in the same locations as *C. elegans* naturally grows.

Avermectins, including abamectin, are the active compounds produced by soil bacterium *Streptomyces avermitilis* and were identified during a screen for natural products with anthelmintic properties (Lasota and Dybas, 1990). Modest resistance to avermectin was previously reported in *C. elegans* wild isolate CB4856 by Ghosh *et al.*, 2012 who also suggested that resistance to avermectins among nematodes may be widespread. However, they only tested the effect of acute (up to 1 hour) exposure to 0.5 µg/ml avermectin (Ghosh *et al.*, 2012), rather than the effect of chronic exposure over a number of days. Our findings suggest that this may not be true as no variation in susceptibilities of wild isolates was observed in the chemical susceptibility screen suggesting that no polymorphisms to confer resistance to chronic avermectin exposure had arisen and spread through the population due to natural exposure resulting in a resistant population. Our results indicate that the natural availability of avermectin has not affected the compounds efficacy in *C. elegans*, a species that in theory inhabits the same environment as *S. avermitilis*, suggesting that low levels of natural resistance affect the efficacy and longevity of the compound in the field. However, close to 280 reports of resistance exist to abamectin with resistance reported in over 20 strains (Table 3.10) suggesting that low levels of natural resistance in the panel of *C. elegans* wild isolates has not meant that, firstly, similarly low levels of natural variation resulting in resistance exist in pests, and, secondly, that resistance has not emerged numerous times in the wild upon the use of abamectin.

Rotenone is a pesticide and a piscicide, but is now withdrawn from some markets due to safety concerns (OJEU, 2008). Naturally available in the roots and stems of *Lonchocarpus* and *Derris* species the compound acts as a strong inhibitor of complex I of the mitochondrial respiratory chain (Heinz *et al.*, 2017). The compound

has been used to control pest populations, such as the Colorado potato beetle, since the 1920s ('Rotenone as an Insecticide', 1933; Brevik *et al.*, 2018). Apart from wider toxicity concerns, rotenone appears to be highly effective, as indicated by avoiding resistance emergence for over 100 generations in the Colorado potato beetle (Brevik *et al.*, 2018). This indicates that any polymorphisms in the target are very unlikely to produce viable biology aligning with our observation of no significant variation in the rotenone susceptibility of *C. elegans* wild isolates.

Ryanodine, a selective insecticide with high mammalian toxicity, has been used since the 1940s and was initially isolated from *Ryania speciosa* (Usherwood and Vais, 1995). While reports of resistance to ryanodine receptor modulators are common, especially in one of the target species *Plutella xylostella* (Sindhu *et al.*, 2017), many related products with lower toxicity profiles have been developed (Usherwood and Vais, 1995). I observed no resistance-conferring natural variation in the panel of *C. elegans* isolates. It has been suggested that resistance has emerged due to insecticide misuse and intensive use as well as particular biological properties of the species including rapid life cycle, high fecundity, and genetic plasticity (Sindhu *et al.*, 2017) therefore indicating that *P. xylostella* is a highly effective pest species, rather than highlighting ryanodine receptor modulators as poor insecticides due to widespread resistance alleles.

Spinosad is an insecticidal compound made by soil fungus *Saccharopolyspora spinosa* which is a nicotinic ACh receptor agonist (Mayes *et al.*, 2003). The compound has been identified as one to see rapid emergence of resistance in the field with a mean years until resistance value of 8.0 (Brevik *et al.*, 2018) and close to 280 reports of resistance logged on the APRD in 2019, with 230 of these specified as field-reported resistance (Mota-Sanchez and Wise, 2019), indicate that resistance is widespread and that many different viable variants exist. Our results show that in *C.*

elegans wild isolates no resistance due to naturally occurring variation is present suggesting that selective pressure is required for resistance-conferring mutations to spread through population as supported by some fitness studies of spinosad-resistant pests (Li *et al.*, 2017).

Overall, across the small sample of bio-pesticides assayed, the theoretical bioavailability of the pesticidal compounds has not resulted in resistant populations of *C. elegans* wild populations. Therefore, based on our model of wild populations using the *C. elegans* wild isolates, pesticides derived from natural sources may represent a group of agrochemicals that target responses where polymorphisms conferring resistance are less likely to arise and spread through populations. The reports of resistance from APRD and FRAC, however, suggest that resistance is also widespread for bio-pesticides, as can be seen in Table 3.10. Considering that resistance management strategies of bio-pesticides can be more limited, for example a number of compounds cannot be used in mixtures due to instability (Siegwart *et al.*, 2015), the very low value of initial frequency of resistance alleles in this model suggests high importance of bio-pesticides in the future of pest control, particularly as the selection of these compounds available diversifies.

3.4.6. *C. elegans* wild isolates as an experimental model to assess the prevalence of natural variation prior to field studies

Overall this chapter provides an example of an experimental method to assess the instance of resistant alleles in wild populations by using a genetically diverse panel of *C. elegans* wild isolates as a model.

While *C. elegans* itself is not a pest species it is used in toxicology studies and identification of new active compounds due to conservation of the target sites of

pesticides used (Leung *et al.*, 2008). As was showed in this chapter, a wide variety of mechanisms of action were active in this species and it was possible to identify the presence of resistance-conferring resistance by a qualitative chemical susceptibility screen. Upon identification of significant resistance levels these can be experimentally assayed with ease outlining the major benefit of establishing the initial prevalence of resistance-conferring polymorphisms using *C. elegans* (Simpkin and Coles, 1981) in comparison to many of the pest species. This model could therefore assist in establishing the occurrence of polymorphisms including ones that confer resistance to currently used or new pesticides in an effort to identify targets associated with a lower likelihood of resistance emergence from standing natural variation. The measure would enable the establishment of the incidence of resistance conferring mutations, which is an important parameter in understanding and managing pesticide resistance. Naturally occurring resistance conferring alleles are less likely to carry a high fitness cost and would thus be maintained in populations upon the loss of the selection pressure of the compound (Ffrench-Constant, 2006; Bourguet *et al.*, 2010; van Westen, Bender and Overington, 2014). Our findings indicate that natural variation in chemical susceptibility is widespread, suggesting that low cost resistance may be more prevalent than previously assumed if our model is a good predictor of the occurrence of resistance-conferring natural variation.

While *C. elegans* is well suited to study the previously under-characterised parameters of pesticide resistance, such as natural prevalence of resistance alleles, the work done with this model organism should be supplemented with field studies utilising pests.

3.4.7. Future work

3.4.7.1. *An increased number of wild isolates would enable the sampling of a larger pool of genetic variation*

One of the caveats of the chemical susceptibility screen was the relatively small number of *C. elegans* wild isolates used, limiting the number of polymorphic backgrounds screened for increased or decreased chemical susceptibility. In our screen I used 25 wild isolates sampled from geographical locations across the world and corresponding with the natural genetic diversity as currently known. However, I was limited in the choice by the lack of diverse locations where *C. elegans* has been isolates from as well as the United Nations Convention on Biological Diversity, which our industrial collaborator Syngenta has agreed to follow.

The current sampling of *C. elegans* has primarily been performed within Western Europe due to the location of the majority of the worm researchers performing the work. As a result there are a number of isolates from France, Portugal, and the United Kingdom. In addition, recent work has seen increase in the number of isolates from the United States of America, in particular from Hawaii due to additional sampling performed in 2017. The selective sampling of *C. elegans* posed a limitation to the number of wild isolates available for use at the start of the project in 2015.

Reports in the past have suggested that genetic variation found within samples from one continent compares favourably and is not far below the diversity across the whole dataset of wild isolates (Haber *et al.*, 2005). However, an increase in the number of isolates utilised would benefit the use of *C. elegans* wild isolates as a model for natural variation as it would increase the amount of variation in terms of unique polymorphisms as well as combinations of polymorphisms included in the

screen. Including more variants from a diverse set of locations, that were not available for this work, would therefore improve the amount of natural variation included in any chemical susceptibility screen (Barriere and Felix, 2005).

3.4.7.2. *Future improvements of the chemical susceptibility screen*

The chemical susceptibility screen only included qualitative assessment of the susceptibility of wild isolates to diverse agrochemicals based on a visual assessment of the worms in the well after a certain length of exposure to the compound. The power of the screen could have been improved by utilising simple quantitative measurements, such as the read-out of a food consumption assay or thrashing, instead of a qualitative susceptibility recording. As suggested by the results using levamisole, the visual assessment used in the chemical susceptibility screen enabled the identification of increased survival after short exposures where the food consumption race indicated no difference. Therefore, utilising a qualitative read-out enabled high degree of coverage of various phenotypes caused by agrochemical exposure such as lethality, developmental delay, movement defects, and fecundity changes. Thus the aim of utilising a simple and high throughput method with limited available technology was achieved.

3.4.7.3. *Screening the susceptibility of compounds could aid in mitigating resistance in the future*

In the chemical susceptibility screen 28 agrochemicals with a range of modes of action were included. This enabled the establishment of *C. elegans* wild isolates as a model for quantifying the prevalence of natural variation that alters chemical susceptibility.

In the future, this model system could be utilised to identify the presence of naturally occurring resistance alleles to novel agrochemicals before their introduction in the field for pest control. This would aid in the development and use of appropriate resistance management strategies from the onset of agrochemical introduction, ultimately aiding in the mitigation of resistance in the wild and ensuring that the pesticides remain effective for longer periods of time.

3.4.7.4. *Identifying the molecular basis of resistance*

Identification of wild isolates resistant to widely used agrochemicals such as carbendazim and aldicarb provides an excellent platform to identify the genetic basis of naturally occurring resistance. Therefore, going forward it would be interesting to identify the molecular basis of the resistance observed to these two agrochemicals.

C. elegans is also known for the ease of utilising it in forward genetic screens that rely on mutations to identify and characterise genes involved in a particular biological process (Kutscher and Shaham, 2014). This means that, alternatively, mutants introduced by an artificial increase in the rate of mutation could be utilised to identify mechanisms of resistance to agrochemicals by screening for animals with decreased susceptibility to the compounds of interest that they were previously sensitive to. These forward genetic screens have proven true to field resistance, for example in the case of *pod-2* being implicated in spirotetramat resistance in a *C. elegans* mutant prior to the identification of a homologous gene as resistance-conferring in the wild in whiteflies as mentioned previously (personal communications Anthony Flemming).

It could be argued that by artificially inflating the rate of mutations, the less frequent and more common mechanisms of resistance can both be identified, while the study of wild isolates enables the identification of resistance alleles that are more

likely to occur naturally and remain in populations in the absence of the selection pressure of a compound. Ultimately, as resistance is predicted to arise to any treatment utilised, the ability to exploit one organism to study a range of resistance conferring alleles can only strengthen the understanding of emergence of resistance and increase our capabilities to mitigate resistance. Indeed, our findings of molecular basis of carbendazim resistance in both *C. elegans* wild isolates and a mutant identified in a chemical mutagenesis screen will be discussed in Chapter 5.

3.4.7.5. Incidence of resistance alleles and resistance emergence

Mathematical modelling has indicated that the higher the incidence of resistance alleles the quicker resistance will emerge. Accurate estimates of frequency of resistance alleles in the field are rarely used due to lack of relevant data (Bourguet *et al.*, 2010). This exemplifies the importance of synergy between mathematical modelling and experimental studies in understanding the multivariate problem of emergence of pesticide resistance. Computational models enable the study of any and all parameters affecting resistance emergence in the field and should thus be utilised to their fullest extent, while also utilising experimental findings in constructing field-realistic assumptions in the models. Reports on current modelling work suggests that the full power of the method is not utilised due to establishing assumptions on limited experimental proof and not studying a wide range of parameters affecting resistance emergence in the wild. *C. elegans* can be manipulated with ease in studies of naturally occurring resistance, in forward genetics to identify further mechanisms of resistance, as well as used in experimental evolution work to study the emergence of resistance in real time. Chapter 4 will outline an experimental example of using *C. elegans* in studying the evolution of resistance.

Chapter 4: Experimental Evolution of Agrochemical

Resistance and Associated Fitness Costs

4.1. Introduction

4.1.1. Investigating the dynamics of resistance emergence using experimental evolution

Experimental evolution, a controlled lab study where the evolutionary change under implemented conditions is studied, is a powerful tool to study evolutionary processes (Gray and Cutter, 2014). While E. Maupas used *C. elegans* in an early evolution experiment to test whether self-fertilisation would result in population extinction (Maupas, 1900), the model organisms that were first widely used in experimental evolution work included fruit flies, mice, yeast, bacteria (Rose and Lauder, 1996; Bell, 2007; Garland and Rose, 2009; Kassen, 2014). It took nearly 90 years for *C. elegans* to re-emerge in experimental evolution research (Teotonio *et al.*, 2017). Recently, experimental evolution in *C. elegans* has been used as a means for gene discovery (Weber *et al.*, 2012; Long *et al.*, 2015), and to study mutation and recombination rates with further examples available in Teotonio *et al.*, 2017. The capability of the method to study the effects of selection thus lends itself to investigate evolution of pesticide resistance in real time rather than having to rely on the analysis of populations that have already acquired resistance under the selection of a combination of unknown parameters. Indeed, rapid adaptation to levamisole has already been observed using *C. elegans* (Lopes *et al.*, 2008).

4.1.2. Fitness cost of resistance

The fitness cost of resistance is an important parameter in the evolution of resistance to pesticides and of great importance in agrochemical research and development (Bourguet *et al.*, 2010). It can be investigated by observing the performance of the evolved line in an environment lacking the selection pressures that drove the evolution (Lopes *et al.*, 2008). A fitness cost associated with resistance to a particular pesticide is the primary selective force that directly counters the selection pressure of the pesticide use (Lenormand *et al.*, 1998). Importantly, this phenomenon underlies many resistance management strategies (Bourguet *et al.*, 2010) where the cost of resistance is assumed to be high and would thus result in reduction of the proportion of resistant animals upon disappearance of the selective force (Ffrench-Constant, 2006).

4.1.3. Understanding the relationship between chemistry and emergence of resistance

Since the understanding in the latter part of the previous century that pesticide resistance was a danger to global food security, various measures have been put in place to limit the emergence of new resistance. The resistance management strategies have over years been optimised to outline the best possible course of action to avoid the emergence of resistance while the agricultural use of currently available pesticides continues (Denholm and Rowland, 1992; Onstad, 2014; Sparks and Nauen, 2015).

Less work has gone into picking apart the relationship between chemistry and emergence of resistance (Brevik *et al.*, 2018) and letting these discoveries prioritise the development of new active compounds. For example, building on the work done in medicinal chemistry, a recent study outlined the potential of proteochemometric

modelling. This computational method that utilises properties of both ligand and target space to make predictions about binding for unknown ligands and protein targets, can be used to predict and quantify target site resistance to pesticides (van Westen, Bender and Overington, 2014).

What is also lacking is the comparison of resistance emergence for different agrochemicals to obtain a value of pesticide durability – the time taken from the introduction of a pesticide until resistance arises in pest control. A study published in 2018 utilised the records of the APRD to identify differences in rates of resistance emergence in pest species and to identify variation in pesticide durability based on field reports. While indications of different rates of evolution emergence were noted in both cases, the approach could be improved by observing resistance evolution in the same species across different mechanisms of action as current data encompasses a patchwork of species and compounds depending on what was available in the database (Brevik *et al.*, 2018). A suitable model to experimentally complete this work without waiting for resistance to emerge in the field in one particular pest species would be *C. elegans* (Gray and Cutter, 2014; Teotonio *et al.*, 2017).

4.1.4. Experimental evolution with *C. elegans*

C. elegans has been used as a model organism in a variety of research areas since the pioneering work by Brenner, but, despite the very early experimental evolution work by Emil Maupas using *C. elegans* (Maupas, 1900), the organism was widely unused in the area until the 1990s. Due to the obvious benefits of this model organism in experimental evolution of short life spans and cycles, the possibility to freeze generations, high fecundity, and the ease of handling large populations, the number of evolutionary biologists utilising *C. elegans* to answer a multitude of

evolutionary questions has grown considerably in recent years (Goussen *et al.*, 2013; Teotonio *et al.*, 2017).

**4.1.4.1. *The highly heterogeneous Portuguese strain, EEV-A₀,
particularly well-suited to study adaptation***

C. elegans is an androdioecious animal and primarily reproduces by self-fertilisation, although males are maintained in populations at low proportions. This limits the adaptation of the species in novel environments as effective segregation and recombination are reduced as selfing effectively decreases heterozygosity and increases linkage disequilibrium between polymorphisms (Teotonio *et al.*, 2012). This makes the standard lab adapted *C. elegans* a poor model to study evolutionary dynamics of pesticide resistance.

It is known that in novel environments outcrossing can facilitate adaptation in the presence of standing genetic variation, with multiple loci that are not fixed within a population. In order to study the evolution of outcrossing in experimental populations, Teotonio *et al.* (2012) created a dioecious *C. elegans* line EEV-A₀ by funnel cross of genetically diverse *C. elegans* wild isolates and by introgressing a sex determination mutant allele, *fog-2(q71)*, transforming hermaphrodites into females (Teotonio *et al.*, 2012). Figure 4.1 shows the wild isolates utilised to create the highly polymorphic strain EEV-A₀ (Portuguese strain). The funnel cross strategy was based on obtaining two-isolate hybrids by crossing the wild isolates in a pair-wise fashion, then repeating the crosses to obtain four-isolate hybrids, eight-isolate hybrids, and finally sixteen-isolate hybrids. The population was maintained for 140 generations without observing significant loss of genetic diversity (Goussen *et al.*, 2013).

The generation of genetic diversity of the Portuguese strain EEV-A₀ was found to be positively correlated with recombination rates, with low diversity observed at the central regions of the chromosomes that have low recombination rates, while the chromosome arms, with high recombination rates, have higher genetic diversity. This pattern has previously been observed in *C. elegans* (Rockman and Kruglyak, 2009). The diversity generated during the derivation of the ancestral dioecious strain was found to encompass more alleles than identified in a worldwide collection of 127 *C. elegans* wild isolates, suggesting that recombination was the primary source of novel diversity. However, 70-80% of the alleles in the derived line were identified as parental suggesting that the overall genetic diversity of this highly polymorphic strain is explained by a combination of standing variation and novel variation from recombination (Teotonio *et al.*, 2012).

Ultimately, this population is highly suitable for selection experiments as it is genetically highly diverse (Teotonio *et al.*, 2012) and comprises of males and females requiring outcrossing to reproduce (Morran *et al.*, 2009).

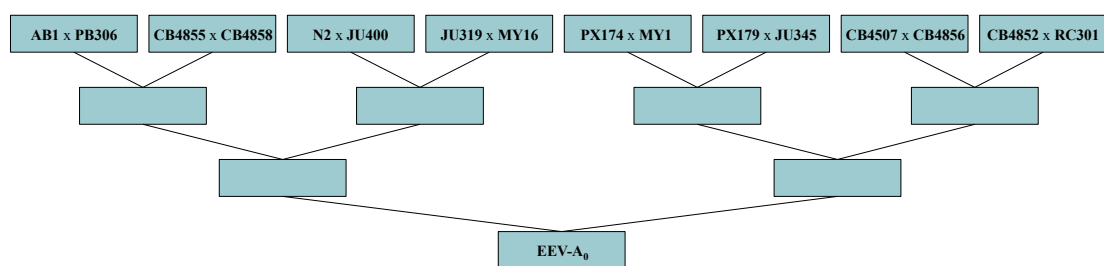


Figure 4.1: Schematic of the funnel cross highlighting the wild isolates used to create EEV-A₀ (Portuguese strain). Figure modified from the supplementary materials of (Teotonio *et al.*, 2012).

4.2. Chapter aims

This chapter aims to investigate the relationship between the emergence and fitness cost of resistance and agrochemical MOA.

Specifically, the work reported in this chapter aims to:

- utilise experimental evolution in proof of principle experiments to investigate adaptation to pesticide selection in a population of modified *C. elegans* strain ('Portuguese strain');
- examine the fitness cost of multi-generational agrochemical selection in a no compound environment under lab conditions;
- establish whether adaptation results in cross-resistance to different pesticide environments;
- investigate whether the mechanism of action affects the rate of emergence of resistance to different agrochemicals.

4.3. Results

4.3.1. Experimental design of selection experiment using *C. elegans*

Six replicate lines were set up in each of four conditions: no compound, aldicarb, carbendazim, and dieldrin, to create a total number of 24 lines per generation. The populations were transferred through 15 generations of selection at 20°C.

The general experimental evolution approach was based on published protocols (Manoel *et al.*, 2007; Lopes *et al.*, 2008) and is depicted in Figure 4.2. On day 1 1,400 L1 worms ('Portuguese strain, EEV-A₀ (Teotonio *et al.*, 2012)) were transferred onto 9cm Petri dishes containing NGM, a lawn of *E.coli* OP50 food source, and compound solution if appropriate. On day 3 all worms were washed off compound and transferred onto 9cm Petri dishes containing NGM and a lawn of *E. coli* OP50 food source as a recovery period. On day 6 individuals were washed off the plates and exposed to a sodium hydroxide and hypochlorite (bleach) solution in which only eggs survive. The eggs were transferred to a 9cm Petri dish with NGM and *E. coli* OP50 and incubated at 20°C for 1 day. The worms were then washed off the plates and the number of larvae estimated by performing counts in 1µl after which 1,400 worms were carried forward to the next generation. This approach also enabled freezing the worms at every generation as the unused young larvae were left to starve and were frozen down when they were early dauer.

Upon completion of fifteen generations of selection the experiment was concluded.

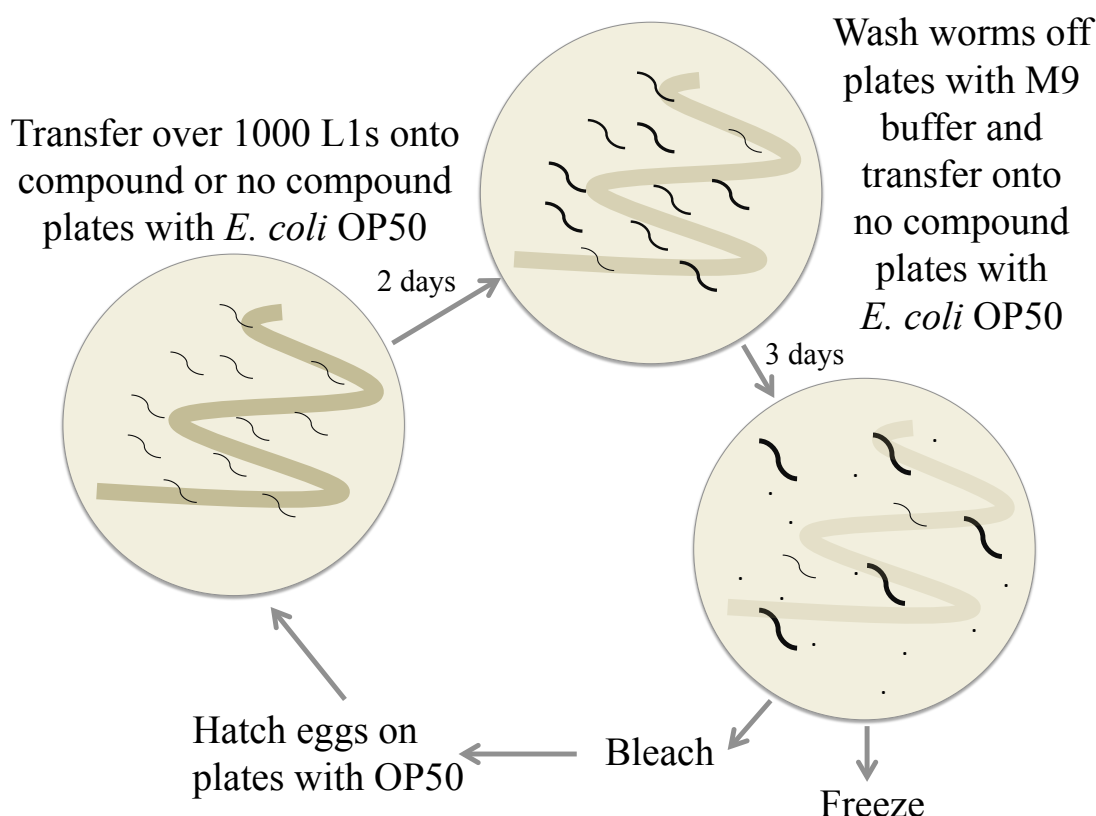


Figure 4.2: Schematic of the experimental design of a single generation of the 15-generation selection experiment of worms on no compound or compound. Generation began with the hatching of eggs on plates with ample *E. coli* OP50 as a food source. Over 1,000 early larval stage worms were then transferred onto compound or no compound plates with OP50 for 2 days. The worms were then washed off plates with M9 and transferred onto no compound plates with OP50 for 3 days to collect progeny. These plates were then bleached to begin a new generation by hatching eggs and once the worms on the plate had entered dauer stage they were frozen down.

4.3.1.1. *Choosing compounds to characterise adaptation to*

agrochemicals with high and low incidence of naturally occurring resistance alleles

In the choice of compounds the prior natural variation in chemical susceptibility observed was key. I used the results of the chemical susceptibility screen to identify compounds with high and low incidence of naturally occurring resistance alleles in the panel of genetically diverse *C. elegans* wild isolates in a way

to observe whether, in an experimental setting, high incidence of resistance in wild isolates correlated with faster emergence of resistance in an initially sensitive strain.

The compounds with high incidence of naturally occurring resistance chosen were aldicarb and carbendazim, and dieldrin was chosen as a compound with low incidence of naturally occurring resistance. The concentration of the compounds to be used was determined by growing the susceptible Portuguese strain in ranging concentrations of the three compounds and establishing a concentration for all that would result in gravid females with plenty of eggs, but still having an effect, upon the same time point in the treatment strategy across three compounds in order to enable selection of all lines under similar conditions and limit the number of differing variables influencing the experiment. The concentrations picked were 90ppm for aldicarb, 30ppm for carbendazim, and 90ppm for dieldrin, sensitivity of the Portuguese strain (EEV-A₀) to which is shown in Figure 4.3. Additionally a no compound selection line would be set up to enable comparison of different selections and associated phenotypes over time.

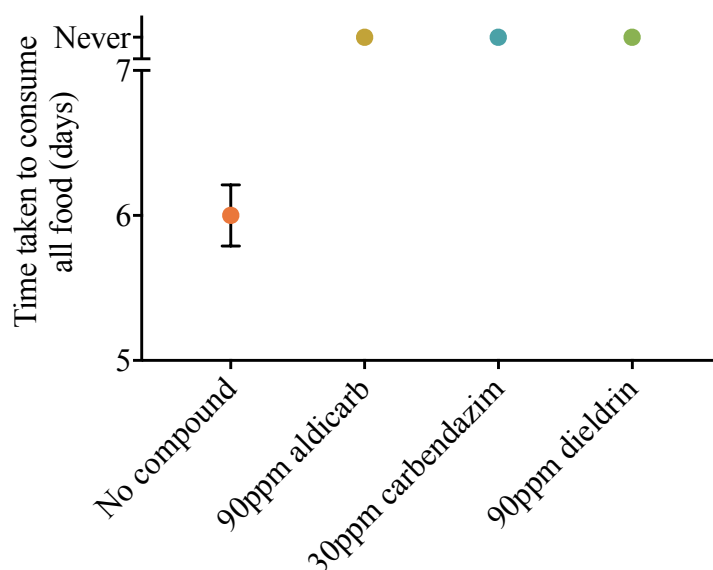


Figure 4.3: Food consumption race showing the sensitivity of the Portuguese strain (EEV-A₀) in the compound concentrations chosen for the selection experiment (n=18). Values are plotted showing the mean $\pm$ SD.

4.3.1.2. *Phenotyping the lines selected on compound and no compound*

The selected lines were then tested in different conditions in food consumption assays outlined in Figure 4.4 below. This enabled me to identify how the overall fitness, described by development, reproduction, movement, and pharyngeal pumping, of the selected lines varied in different environments.

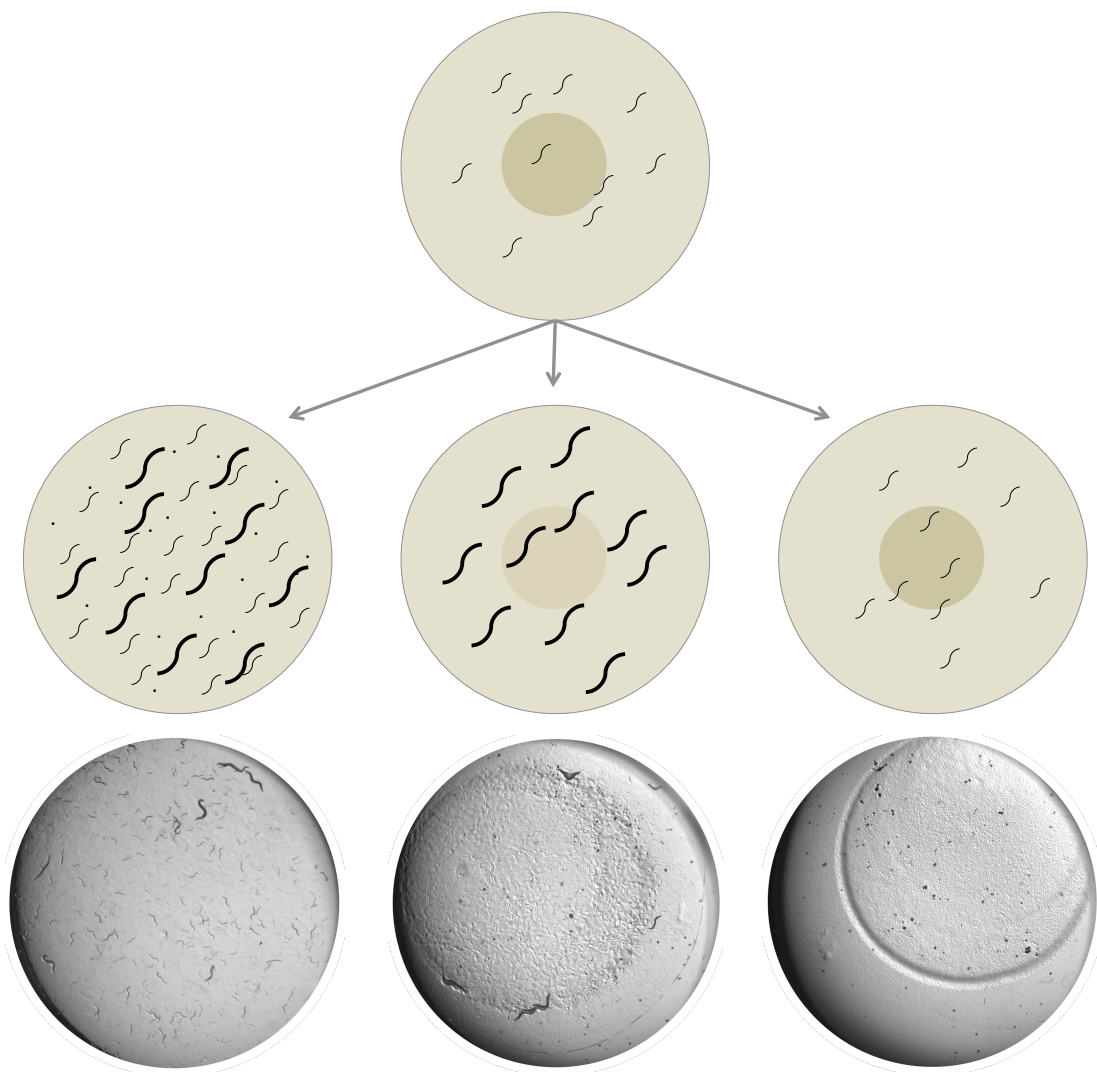


Figure 4.4: Schematic of the food consumption assay. Ten synchronised L1s of a population are placed on a plate with a constant amount of food and the days counted until all food is consumed. As the population will be differently affected, in terms of development, fecundity, movement, and pharyngeal pumping, by the conditions they are placed in, the time taken until all food is consumed will diverge.

4.3.2. Rapid selection for carbendazim-resistance

Results of the food consumption race in 30ppm carbendazim of lines selected on no compound or carbendazim, shown in Figure 4.5, indicate that lines that were selected in 30ppm carbendazim became increasingly adapted to the toxic environment over 15 generations. The ancestral line, shown as generation 0, is highly sensitive to 30ppm carbendazim in the presence of which it may survive some time but not for long enough to consume all food placed in the well. The lines selected in the presence of no compound remain sensitive to carbendazim and also never finish the food in compound. The lines selected in the presence of carbendazim have adapted to the presence of the compound sufficiently to finish food by generation 5 and remain capable of finishing food across generations 10 and 15. This indicates a very rapid adaptation to the compound in the ‘Portuguese strain’.

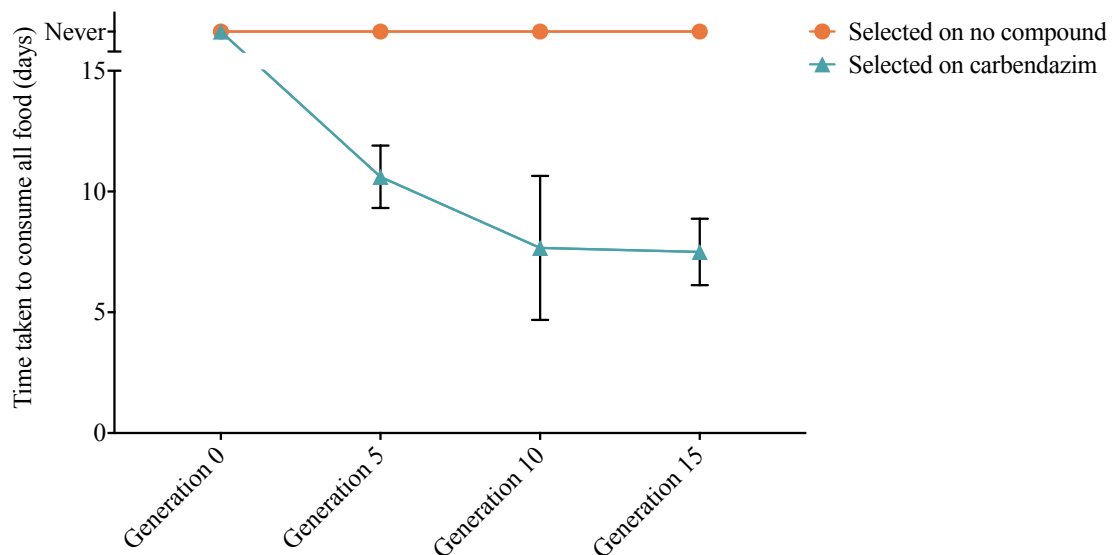


Figure 4.5: Food consumption race of lines selected on no compound (orange) or carbendazim (blue) for 15 generations in 30ppm carbendazim (n=18). Results indicate rapid emergence of carbendazim resistance in lines selected on 30ppm carbendazim. Values are plotted showing the mean of the responses across replicate lines $\pm$ SD.

The results of the food consumption assay suggested that the lines selected on carbendazim had adapted to the toxic environment very quickly over five generations of selection. I was interested in identifying what in particular was changing in the carbendazim-selected lines that was resulting in this continued across generations improvement in the food consumption rate. By quantifying one of the influencers of performance in the food consumption rate, fecundity, I was able to identify that the number of eggs laid by the lines selected on 30ppm carbendazim increased significantly, in comparison to generation 0, by generation 10 (p value=0.0004) of selection and remained highly significantly different at generation 15 (p value <0.0001), as determined by one-way ANOVA in GraphPad Prism.

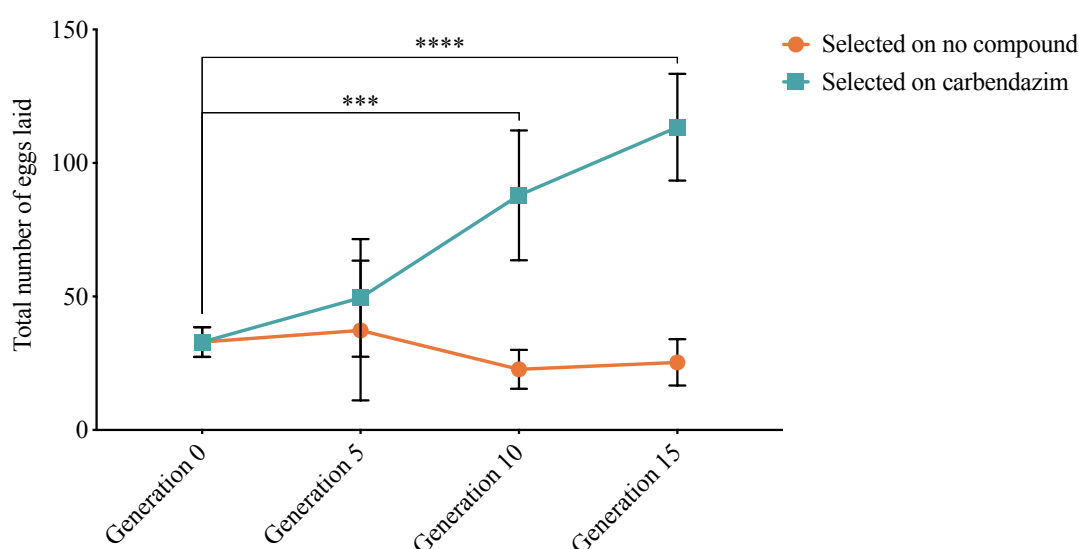


Figure 4.6: Fecundity in 30ppm carbendazim (n=18) of lines selected on no compound (orange) and 30ppm carbendazim (blue). Results indicate that the lines selected on carbendazim lay significantly more eggs than the ancestral population by generation 10 of selection. Values are plotted showing the mean of the responses across replicate lines $\pm$ SD.

While an obvious difference in performance in the food consumption rate was noted after five generations of selection on compound, the difference in egg-laying did not become significant until generation 10. This suggests that a slight increase in

the proportion of resistance alleles in the population during the selection experiment was able to confer the resistance phenotype in the food consumption race with the resistant worms being able to progress through development and reproduce faster, resulting in rapid food clearing. However, a noticeable proportion of susceptible worms remained in the population at that point in the selection experiment, resulting in no significant increase in the average number of eggs laid by all worms in 30ppm carbendazim.

4.3.3. Resistance is not lost upon removal of selection pressure

Upon establishing that rapid adaptation to the agrochemical carbendazim had been observed, I wanted to identify what would happen if the selective pressures of the compound were lost and the carbendazim-selected lines were exposed to no compound selection. Using the experimental design outlined in the beginning of this chapter, I used lines selected in carbendazim for 10 generations, as strong resistance was present by this generation, and continued the selection experiment with them in no compound for a further 5 generations. When compared, the carbendazim-selected lines that had been exposed to no compound for 5 generations showed no loss of resistance in comparison to the initial levels of resistance as shown in Figure 4.7. Statistical significance and p value in this case were assessed by an unpaired t-test, which confirmed no significant difference (p value=0.2447, ns). Firstly, this suggests that adaptation is not lost upon loss of the selective force on the population, which, secondly, suggests that the adaptation to carbendazim-exposure is likely to carry very little or no cost in the no compound environment.

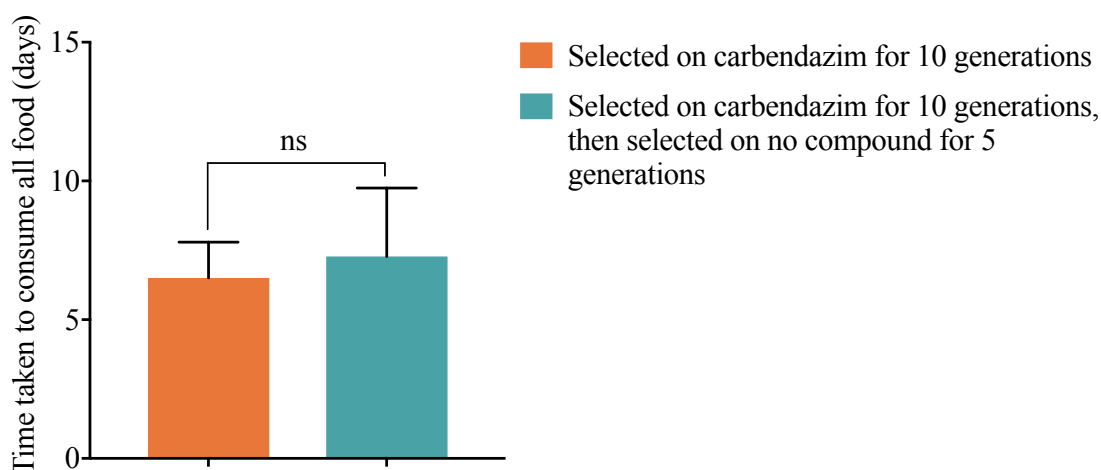


Figure 4.7: Food consumption assay results comparing the time taken to finish all food in 30ppm carbendazim by lines that have been selected in carbendazim for 10 generations (n=18) and lines that have been selected in carbendazim for the same time and then selected in no compound for 5 generations. The statistical significance was assessed by an unpaired t-test and found to be not significant (p value=0.2447, ns). Values are plotted showing the mean of the replicates $\pm$ SD.

4.3.4. Characterising fitness in no compound reveals no cost of carbendazim resistance

The fact that evolved benzimidazole resistance did not disappear upon removal of the selection pressure of the compound indicated a low fitness cost of resistance. In order to assess this further, I compared the responses of lines selected on no compound and on 30ppm carbendazim in their ancestral environment where no compound was present.

The results of this comparison as a food consumption race, when visually and statistically assessed on Figure 4.8, indicated that there was no fitness cost for benzimidazole resistance.

I performed ordinary one-way ANOVA with Dunnett's multiple comparisons test to identify differences between generation 0 and 5, 10, and 15 within the no compound and carbendazim selected lines. This showed no differences between generation 0 and later generations in no compound selected lines and for the lines

selected on carbendazim. This indicated that multigenerational selection on carbendazim was affecting the fitness of the populations in ancestral no compound environment similarly as multigenerational selection on no compound.

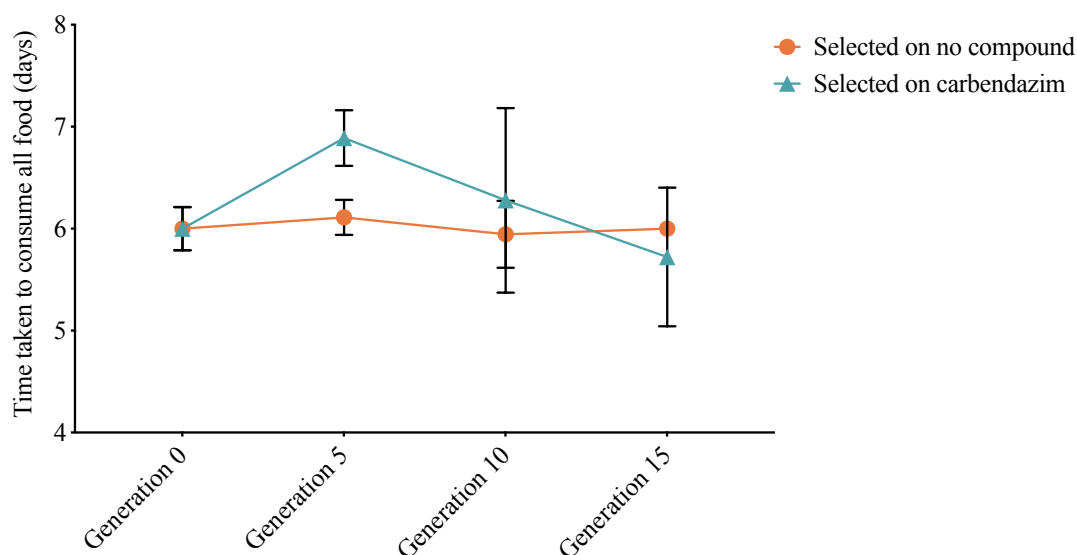


Figure 4.8: Food consumption rate of lines selected on no compound (orange) or carbendazim (blue) for 15 generations in no compound (n=18). Results indicate that carbendazim resistance carries no fitness cost in no compound as no significant changes over generations were detected in statistical analysis. Values are plotted showing the mean of the responses across replicate lines $\pm$ SD.

4.3.5. Adaptation to carbendazim results in cross-resistance to other benzimidazoles

Having identified carbendazim-resistance in the lines selected on carbendazim and establishing that the resistance was not lost when lines were transferred off compound selection, I wanted to identify whether the populations were also resistant to other benzimidazoles. Cross-resistance of benzimidazoles would suggest a resistance mechanism effective in conferring decreased susceptibility across the compound class either by target site resistance or a more general mechanism, such as metabolic resistance. In addition, cross-resistance to other benzimidazoles in the

selected lines would provide insight to the likely genetic basis of resistance, with a number of genetic loci on chromosomes III, IV, and V known to be associated with the trait (Driscoll *et al.*, 1989; Zamanian *et al.*, 2018).

I performed food consumption races to compare the adaptation of lines selected on no compound or carbendazim in 30ppm albendazole, and 60ppm thiabendazole. The results of the food consumption race in 30ppm albendazole, shown in Figure 4.9, indicate that the carbendazim-selected lines are able to finish the food, while the lines selected on no compound are not. Therefore, the selection on 30ppm carbendazim has resulted in cross-resistance to this benzimidazole. The outcome suggesting cross-resistance to benzimidazoles is supported by the finding that the lines selected on carbendazim are able to finish food in 60ppm thiabendazole, as demonstrated in Figure 4.10, while the lines selected on no compound do not survive for long enough to consume all food.

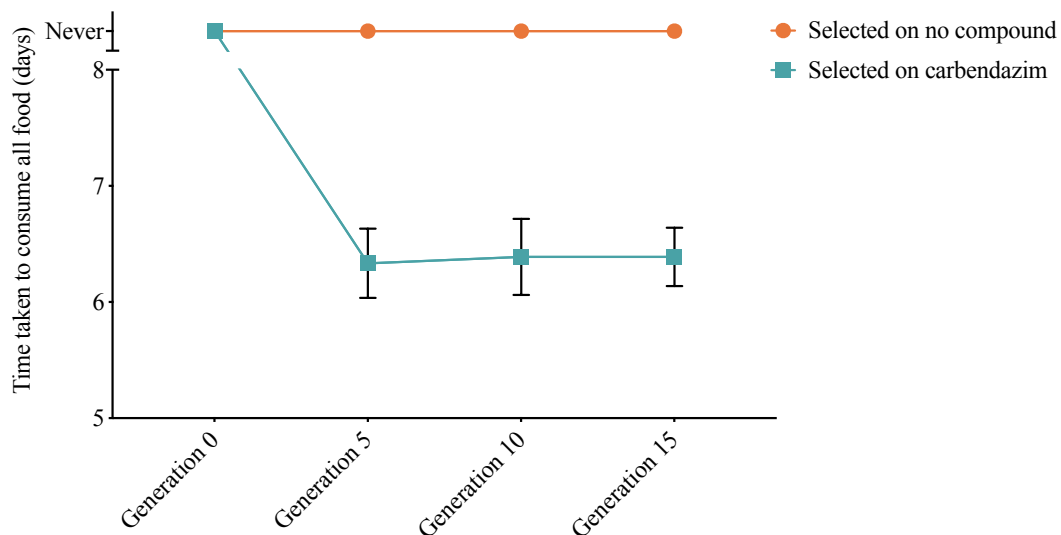


Figure 4.9: Food consumption race of lines selected on no compound (orange) or carbendazim (blue) for 15 generations in 30ppm albendazole (n=18). Results indicate that selection on carbendazim resulted in adaptation to albendazole by generation 5, which did not improve with further generations of selection. Values are plotted showing the mean of the responses across replicate lines $\pm$ SD.

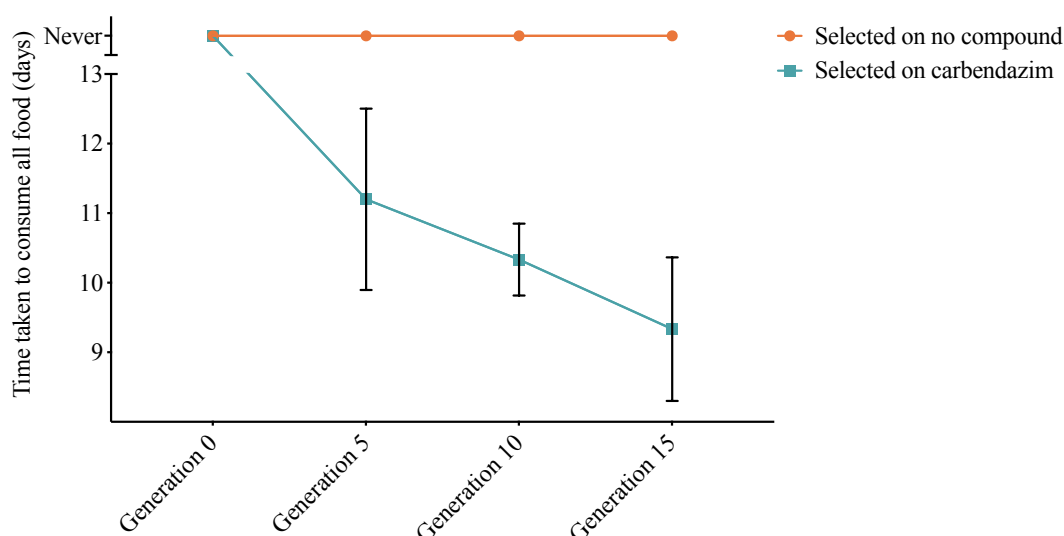


Figure 4.10: Food consumption rate of lines selected on no compound (orange) or carbendazim (blue) for 15 generations in 60ppm thiabendazole (n=18). Results indicate a gradual increase in resistance to thiabendazole from generation 0 to 15 in the lines selected on carbendazim. Values are plotted showing the mean of the responses across replicate lines $\pm$ SD.

Additionally, it appears that the results of the food consumption assay have slightly different dynamics in thiabendazole, where sensitivity to the compound incrementally decreases in the lines selected on carbendazim from generation 5 to generation 15, in comparison to albendazole and carbendazim, which strongly resemble one another and where lines selected on carbendazim for 5 and 15 generations respond similarly in the assay. The food consumption takes longer to conclude in 60ppm thiabendazole using lines selected on carbendazim for 15 generations, around 9 days, while all food is consumed between six and seven days in 30ppm albendazole and carbendazim. This suggests that thiabendazole has an alternative MOA to the other benzimidazoles used, with the 15-generation selection experiment resulting in only partial resistance to the compound. As well as disrupting the assembly of tubulin like other benzimidazoles, thiabendazole has been reported to

inhibit respiration in fungi (Allen and Gottlieb, 1970), suggesting that the lines selected on carbendazim adapted in way that conferred specific resistance to the tubulin disrupting effect of the compounds rather than by metabolic or penetration resistance conferring non-target site resistance.

What is striking with this adaptation is the speed with which a sensitive population has become resistant; with lines selected on 30ppm carbendazim showing adaptation to the harsh compound environments after 5 generations.

4.3.6. Carbendazim resistance does not confer resistance to non-benzimidazole tubulin disruptors

While I had established cross-resistance to other benzimidazoles in the lines selected on carbendazim, I had not investigated whether resistance to other tubulin disruptors was also present. As the food consumption assay results in thiabendazole indicate, shown in Figure 4.10, the mechanism of resistance appears to be specific to the way benzimidazoles disrupt the assembly of tubulin. To test this I performed another food consumption race in 450ppm colchicine (Leung *et al.*, 2015) comparing the lines selected on carbendazim and no compound.

Results, shown in Figure 4.11, indicated that, as no difference was observed in food consumption in colchicine between the two selection regimes, the lines selected on carbendazim were not resistant to colchicine. This result demonstrates that the resistance observed in the lines selected on carbendazim is specific to benzimidazoles, suggesting that the tubulin-specific resistance mechanism does not confer resistance to non-benzimidazole tubulin disruptors and therefore is likely to be specific to the binding site of benzimidazoles.

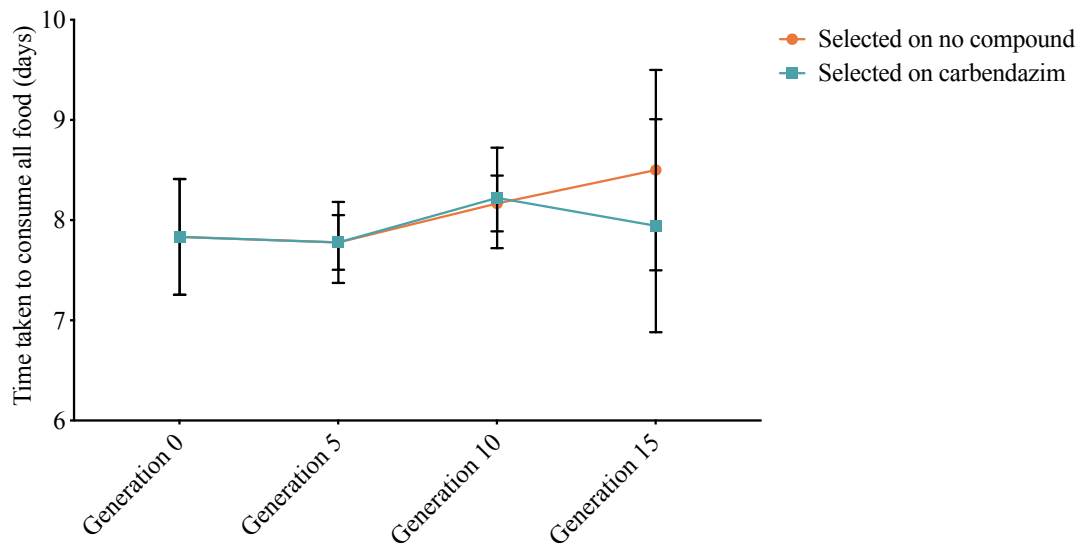


Figure 4.11: Food consumption rate of lines selected on no compound (orange) or carbendazim (blue) for 15 generations in 450ppm colchicine (n=18). Results indicate no difference in the ability of the lines from the two different selection treatments to clear food. Values are plotted showing the mean of the responses across replicate lines $\pm$ SD.

4.3.7. Summary of evolved carbendazim resistance in modified *C.*

elegans strain EEV-A₀

Overall, I have observed the rapid emergence of carbendazim and general benzimidazole resistance, with the lines selected on carbendazim significantly better-off by generation 5 than the lines selected on no compound in a food consumption assay and able to lay more eggs than the lines selected on no compound when exposed to carbendazim after longer selection, by generation 10 while there were no significant differences in egg-laying after five generations of selection on carbendazim. This resistance appears to carry no fitness cost in the tested environment.

4.3.8. Selection experiment results in no adaptation to aldicarb but

introduces a fitness cost in the ancestral no compound environment

Changes in the fitness of lines obtained from 15-generations of selection in 90ppm aldicarb was performed by food consumption assay in compound as well as by quantifying the number of eggs laid in three days upon a two-day exposure to 90ppm aldicarb.

The results of the food consumption assay in 15ppm concentration of aldicarb, where the lines selected in no compound and 90ppm aldicarb were compared, indicated absence of adaptation to the agrochemical environment, as shown in Figure 4.12. The food consumption race was performed at a 15ppm concentration after establishing this as a concentration high enough to exhibit an effect on lines selected on aldicarb and no compound and low enough to ensure the survival of worms for the long periods of time required to clear food in the food consumption race. While a number of compound concentrations were tested for suitability in a food consumption race, ranging from 5ppm to 50ppm, they were either too low, to observe any differences in time taken to finish food, or too high, with similar lethality observed in the lines selected on aldicarb and on no compound. This indicated either that the food consumption assay was not the best method to investigate the adaptation in lines selected in 90ppm aldicarb or that no adaptation to aldicarb had occurred over 15 generations of selection in the compound.

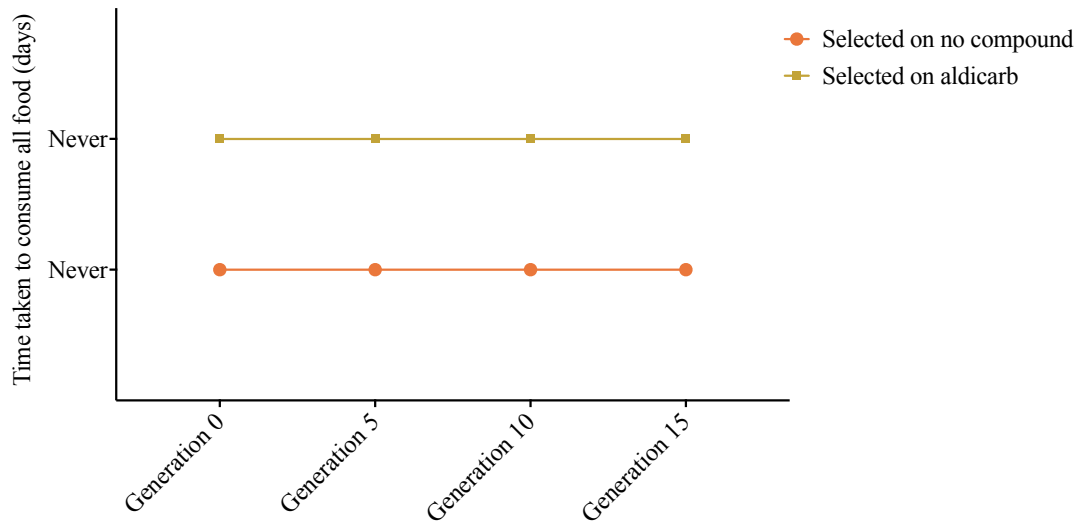


Figure 4.12: Food consumption rate of lines selected on no compound (orange) or aldicarb (yellow) for 15 generations in 15ppm aldicarb (n=18). Results indicate no adaptation to aldicarb environment in lines selected on aldicarb or no compound. Values are plotted showing the mean response across replicate lines.

To decide which of the two options was true, I characterised the responses of the lines selected on no compound and on 90ppm aldicarb to aldicarb exposure by measuring the number of eggs laid in 3 days upon a two-day exposure to 90ppm aldicarb. The results, shown in Figure 4.13, suggest that no adaptation occurred in lines selected on no compound or on aldicarb as no changes in the fitness of the lines, as observed by number of eggs laid upon exposure to aldicarb, is observed within 15 generations.

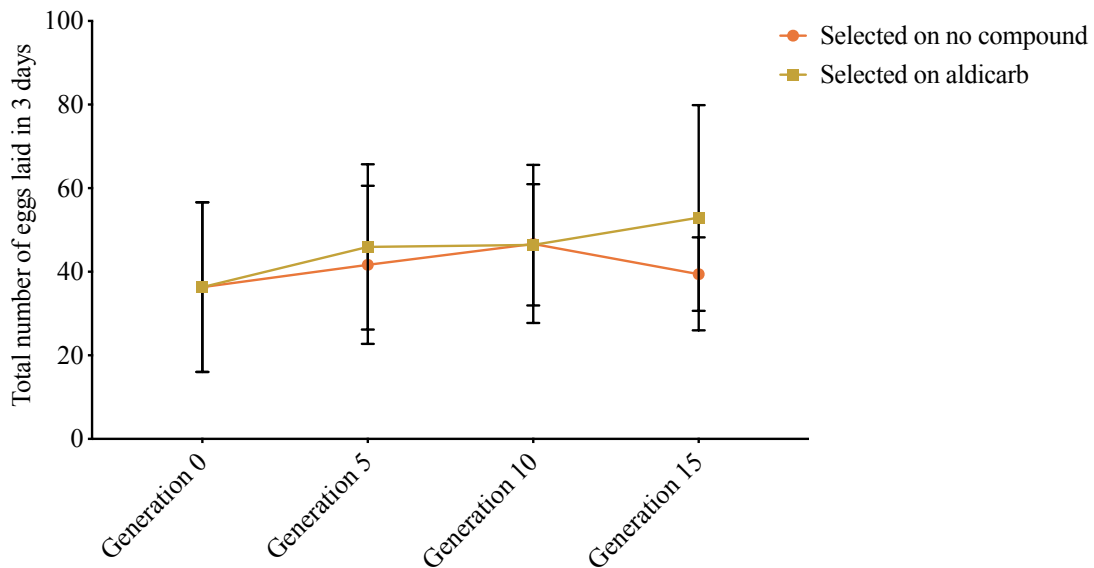


Figure 4.13: Number of eggs laid in 3 days after a 2-day exposure to 90ppm aldicarb by lines selected on no compound and on 90ppm aldicarb for 15 generations (n=18). Results indicated no significant changes to aldicarb sensitivity across the selection experiment, suggesting that no adaptation had occurred. Values are plotted showing the mean of the responses across replicate lines $\pm$ SD.

While I observed no adaptation to aldicarb in the lines selected in aldicarb or no compound, I wanted to identify whether a fitness cost in no compound environment was introduced by the selection treatments. Even though I did not observe adaptation using the food consumption rate or egg laying, the results of the latter indicated possible changes across generations with the number of eggs laid after a two-day exposure to aldicarb increasing (although not significantly) for lines selected on 90ppm aldicarb. Therefore, testing the performance of both of the lines selected on no compound and aldicarb in no compound could further illuminate the changes occurring across generations of selection. For this I compared the lines selected on no compound and 90ppm aldicarb in a food consumption assay in the absence of compound, results shown in Figure 4.14. Additionally I compared the egg-laying capabilities of the lines upon a two-day incubation in no compound, shown in Figure 4.15.

The results of the food consumption race in no compound indicated no significant differences between the different treatments, as determined by multiple t tests, or different generations within particular treatments, as determined by one-way ANOVA with Dunnett's multiple comparisons test. I used multiple unpaired t tests to confirm that the responses of the lines from the two selection regimes did not differ at 5, 10, or 15 generations and all comparisons were identified as not significant. The number of eggs laid in 3 days after a two-day exposure to no compound indicated some changes in egg-laying across the 15-generation selection experiment. For both, the lines selected on no compound and on aldicarb, the number of eggs decreased by generation 15 in comparison to number of eggs laid at generation 0, where the selection experiment had not begun.

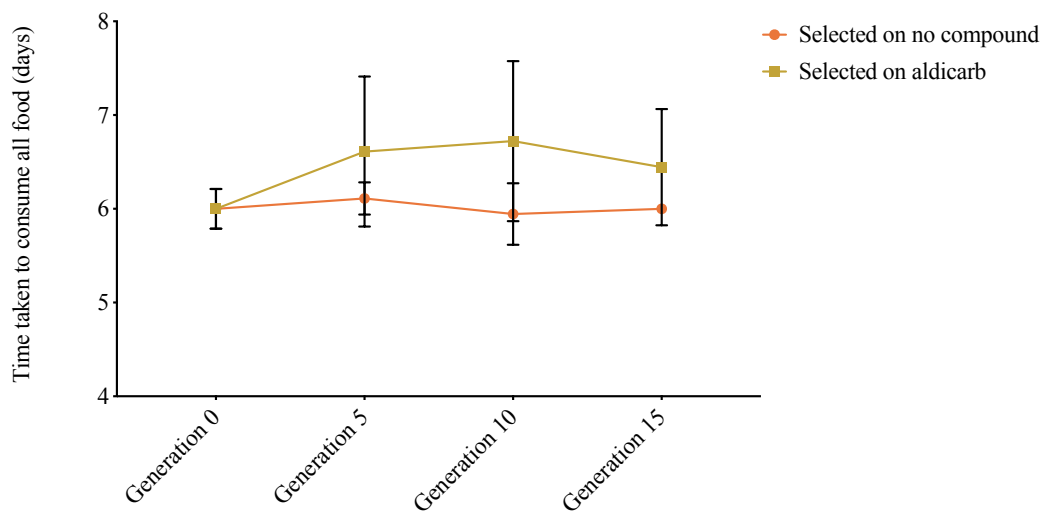


Figure 4.14: Food consumption rate of lines selected on no compound (orange) or aldicarb (yellow) for 15 generations in no compound (n=18). Results indicate that multi-generational selection on aldicarb or no compound did not significantly impact the ability of the lines to clear food in a no compound environment. Values are plotted showing the mean of the responses across replicate lines $\pm$ SD.

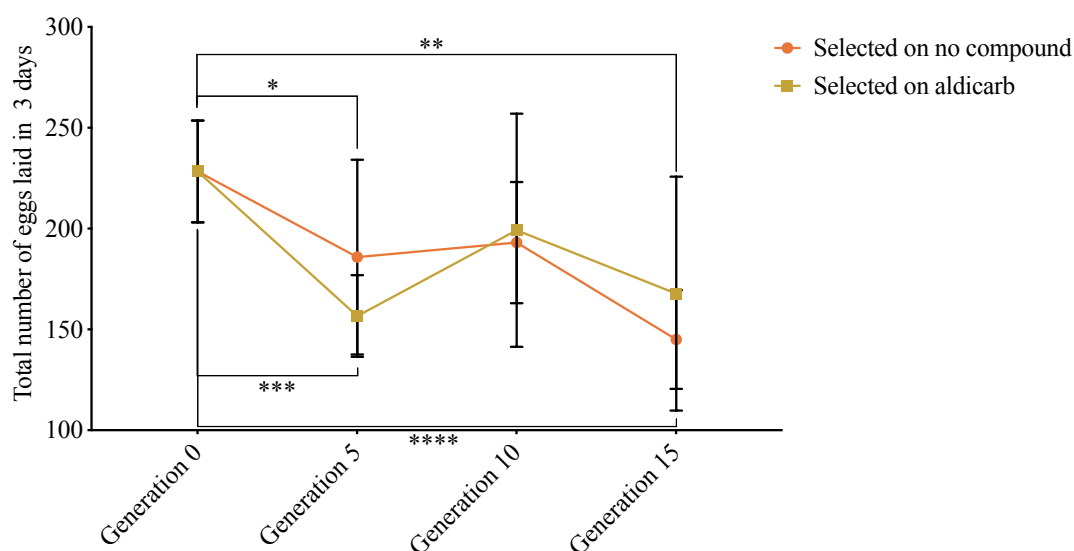


Figure 4.15: Number of eggs laid in 3 days after a 2-day incubation in no compound by lines selected on no compound or on 90ppm aldicarb (n=18). Results of fecundity in no compound indicated that the multi-generational selection on compound and aldicarb affected the populations in unexpected ways, resulting in an overall decrease in the number of eggs laid in the time period observed. Values are plotted showing the mean of the responses across replicate lines $\pm$ SD.

Overall this indicates that selection in 90ppm aldicarb was ineffective in increasing adaptation to aldicarb in the polymorphic Portuguese strain. The results of the fitness cost in the tested environments imply that the treatment on both no compound and 90ppm aldicarb selected for worms that laid less eggs in the ancestral environment.

4.3.9. Multi-generational selection on dieldrin results in no adaptation to the compound and reduces fitness in no compound

In order to identify whether multi-generational exposure to 90ppm dieldrin selected for increased adaptation to the compound I performed a food consumption assay in 15ppm dieldrin and measured the number of eggs laid upon a two-day exposure to 90ppm dieldrin. The food consumption assay was performed at 15ppm concentration as doses lower than that did not appear to have any effect on the worms

as no difference was observed between compound and no compound wells. I performed the egg counts after a two-day exposure to 90ppm dieldrin to investigate egg laying in an assay that resembled the selection experiment in order to identify whether the multigenerational treatment had resulted in differences across generations in the number of eggs laid after compound exposure.

The results of the food consumption race, shown in Figure 4.16, clearly indicated that no clear selection to the toxic agrochemical had occurred within the 15-generation experiment. The lines selected on no compound and 90ppm dieldrin did not survive for sufficiently long upon chronic exposure to 15ppm dieldrin to finish the food placed in wells.

The number of eggs laid in three days upon a two-day exposure to 90ppm dieldrin, results shown in Figure 4.17, also suggests that no adaptation to the dieldrin environment has occurred in the lines selected on 90ppm dieldrin as no significant differences are identified between generations within treatment or between treatments. The number of eggs laid after the two-day dieldrin exposure by the lines selected in no compound decrease significantly at generation 15 (p value=0.0024, **) suggesting that continuous selection in no compound results in reduced egg-laying in the three days after compound exposure.

Together, this suggests that no adaptation to dieldrin has occurred in the modified *C. elegans* strain upon a multigenerational selection regime in dieldrin, but the selection in no compound for multiple generations has resulted in a reduction in numbers of eggs laid by generation 15.

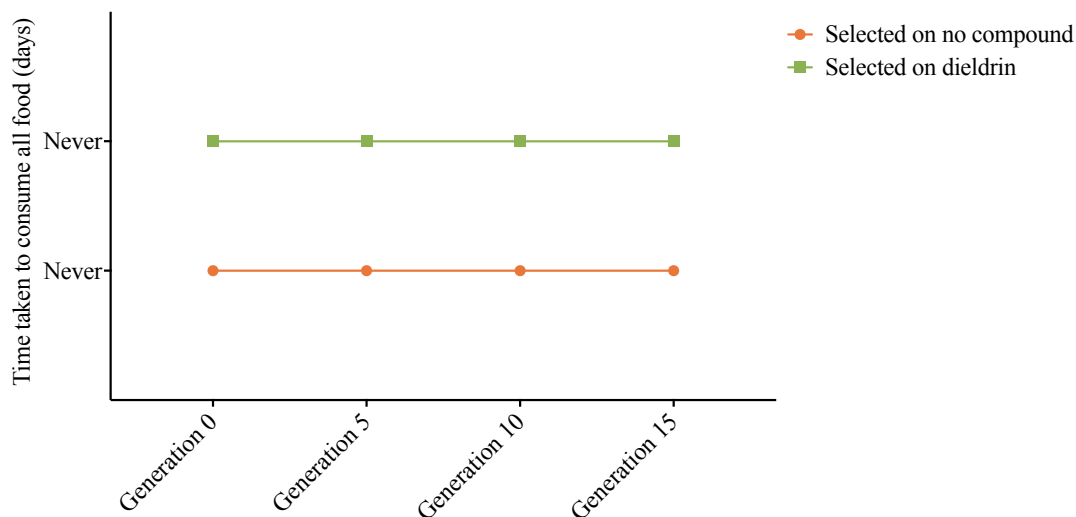


Figure 4.16: Food consumption rate of lines selected on no compound (orange) or 90ppm dieldrin (green) for 15 generations in 15ppm dieldrin (n=18). Results indicate that selection on 90ppm dieldrin did not result in the adaptation of the populations to the chemical environment. Values are plotted showing the mean of the responses across replicate lines.

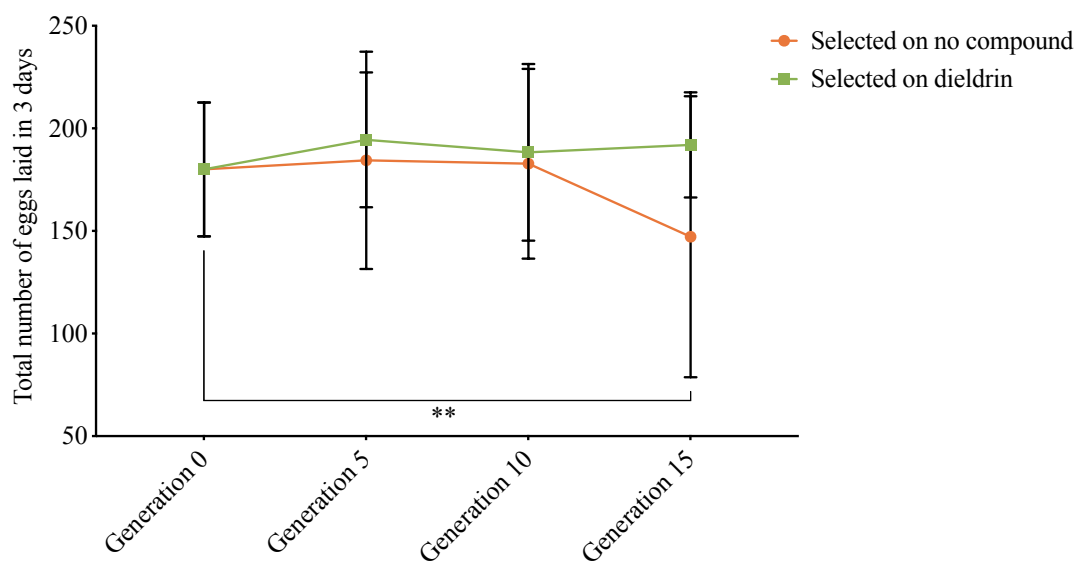


Figure 4.17: Number of eggs laid in 3 days after a 2-day exposure to 90ppm dieldrin by lines selected on no compound or on 90ppm dieldrin (n=18). Results indicate that the multi-generational selection on dieldrin did not result in a noted improvement in the number of eggs laid, but that the selection on no compound resulted in a decrease in the number of eggs laid by generation 15. Values are plotted showing the mean of the responses across replicate lines $\pm$ SD.

No adaptation to the dieldrin environment was observed in the lines selected on no compound or on 90ppm dieldrin, but I wanted to confirm, like in the case of

aldicarb selection, whether a fitness cost had been introduced to the lines during the selection regime that only became apparent when the lines were returned to the ancestral no compound environment. Therefore, I performed a food consumption race and counted the number of eggs laid in three days upon a two-day exposure to dieldrin.

The results of the food consumption race, shown in Figure 4.18, indicated no significant differences within (as tested by one-way ANOVA) or between (as tested by multiple t tests) compound and no compound treatments. Analysis of the number of eggs laid by lines selected on no compound or on dieldrin across generations showed a significant decrease in the number of eggs laid by lines selected in both treatments by generation 15, as can be seen in Figure 4.19.

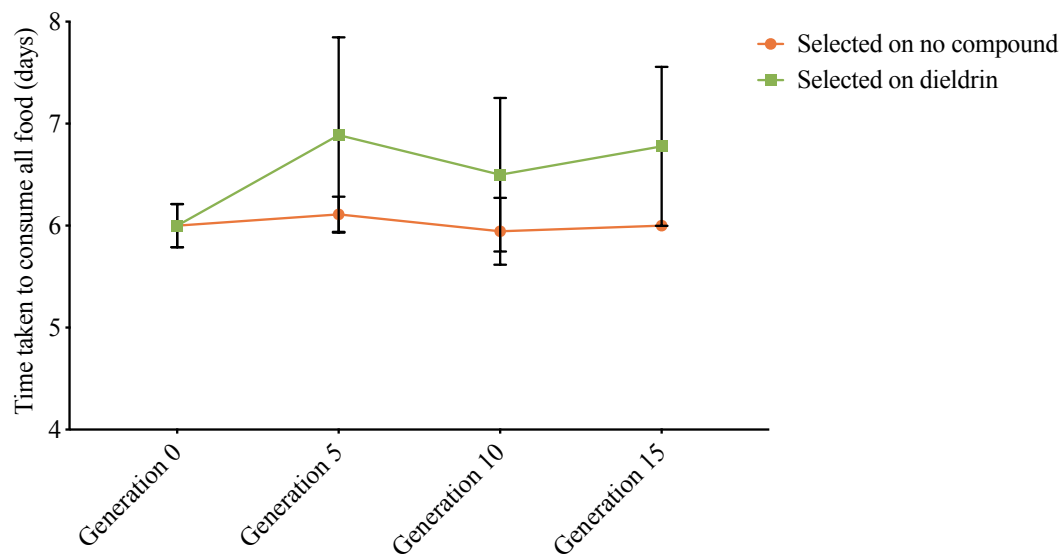


Figure 4.18: Food consumption race of lines selected on no compound (orange) or 90ppm dieldrin (green) for 15 generations in no compound (n=18). The findings indicate that the multi-generation dieldrin or no compound selection did not result in a significant decrease in the time taken to clear food in no compound. Values are plotted showing the mean of the responses across replicate lines $\pm$ SD.

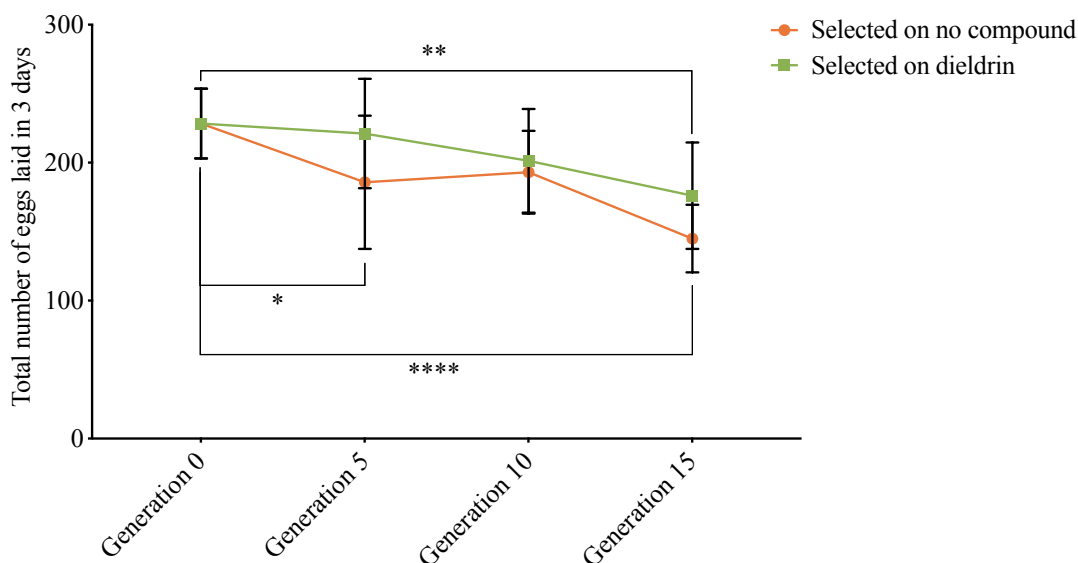


Figure 4.19: Number of eggs laid in 3 days after a 2-day incubation in no compound by lines selected on no compound (orange) or on 90ppm dieldrin (green) (n=18). The results indicate that the multi-generational selection on dieldrin resulted in a significant decrease in the number of eggs laid in no compound by generation 15. Statistical significance assessed by ordinary one-way ANOVA followed by Dunnett's multiple comparisons test. Significant comparisons were: selected on dieldrin generation 0 versus generation 15 (p value=0.0024); selected on no compound generation 0 versus generation 5 (p value=0.0216); selected on no compound generation 0 versus generation 15 (p value=0.0001). Multiple t tests did not identify any significant differences between treatments. Values are plotted showing the mean of the responses across replicate lines $\pm$ SD.

Overall these results suggest that multi-generational selection in 90ppm dieldrin did not result in higher fitness of the selected lines in the agrochemical environment, but did introduce a fitness cost of dieldrin exposure with worms laying fewer eggs after 15 generations of selection than prior to any selection at generation 0. As a reduction of eggs laid by lines selected on no compound was seen in both the no compound (Figure 4.19) and dieldrin (Figure 4.17) egg-laying results, it is possible that the selection regime was not well-suited to select for healthiest worms in every generation due to the long span of exposure and recovery period. While the combination of exposure and recovery period enabled me to collect progeny after a developmental delay introduced by the compound, utilising the same regime in no compound would have resulted in selection of worms laid at a relatively later stage in

egg laying, influencing the qualities of the worms carried on to the next generation under no compound conditions.

4.3.10. Summary of the results of the selection experiment

Overall the analysis of the lines selected in no compound, 90ppm aldicarb, 30ppm carbendazim, and 90ppm dieldrin identified that only the lines selected in carbendazim became adapted to the compound environment with no detectable adaptation to compound environments present from the other two compound selection streams. However, changes resulting from selection were observed in the cases of all four treatments (no compound, aldicarb, carbendazim, dieldrin). In the case of carbendazim-selected lines, the fitness in no compound was slightly increased as these lines outperformed the pre-selection ancestral strain (generation 0) in a food consumption race. In the case of the lines selected in no compound, aldicarb, and dieldrin, the selection regime appeared to introduce a cost as fewer eggs were laid by the selected lines than by the ancestral strain. These results are outlined in Table 4.1. None of the selected lines exhibited cross-resistance to other compounds used in the screen (data not shown).

Table 4.1: Changes observed as a result of the 15-generation selection experiment highlighting whether adaptation to the compound environment was observed and whether a fitness cost in the ancestral no compound environment was observed as a result of the selection treatment.

<i>Selection on:</i>		<i>Adaptation to compound environment</i>	<i>Fitness cost of selection in no compound</i>
	<i>no compound</i>	None	Decrease
	<i>90ppm aldicarb</i>	None	Decrease
	<i>30ppm carbendazim</i>	Yes	Increase
	<i>90ppm dieldrin</i>	None	Decrease

**4.3.11. Rapid emergence of carbendazim resistance is likely caused by
incorporation of parental resistance alleles in the Portuguese strain
EEV-A₀**

As previously stated, only lines selected in carbendazim saw rapid adaptation to the agrochemical environment within the 15-generation selection experiment. The extremely quick selection possibly emerged from genetic diversity within the ‘Portuguese strain’ with resistance-conferring polymorphisms or alleles already present in the population subjected to the selection. There is also a possibility that novel resistance-conferring mutations occurred within the population between its derivation in 2012 and use in the selection experiment in 2018.

I wanted to identify whether resistance could be observed in the ‘Portuguese strain’ population prior to any selection by exposing single gravid females to 30ppm carbendazim in a 24-well plate format. I wanted to use a large enough number of worms to capture low levels of standing resistance in the population. 216 (nine 24-well plates of) worms were tested in this way and no resistance was observed suggesting that less than 1/216 or less than 0.46% of the population was naturally resistant to the pesticide highlighting just how quickly this proportion increased under selection. It is also significant that the similar rapid outcome was observed in all six replicate populations of the selection experiment, as seen in Figure 4.20.

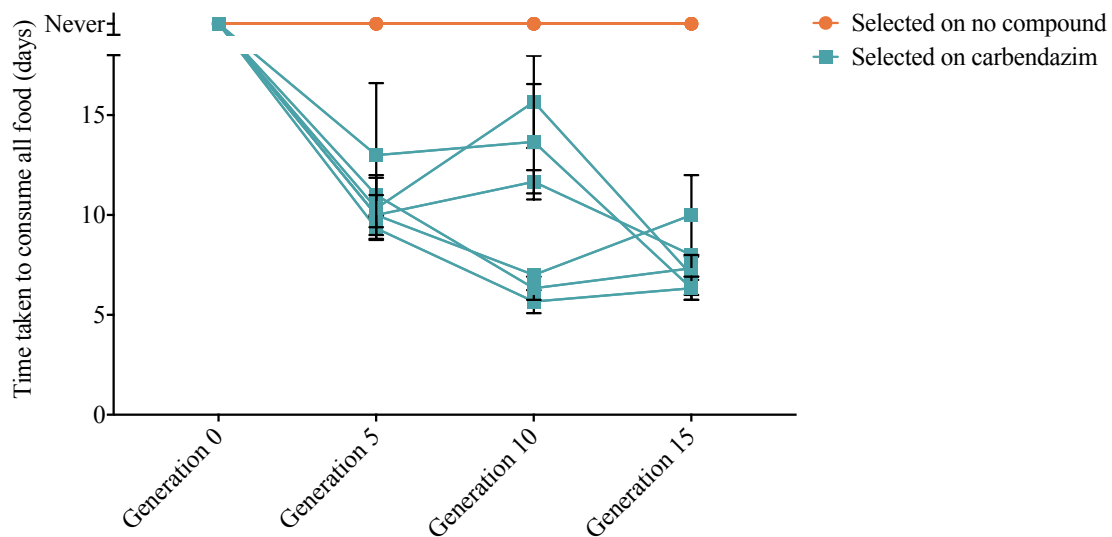


Figure 4.20: Food race in 30ppm carbendazim of all six lines selected on carbendazim (blue) shown separately with the performance of lines selected in no compound (orange) added for indication of initial susceptibility to compound (n=3). Values are plotted showing the mean of the responses for a single replicate line $\pm$ SD.

4.4. Discussion and future work

4.4.1. Observing the evolution of benzimidazole resistance in real time

In this chapter the effects of multi-generational agrochemical exposure and associated selection regimes on a highly polymorphic and dioecious *C. elegans* strain were assessed. The results indicated that selection in 30ppm carbendazim led to the rapid emergence of resistance to benzimidazoles observed within 10 generations with no detectable fitness cost in the tested environment. The speed of resistance emergence implied that the resistance likely arose from standing genetic variation within the Portuguese strain rather than as a result of novel mutations that occurred within the first generations of the selection experiment. I was not able to observe the presence of resistance alleles in the sample of 216 female worms tested. This is well under the 1400 worms subjected to selection every generation as per the experimental design so the possibility that resistance arose from standing genetic variation remains. This idea is supported by the suitability of the Portuguese strain to study the adaptation to novel environments (Teotonio *et al.*, 2012) and the fact that the creation of the Portuguese strain in a funnel cross of 16 *C. elegans* wild isolates means that benzimidazole-resistance variants may have been incorporated to the population (Driscoll *et al.*, 1989; Teotonio *et al.*, 2012; Hahnel *et al.*, 2018) as suggested by our survey of wild isolates in Chapter 3 which revealed several carbendazim-resistant strains. The screen of chemical susceptibility, which assessed the prevalence of natural resistance causing alleles in *C. elegans* wild populations, identified carbendazim as a compound with high variation in susceptibilities and naturally resistant strains within the wild isolates panel. Therefore, while none of the resistant wild isolates identified in my screen were utilised in the derivation of the Portuguese strain EEV-A₀, alternative resistant wild isolates may have been incorporated.

However, with the selection experiment performed as six parallel populations and, with adaptation to the carbendazim environment seen in all, it will be interesting to identify the genetic basis of resistance in all six cases. As Chapter 5 will be focusing on the genetic basis of carbendazim resistance, the origin of resistance alleles will be further investigated there.

4.4.2. Large scale implications of rapid emergence of no cost benzimidazole resistance

The implications of rapid emergence of no cost resistance for global agriculture are vast, as benzimidazoles are broad-spectrum anthelmintics widely used for pest control in agriculture, livestock, domesticated animals, and humans (Jones, Buckingham and Sattelle, 2005). The possibility of 5-10 generations of compound exposure resulting in resistance emergence from standing variation, as seen in our selection experiment, creates a rather concerning picture of the future of benzimidazoles in pest control unless they are used within very stringent resistance management strategies. This recommendation is supported by multiple reports of albendazole (Geerts and Gryseels, 2000; Krücken *et al.*, 2017), carbendazim (Liu *et al.*, 2014; Xu *et al.*, 2015), and thiabendazole (Cabanas *et al.*, 2009; Kotze *et al.*, 2012) resistance caused by a variety of molecular mechanisms ranging from polymorphisms within β -tubulin genes to drug efflux pathways (Kotze *et al.*, 2012). Based on these reports, a relationship between MOA and likely fitness cost appears with some of these mechanisms of resistance more commonly identified than others. Alterations in drug efflux pathways can easily be imagined to confer a high fitness cost, while reports in *C. elegans* suggest that missense mutations in particular β -tubulin genes are well tolerated and carry a low fitness cost (Driscoll *et al.*, 1989).

Fortunately, these concerns regarding the rapid emergence of benzimidazole emergence have been met to some extent with appropriate responses in terms of resistance management. In Great Britain and Ireland, for example, 80% of farmers questioned reported that they rotate their nematode control agrochemicals (Morgan *et al.*, 2012). It is important to note, however, that due to the low cost of resistance observed in this case, rotating pesticides would have little benefit for as long as benzimidazole is still included in the rotation. The low fitness cost of the allele in a no compound environment means that it can remain in the population at a stable proportion indefinitely, a notion that can be experimentally investigated using wild isolates of *C. elegans* as a model for natural variation resulting in resistance. I identified no adaptation to exposure of non-benzimidazole compounds suggesting that other compounds remain effective upon emergence of high resistance to benzimidazoles. Therefore, in practice, benzimidazoles should be used in a limited amount and pest populations controlled with alternative agrochemicals where possible.

4.4.3. No adaptation to aldicarb or dieldrin observed experimentally despite widespread field resistance

The chapter also outlined the results of a multi-generational selection experiment in AChE inhibitor aldicarb and GABA receptor antagonist dieldrin which indicated that no adaptation to either compound environment occurred within populations exposed to the compound for 15 generations. I had chosen to identify the result of a multi-generation selection experiment with aldicarb as this was identified as a compound with significant natural variation in chemical susceptibility in *C. elegans* wild isolates in chemical susceptibility screen, similar to carbendazim, and as

dielldrin, in contrast, was found to have a very uniform responses across the same panel. No adaptation to either compound environment suggests that, firstly, no resistance-conferring natural variation to either compound is present within the population of the Portuguese strain and that, secondly, occurrence of genotypes resulting in lower chemical susceptibility are rare in the MOA targets of both compounds.

Pesticide durability findings have identified that on average it takes just under 18 years to see emergence of resistance to dielldrin in the field. While no data is available on aldicarb, other carbamate insecticides methomyl, carbaryl, and carbofuran have, in the past, seen emergence of resistance on average between 13 and 18 years based on the same study (Brevik *et al.*, 2018). The APRD indicates that 5 reports of resistance in the field exist for aldicarb and 16 dielldrin-resistant species have been isolated in the wild (Mota-Sanchez and Wise, 2019). These reports fall in line with the results of the selection experiment indicating that aldicarb and dielldrin see very slow emergence of pesticide resistance both in the lab and in the field. In comparison, resistance to benzimidazoles is widespread as suggested by data collected by FRAC, with reports of resistance emerging within approximately 5 years of the introduction of a new compound of the class based on reports from the 1970s (FRAC, 2018), indicating that our observation of rapid emergence of resistance to carbendazim in the selection experiment is akin to the speed of emergence of field resistance with carbendazim seeing resistance emerging faster than aldicarb or dielldrin.

The finding is interesting as a number of viable aldicarb-resistant mutants have been described in *C. elegans* with the compound extensively used to study cholinergic neurotransmission in the model organism (Rand, 2007). Aldicarb

resistance can be conferred by mutations in both essential and non-essential genes and involve a range of targets, but, explaining the lack of their natural spread, these mutations often carry a strong fitness cost in a no AChE inhibitor environment with the mutants exhibiting moving uncoordination or slow growth (Nguyen *et al.*, 1995). This suggests that a high fitness cost excludes the mutations from standing genetic variation if they do occur and explains why no adaptation to aldicarb was observed.

Meanwhile, dieldrin resistance appears to be caused by specific mutations within the GABA receptor subunit that remain in the population upon loss of selection (Brooke *et al.*, 2006) suggesting a far lower fitness cost of resistance in no compound but additionally a lower number of possible targets altered.

In the case of carbendazim, where rapid adaptation was observed in the selection experiment, general resistance-conferring mutations converge on a single molecular function, with tubulin genes implicated in resistance (while other reports also exist), and result in a low fitness cost of the alterations in no compound that result in decreased susceptibility.

This highlights the two parameters, amount of variation tolerated at specific physiology targeted and fitness cost of resistance alleles in a no compound environment, controlling likelihood of emergence of pesticide resistance both under experimental conditions as well as in the field.

4.4.4. Anticipating the emergence of resistance by better characterisation of compound chemistry

The results of the selection experiment outlined in this chapter indicate how the prevalence of resistance alleles in experimental polymorphic populations, field populations, or mutagenesis experiments, based on published reports, and their fitness

landscape can be used to indicate which compounds are more likely to see emergence of resistance. Predicting the emergence of resistance purely from chemistry still remains challenging, but it is worth investigating my results in the light of known modes of action as well as field relevant chemical attributes of active compounds used.

Compounds targeting muscle and nerve physiology or growth and development were used in the experimental selection. Adaptation was only seen in response to selection on carbendazim, a growth and development perturbing agent, while selection on aldicarb and dieldrin did not result in increased fitness within the experimental evolution timeframe of 15 generations in the environments tested. Therefore, muscle and nerve targeting pesticides appear to have a more robust profile as pesticides than those perturbing growth and development but the very limited sample size of compounds in this comparison must be kept in mind. Further studies utilising a larger sample of agrochemicals would strengthen the association between particular physiology targeted by agrochemicals and rapid emergence of resistance experimentally.

The environmental persistence of a pesticide is also important to consider. Long and short half-life, the time taken for the amount of compound to half in the environment, both come with their theoretical concerns if pesticide resistance is considered. A short half-life means that a compound has to be more frequently sprayed, with the field concentration of the compound changing rapidly in a short timeframe. This may result in resistance mechanisms that provide the pests with a slight but crucial advantage in a ranging concentration. A long half-life at the same time means that the compound persists in the environment over multiple generations making it difficult to implement strategies of pesticide resistance management where

active compounds are rotated in a certain sequence and could be particularly detrimental in cases where natural resistance alleles are present (Taylor and Georghiou, 1982).

Table 4.2: Environmental half-life of the pesticides used in the chemical susceptibility screen.

Compound	Environmental half life	Introduced
<i>Aldicarb</i>	1 day to a few months	1970
<i>Carbendazim</i>	1-25 months	1974
<i>Dieldrin</i>	5 years	1949

Most compounds used in the field have a short half-life due to the increased environmental and ecological toxicity of compounds with a long half-life (multiple years) but how does that affect the emergence of resistance? As seen in Table 4.2, which details the environmental half-life of the pesticides used in the selection experiment, aldicarb has an incredibly short half life while dieldrin and carbendazim are far more persistent. Little experimental work has been done so far on the direct relationship between compound degradation and the emergence of resistance but mathematical models have predicted that short-lived compounds delay emergence of resistance (Taylor and Georghiou, 1982), while a recent report analysing the entries of APRD suggested that the speed of resistance emergence to a compound is species specific and is not affected by the chemistry of the compound (Brevik *et al.*, 2018). Our results support the latter report, suggesting that a long half-life in itself is not necessarily associated with faster emergence of resistance and that the effect of alterations, in terms of physiological viability and fitness cost in no compound, on the targeted mechanism of the pest provides a better estimate.

4.4.5. Future work

This chapter has outlined the results of a proof-of-principle selection experiment where *C. elegans* is utilised to study the dynamics of adaptation to pesticide selection. The adaptation of lines selected on carbendazim to the agrochemical environment enabled us to investigate the fitness cost of resistance, identifying this particular case of carbendazim resistance as having a low fitness cost in a no compound environment. This discovery has its implications from a viewpoint of identifying the pesticides with the longest lifetime.

Future work utilising the lines selected in carbendazim will go into identifying the genetic basis of this resistance in order to establish how this rapid emergence of resistance could be observed. As Chapter 3 outlined results detailing the identification of three naturally carbendazim-resistant *C. elegans* wild isolates it will be interesting to establish whether the genetic basis of the resistance overlaps between these different biological samples. The genetic basis of resistance to carbendazim in wild isolates as well as in the lines selected on carbendazim will be investigated in Chapter 5.

Chapter 5: Identification of Alleles Conferring Resistance to Carbendazim in Wild Isolates, Chemically Induced Resistant Mutants, and Evolved Resistant Lines

5.1. Introduction

5.1.1. Proposed mode of action of carbendazim

Carbendazim is a member of the benzimidazole class of agrochemicals, which have been widely used as fungicides and nematicides since their introduction in the early 1970s. Benzimidazoles are potent inhibitors of tubulin polymerisation. They act by targeting the β -tubulin subunit of the microtubules and cap the microtubules at the associating end while the microtubules continue to dissociate at the opposite end, leading to the loss of integrity and arrest of microtubule formation. Ultimately this results in failure of cell division, leading to cell death (Lacey, 1990; Saunders *et al.*, 2013; Vela-Corcía *et al.*, 2018).

5.1.2. Genetic basis of resistance to carbendazim and other benzimidazoles

Due to regular use of benzimidazoles in agriculture, as well as veterinary and human medicine (Jaeger and Carvalho-Costa, 2017; Krücken *et al.*, 2017), reports of benzimidazole resistance are widespread in the literature, with resistant pest species having been isolated in the wild as well as from mutagenesis screens in the lab. These studies have revealed that specific missense mutations of β -tubulin genes often explain the resistance that has emerged in the field. In particular, through the literature, the contribution of three polymorphisms is emphasised: F200Y, F167Y,

and E198A (Kotze *et al.*, 2012, 2014). Additional, non-target mechanisms, such as detoxifying enzymes or drug efflux pumps, have also been shown to contribute (Hahnel *et al.*, 2018).

The specific contribution of polymorphisms in β -tubulin on the one hand and metabolic resistance on the other has often remained unclear. Reports suggest that the resistance phenotype is often not explained entirely by alterations in β -tubulin (Hahnel *et al.*, 2018) and that in some cases polymorphisms in β -tubulin may not be implicated at all in conferring benzimidazole resistance (Krücken *et al.*, 2017). Identification of the benzimidazole bio-transformation pathway in nematodes, whereby thiabendazole is found to bind the nuclear hormone receptor NHR-176, required to induce the cytochrome P450 (CYP) encoded by *cyp-35d1*, suggests that modifications of benzimidazole metabolism can result in reduced susceptibility to the compound class. Particularly as the knockdown of *cyp35d1* and its transcription factor result in increased sensitivity in both wild type and otherwise resistance conferring β -tubulin (*ben-1*) deletion backgrounds in *C. elegans* (Jones, Flemming and Urwin, 2015).

The results of Chapters 3 and 4 outlined the discovery of two instances of carbendazim-resistance: one in *C. elegans* wild isolates and another in a modified *C. elegans* strain upon a selection experiment. In addition, prior work in the lab resulted in the isolation of a carbendazim-resistant mutant from a chemical mutagenesis screen using ethyl methanesulfonate (EMS). This provides us with a unique set of resources to further understand the nature of benzimidazole resistance and how this might impact field application.

5.2. Chapter aims

This chapter aims to compare the carbendazim resistance conferring alleles from three separate biological sources in terms of genetic basis and relative fitness.

Specifically, the work presented in this chapter will:

- compare the carbendazim susceptibility of a chemically induced carbendazim-resistant *C. elegans* strain (AW1783) to a naturally occurring resistant strain (ED3005) of the same species;
- identify the genetic basis of resistance in *C. elegans* wild isolates, in a chemically induced carbendazim-resistant strain, as well as in carbendazim-resistant lines from a multi-generational selection experiment where a highly polymorphic *C. elegans* strain was used;
- use the CRISPR-Cas9 approach to confirm that the identified variation explains resistance seen in *C. elegans* wild isolate ED3005;
- compare the fitness of the chemically induced carbendazim resistant mutant (AW1783) to a sensitive strain marked with *myo-1::GFP* in different carbendazim and no compound environments across generations to characterise the influence of multiple variables on the dynamics of resistance emergence.

5.3. Results

5.3.1. The EMS induced mutant demonstrates high resistance to carbendazim

The work described in Chapters 3 and 4 outlined the discovery of two cases of carbendazim-resistance. First, a set of *C. elegans* wild isolates was found to be naturally resistant to the agrochemical. Second, upon a selection experiment, a modified *C. elegans* strain was obtained that was adapted to different benzimidazole environments, exhibiting cost-free resistance to carbendazim, albendazole, and thiabendazole. In addition, a project student in the lab (Chloe Casey), utilising the Bristol N2 as the starting population, isolated a carbendazim-resistant line from an EMS mutagenesis screen (AW1783). This presented me with a unique possibility to characterise and compare three separate cases of resistance in terms of the genetic basis as well as fitness in relation to other sensitive strains in a number of environments.

No further tests had been performed with AW1783 after isolation of the mutant (work done by Chloe Casey, not published). Therefore, I wanted to quantify the level of carbendazim-resistance present in comparison to sensitive strains, such as N2 and CB4856, as well as another resistant strain, such as ED3005. The results of a food consumption assay in 0, 5, 30, 50, 90, 160, 300ppm carbendazim, shown in Figure 5.1, indicate that AW1783 is highly resistant to carbendazim. While the sensitive strains N2 and CB4856 become unable to finish food in the assay when the compound concentration reaches 90ppm, AW1783 and ED3005 remain capable of finishing food at higher concentrations.

Based on multiple t tests, comparing the responses of ED3005 and AW1783 in the food consumption assay, the difference between the two strains becomes

significant (p value <0.0001) at 300ppm concentration suggesting that AW1783 may be even more resistant to the compound. This is notable as it implies that the resistance in the two carbendazim-resistant strains may not be explained by the same mechanism. This is likely as the genetic background of the two resistant strains is different, with ED3005 likely harbouring more unique polymorphisms than AW1783, with this strain having been derived from the Bristol N2 in a chemical mutagenesis experiment. However, there are likely many differences between Bristol N2 and AW1783 due to the elevated rate of mutation in an EMS experiment and AW1783 not being backcrossed to N2.

Establishing the level of carbendazim-resistance in the chemically induced mutant, as well as wild isolate ED3005, was important as it meant that strong resistance to carbendazim could be used in mapping approaches to identify the genetic basis of resistance in the two strains.

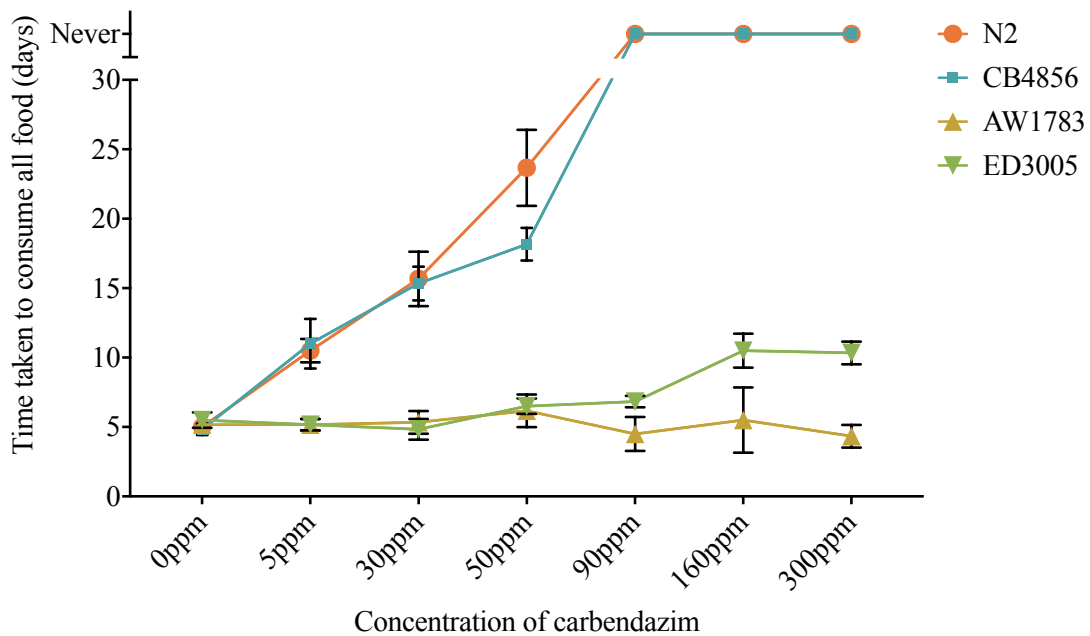


Figure 5.1: Food consumption rate of N2 (orange), CB4856 (blue), AW1783 (yellow), and ED3005 (green) in 0, 5, 30, 50, 90, 160, 300ppm concentrations of carbendazim ($n=3$). Results indicate that AW1783 and ED3005 are both resistant to carbendazim while N2 and CB4856 are sensitive. Additionally, resistance to higher concentrations is observed in AW1783 suggesting the presence of additional resistance mechanisms. Values are plotted showing the mean of the responses $\pm$ SD.

5.3.2. Design of the SNP linkage approach to identify the genetic basis of benzimidazole-resistance in ED3005 and AW1783

Gene mapping by SNP linkage has been used extensively in *C. elegans* (Wicks *et al.*, 2001; Davis *et al.*, 2005; Doitsidou *et al.*, 2010) which enabled us to modify the published methodology (Doitsidou *et al.*, 2010) to suit the mapping of resistance conferring alleles in the *C. elegans* wild isolate ED3005 and the chemically mutagenised strain AW1783.

The method was set up to establish the chromosomal location of the mutation conferring resistance to carbendazim in the strains of interest by utilising the polymorphic strain CB4856 and is illustrated in Figure 5.3. Briefly, the males of CB4856 were crossed with the hermaphrodites of the resistant strain (ED3005 or AW1783) and cross-progeny confirmed in F1 by single worm PCR. Chromosome IV mapping primers M117_fwd and M117_rev (detail in Materials & Methods) were used, with ED3005 and AW1783 producing a single band of 200bp, while CB4856 shows a band at 100bp on a 1% agarose gel, upon EcoRI restriction digest of the PCR product, as can be seen in Figure 5.2, due to the presence of a SNP in CB4856 which has created a suitable restriction site.

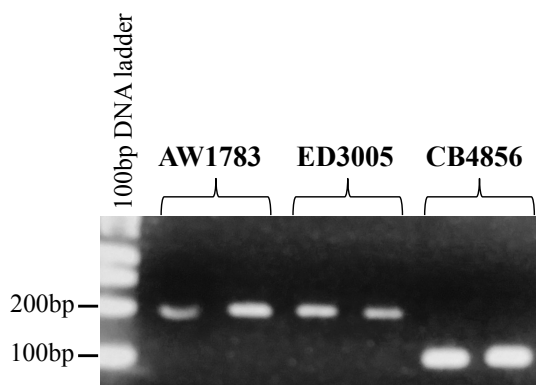


Figure 5.2: Image of 1% agarose gel of the restriction digest of the PCR product obtained using M117 primers showing the signatures expected with AW1783, ED3005, and CB4856.

The F1 cross-progeny were then allowed to self on both no compound and 30ppm carbendazim plates. Allowing cross-progeny to self on no compound enabled me to identify the control distribution of SNPs as polymorphisms from both parents were expected to be equally likely to be included in the absence of compound selection. Selfing of F1 progeny on compound was required to identify the fittest animals in the compound and enable, further down the line, the identification of genomic regions close to the mutation of interest by decreased incidence of CB4856 and increased incidence of resistant strain SNPs. The identification of semi-dominance of carbendazim-resistance in AW1783 and ED3005, which complicated the collection of cross-progeny homozygous for resistance-conferring allele, is detailed below under heading 5.3.2.1.

The no compound and carbendazim selections were followed through F2 to minimise the number of worms collected from carbendazim-selection that were false positives for the homozygous presence of the resistance-conferring allele. The healthiest F3 progeny of singled F2s selected on either no compound or carbendazim plates were then picked to self to enable preparation of genomic DNA. A whole genome sequencing and SNP mapping approach was then used to establish the ratios of CB4856 SNPs as well as those of the resistant strain across chromosomes in order to identify locations with low CB4856 SNP incidence due to selection of the resistance-conferring locus. In areas unimportant to the phenotype of interest the ratio of SNPs from both parents would remain at an equal level.

Results of the SNP linkage mapping of carbendazim resistance are discussed under heading 5.3.3 for ED3005 and heading 5.3.6 for AW1783.

5.3.2.1. *Semi-dominance of carbendazim resistance alleles in ED3005 and AW1783*

The alleles conferring carbendazim resistance in both ED3005 and AW1783 were identified as semi-dominant in a food consumption assay in 30ppm carbendazim by comparing their cross-progeny with the sensitive CB4856. The cross-progeny cleared the food faster at 30ppm carbendazim than the sensitive CB4856, suggesting that the trait displayed some level of dominance (data not shown), with the cross-progeny heterozygous for the allele conferring carbendazim resistance able to clear food similarly but not as fast as resistant strains (ED3005, AW1783) in the presence of the compound, hence indicating semi-dominance. Therefore, early selection on compound was required to select for the fittest animals and enable selection for cross-progeny homozygous for the carbendazim resistance allele in the F2 progeny, by excluding plates where sensitive worms emerged in the next generation.

Dominance and semi-dominance of benzimidazole resistance conferring mutations has been previously shown with chemically induced mutants. The semi-dominance was in that case determined to be conditional on temperature and concentration. The study observed no heterozygote resistance at 15°C or at concentrations of benomyl (a benzimidazole compound) that were higher than 12.5 µM (Driscoll *et al.*, 1989). While a lower temperature would have resulted in much longer screening times, due to the slower rate of development of *C. elegans* at 15°C, I could have alternatively utilised a much higher dose of carbendazim to identify cross-progeny homozygous for the resistance-conferring allele in F2.

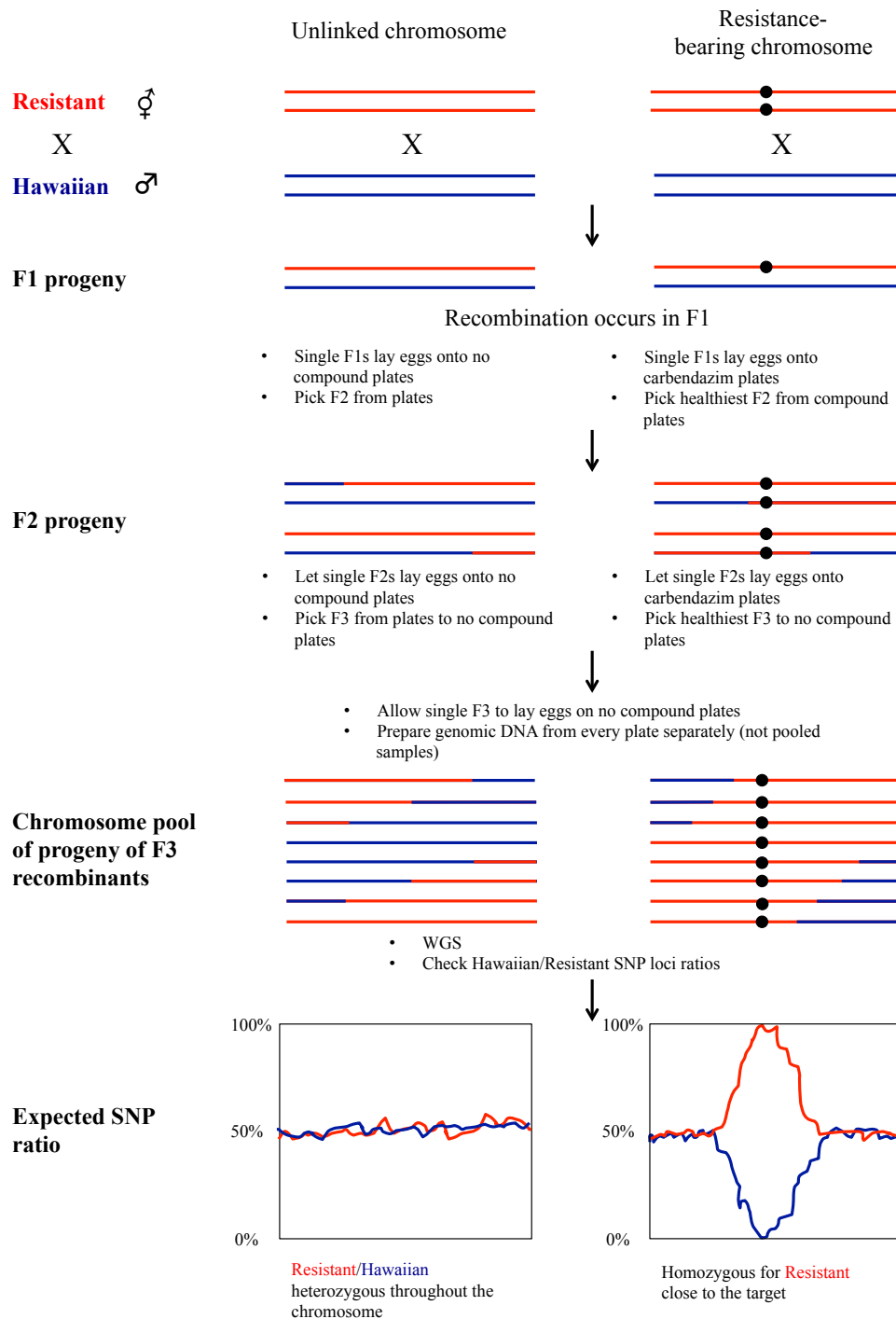


Figure 5.3: SNP linkage mapping approach to identify the genetic basis of carbendazim-resistance in ED3005 and AW1783 by utilising the carbendazim-sensitive CB4856. First, a resistant hermaphrodite was crossed with a sensitive male. Cross-progeny were selected on both compound (shown in right column), to identify genomic location important for resistance, and no compound (shown in left column), to identify normal segregation of genetic material. The healthiest cross-progeny on compound plates and on no compound plates were singled at F1 and F2 with F3 individuals allowed to self prior to DNA preparation. SNP frequencies in the DNA preps of cross-progeny from no compound and carbendazim plates were established. An unlinked chromosome would show an equal distribution of SNPs from both parents but a resistance allele-carrying chromosome location would only have the SNPs of the resistant parent. Figure is modified from (Doitsidou *et al.*, 2010).

5.3.3. SNP linkage highlights a region of chromosome III in ED3005

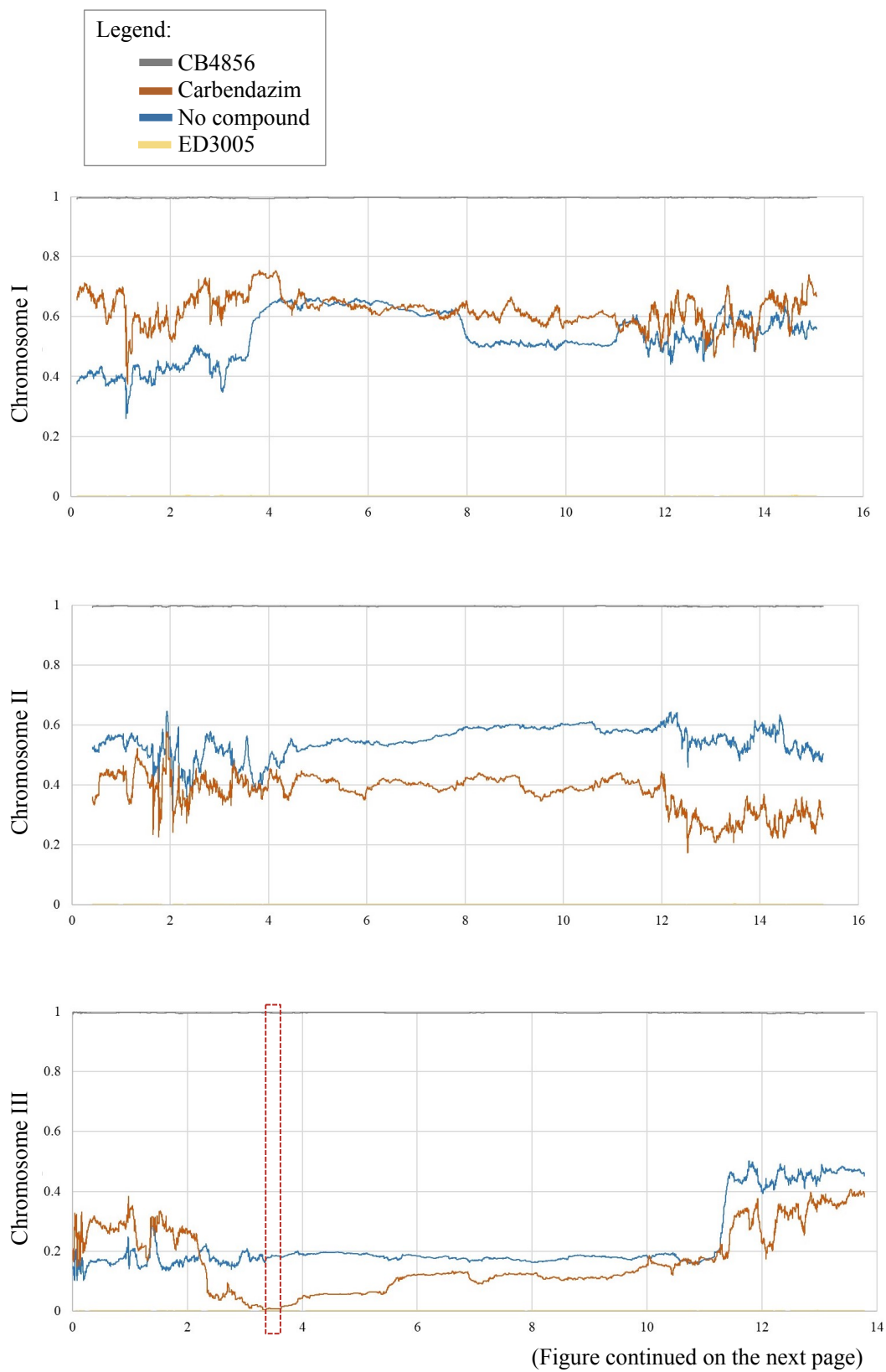
The identified SNP frequencies in the ED3005 and CB4856 selected progeny highlight the importance of a region of Chromosome III in conferring the carbendazim-resistance phenotype.

The SNP ratio data obtained from whole genome sequencing and following data analysis was further filtered to produce SNP linkage maps shown in Figure 5.4. Locations where the CB4856 SNP ratios were ≥ 0.95 and ED3005 SNP ratios ≤ 0.05 were included in the linkage plots. This meant that only the ratios of unique CB4856 SNPs in all four samples were included in the filtered plots. The SNP ratios of cross-progeny selected on no compound or carbendazim were not filtered. Plotting the running average across 255 SNP ratios of the four datasets created the SNP linkage map. Figure 5.4, which shows the results, illustrates the running averages of unique CB4856 SNP ratios across all six chromosomes of CB4856 (grey), ED3005 (yellow), carbendazim-selected cross-progeny (orange), and the no compound selected cross-progeny (blue).

In the experimental design I outlined that I expected to observe roughly half of SNPs from either parent in genomic regions unlinked to the carbendazim-resistance phenotype. This means that in the no compound selected cross-progeny, the frequency of SNPs observed from either parent should be similar. Indeed, this was the result observed with the ratio of CB4856 SNPs across all chromosomes remaining constant in the absence of carbendazim-selection.

In the presence of carbendazim, I see a total absence of CB4856 SNPs in a region of chromosome III highlighting that the area is associated with carbendazim-resistance in ED3005. No other areas have a decrease in the CB4856 SNP ratios.

A heavily fluctuating running average of CB4856 SNP ratios is observed on chromosome arms. This is consistent with reports of areas of differing recombination rates within *C. elegans* autosomes and sex chromosome. Every chromosome is characterised as having two tips of extremely low recombination, two high recombination arms, and a low recombination centre (Rockman and Kruglyak, 2009).



(Figure continued)

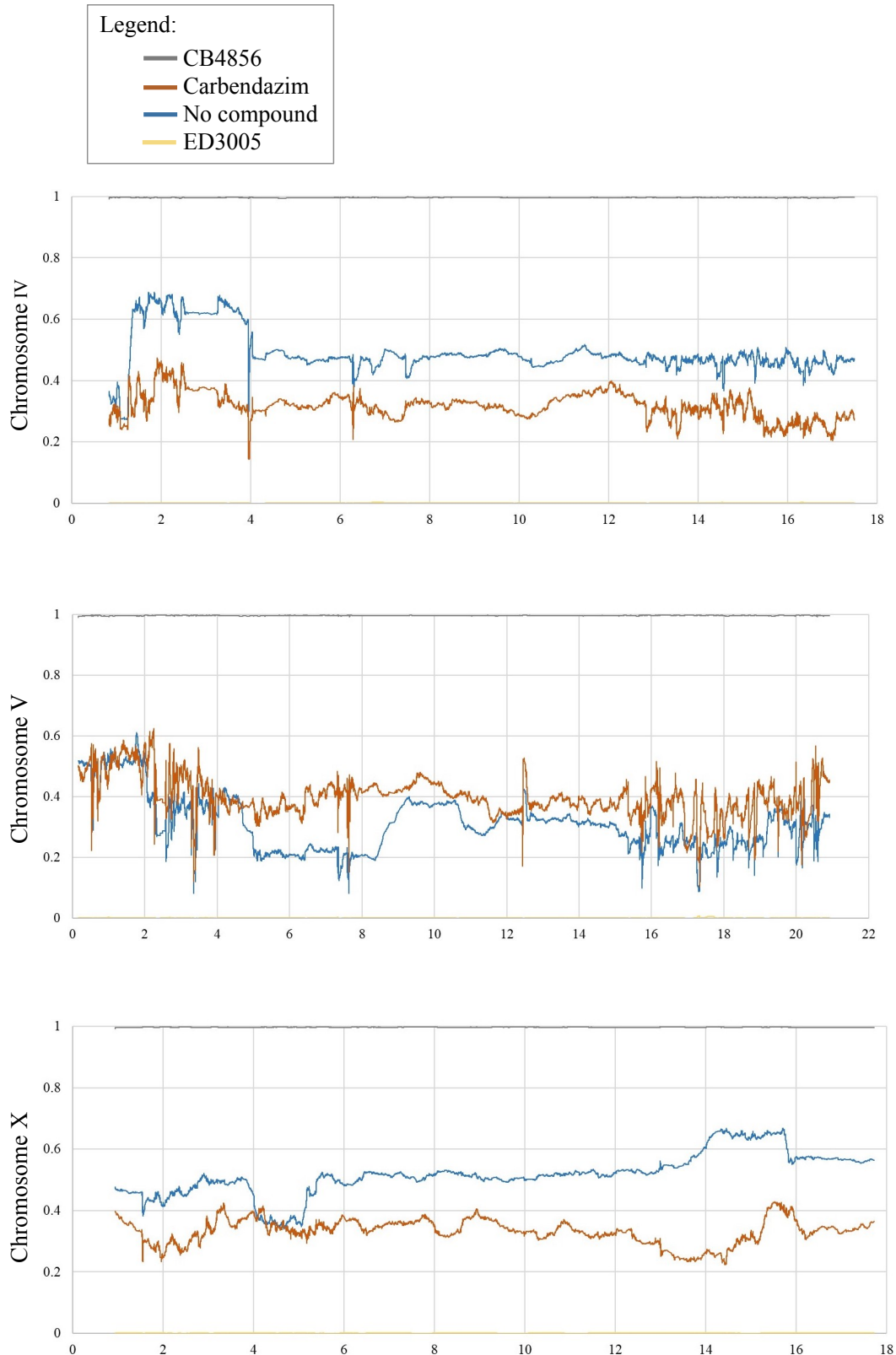


Figure 5.4: SNP linkage map of CB4856 SNP frequencies in 4 different samples: CB4856 (grey), ED3005 (yellow), no compound selected cross-progeny (blue), carbendazim selected cross progeny (orange). The results highlight chromosome III as important in carbendazim-resistance in *C. elegans* wild isolate ED3005 (highlighted with a red dashed line). SNP frequencies are plotted as running averages of every 255 frequencies.

5.3.4. Resistance to carbendazim in wild isolates is conferred by different polymorphisms in a *C. elegans* β -tubulin

The linkage area on chromosome III identified 19 potential targets, listed in Table 5.1. Genes among the ones identified encompass a variety of functions. Importantly *ben-1*, a β -tubulin gene of *C. elegans*, which has been implicated in carbendazim and benzimidazole resistance before in both *C. elegans* and pest species (Driscoll *et al.*, 1989; Cabanas *et al.*, 2009; Xu *et al.*, 2015; Hahnel *et al.*, 2018) is among the targets identified.

Table 5.1: Targets identified from SNP linkage map for carbendazim-resistance in ED3005.

Gene name	Description
<i>ben-1</i>	Tubulin β chain [Source:UniProtKB/TrEMBL;Acc:Q18817]
<i>C34C12.1</i>	Uncharacterised
<i>C34C12.2</i>	Uncharacterised
<i>cutl-25</i>	CUTiclin-Like [Source:UniProtKB/TrEMBL;Acc:Q7KWZ4]
<i>golg-4</i>	GOLGi associated coiled-coil protein homolog [Source:UniProtKB/TrEMBL;Acc:G5EEK2]
<i>H06I04.3</i>	Putative rRNA methyltransferase [Source:UniProtKB/TrEMBL;Acc:Q5CCJ2]
<i>H06I04.5</i>	Protein-tyrosine-phosphatase [Source:UniProtKB/TrEMBL;Acc:Q9N5P3]
<i>H09G03.1</i>	Uncharacterised
<i>hecw-1</i>	HECT, C2 and WW domain-containing ubiquitin ligase [Source:UniProtKB/TrEMBL;Acc:G5EEJ0]
<i>K01A11.3</i>	Uncharacterised
<i>phf-15</i>	PHd Finger family [Source:UniProtKB/TrEMBL;Acc:Q8IAA4]
<i>rpl-37</i>	60S ribosomal protein L37 [Source:UniProtKB/Swiss-Prot;Acc:P49622]
<i>Y53G8AL.1</i>	FHA-domain containing protein L37 [Source:UniProtKB;Acc: Q9N3H4]
<i>Y53G8AL.2</i>	NADH Ubiquinone oxidoreductase Fe-S protein [Source:UniProtKB;Acc: Q9N3H3]
<i>Y53G8AM.8</i>	HTH CENPB-type domain-containing protein [Source:UniProtKB;Acc: Q9BL70]
<i>Y53G8AR.1</i>	Uncharacterised
<i>Y53G8AR.7</i>	MFS domain-containing protein [Source:UniProtKB;Acc: Q9N3G2]
<i>Y53G8B.3</i>	Uncharacterised

No reports of changed benzimidazole susceptibility exist for any of the targets identified apart from the aforementioned *ben-1*, therefore as a next step I decided to sequence the *ben-1* gene in ED3005 to identify whether any sequence changes in comparison to the sensitive N2 strain were present.

As the chemical susceptibility screen, outlined in Chapter 3, had indicated the presence of two further carbendazim-resistant wild isolates in the panel used, ED3012 and JU363, I decided to also sequence the *ben-1* locus in these strains.

I utilised a method of PCR coupled with Sanger sequencing to sequence the gene in all three wild isolates, identifying polymorphisms in *ben-1* in each case, resulting in frameshift mutations in all three wild isolates. The frameshift mutations in all three cases result in premature stop codons. In ED3005 and ED3012 the shift resulting in Lys58fs and Gly93fs respectively, is introduced in exon 2 and in JU363 the mutation, causing Gly319fs, lies in exon 4 as can be seen in Figure 5.5. The full sequences of *ben-1* in JU363, ED3012, and ED3005 can be viewed in Appendix II.

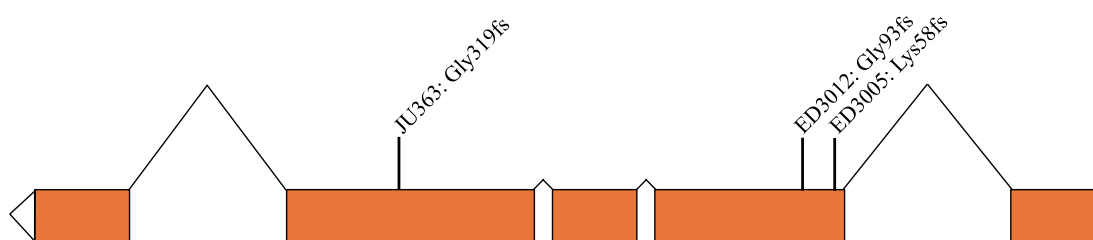


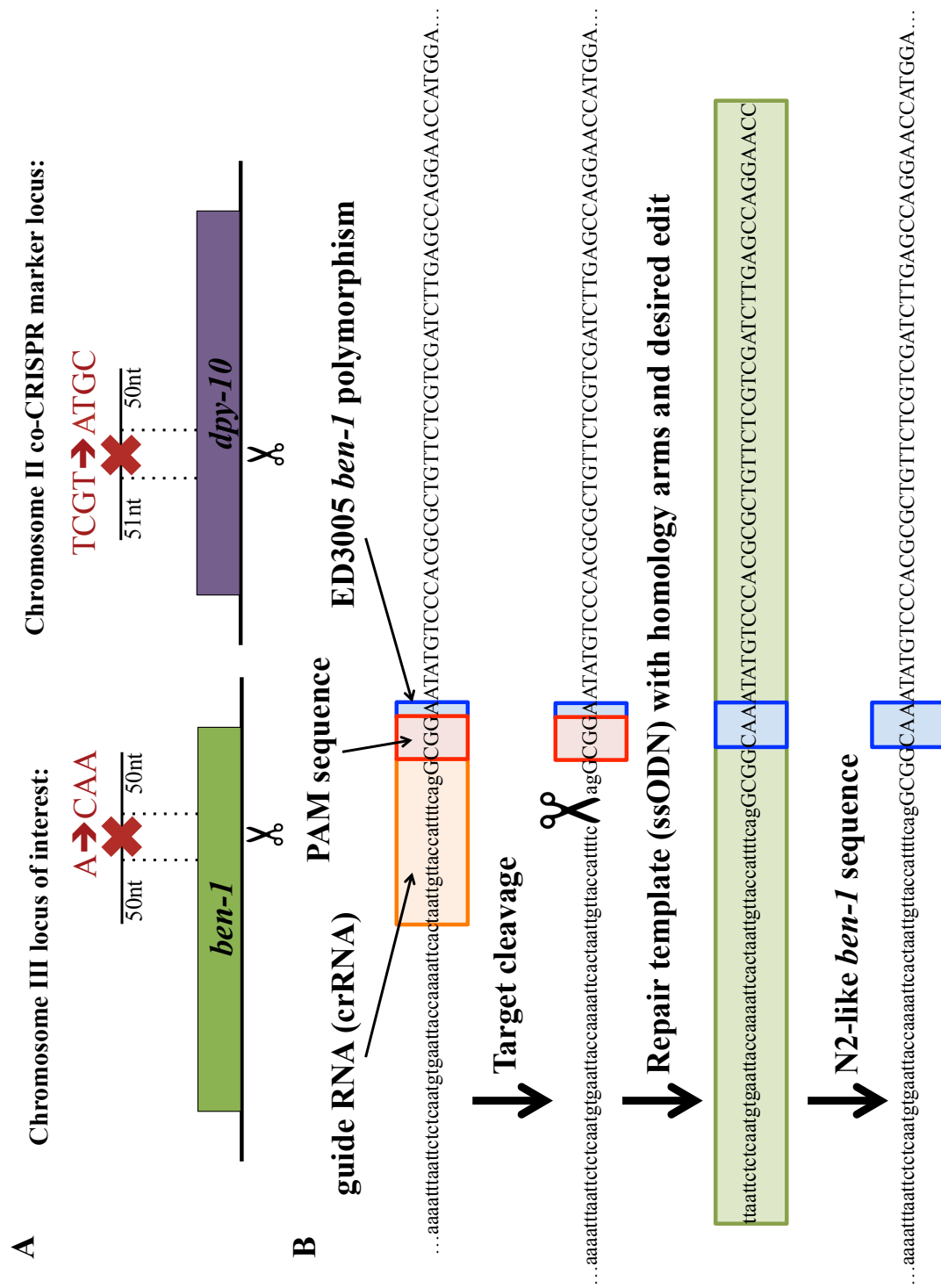
Figure 5.5: Schematic of the coding sequence of *ben-1* with mutations conferring carbendazim-resistance in JU363, ED3012, and ED3005 highlighted. 5' and 3' UTR are shown as white rectangle and triangle respectively. Five exons are shown as orange blocks, and introns as thin lines between blocks.

5.3.5. Genome editing to assess the contribution of a single polymorphism to carbendazim-resistance in ED3005

5.3.5.1. Design of experiment

In order to confirm the Lys58fs polymorphism in ED3005 as a sole contributor of the carbendazim-resistance phenotype, I designed a genome editing experiment using the CRISPR-Cas9 system to modify the ED3005 polymorphism, resulting in the Lys58fs, into the N2-like sequence at the locus, resulting in an A to CAA change in the sequence of *ben-1* (i.e. insertion of two bases).

I decided to utilise a *dpy-10* co-CRISPR strategy (Paix *et al.*, 2015) due to the increased ease of tracking edits. The design of the genome editing experiment is shown in Figure 5.6. In this experimental design I co-targeted, in the wild isolate ED3005, the *dpy-10* locus with a single-stranded oligonucleotide (ssODN) repair template that would introduce a missense mutation leading to a dominant roller phenotype (Arribere *et al.*, 2014) and the *ben-1* exon 2 resistance-conferring polymorphism locus with a ssODN repair template to change the site to the N2 *ben-1* sequence. The gonads of 60-80 hermaphrodites were injected and the broods examined for the presence of rollers (*dpy-10* edits). Jackpot broods, the broods with the highest number of rollers, were then identified. Edits at the *ben-1* locus were expected to be present within the broods with rollers and in both roller and non-roller worms. The presence of edits at the *ben-1* locus were to be determined using PCR primer pair where the forward primer was specific to the edited N2-like *ben-1* sequence and reverse primer annealed in an unedited locus producing a 200bp fragment. The worms with confirmed edits would then be allowed to self in order to obtain lines homozygous at the site of interest.



(Figure continued on the next page)

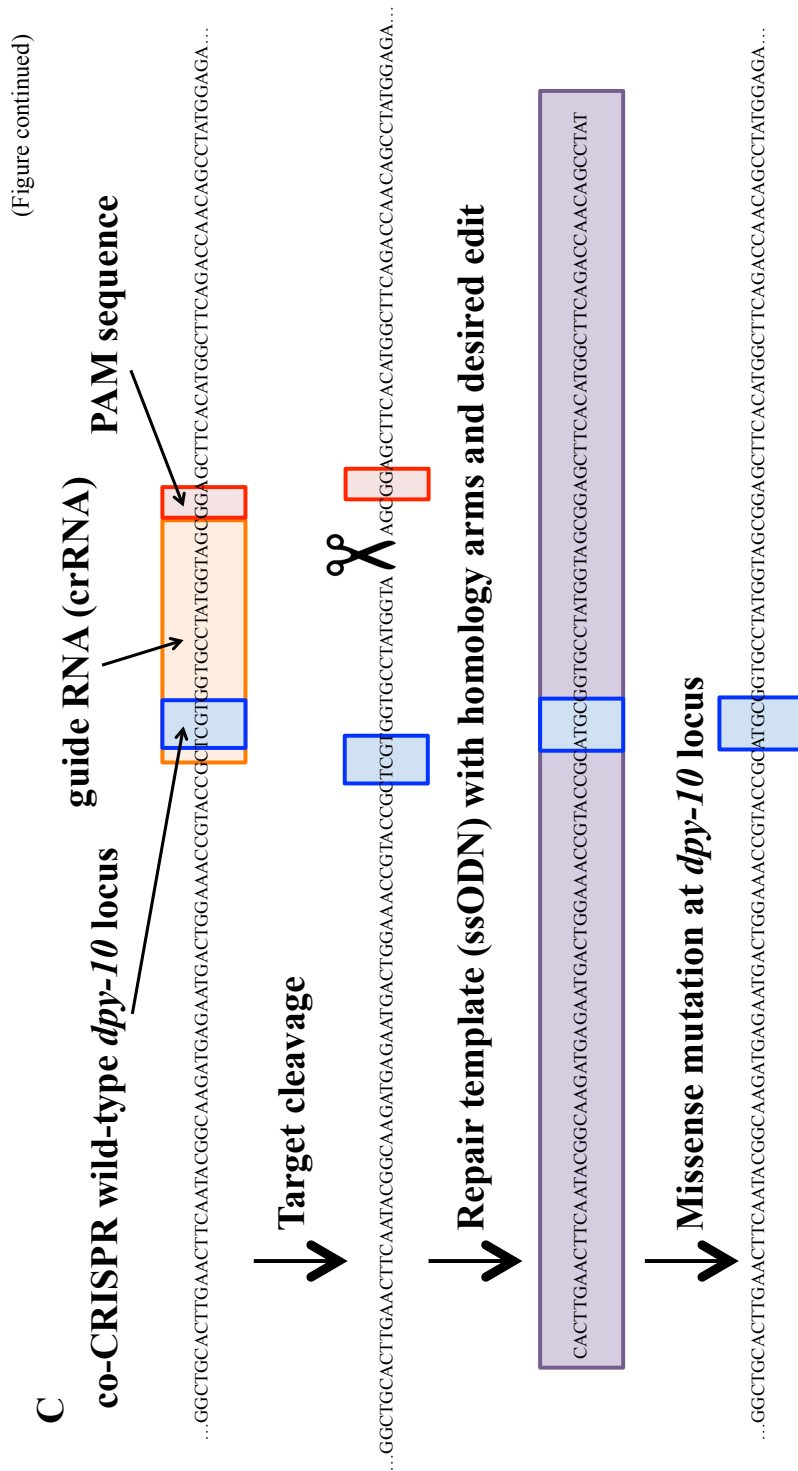


Figure 5.6: Outline of the genome edits introduced using CRISPR-Cas9 ribonucleoprotein complexes at *ben-1* and *dpy-10* sited in ED3005 (A). Edits performed at the *ben-1* site are shown in B, with red indicating the PAM sequence used, orange the sequence recognised by the guide RNA, blue indicating the locus edited, and green showing the repair template used to introduce the edit. Strategy for edits at *dpy-10*, the co-CRISPR site, are shown in C, with red showing the PAM sequence used, orange the sequence recognised by the guide RNA, blue the locus edited, and purple the repair template used to introduce the edit.

5.3.5.2. Results

The genome editing experiment yielded multiple broods with roller worms indicating that edits at the *dpy-10* site had occurred. Within these broods I expected to be able to identify some worms with edits in our gene of interest, *ben-1*. Unfortunately, the PCR-screen did not detect any N2-like edits at the locus of interest in over 70 worms screened utilising primers where one was designed to anneal to the edited *ben-1* site and another to a downstream not edited site. They were confirmed, by PCR, to be unedited and contain the ED3005 polymorphism at the site using primers specific to unedited *ben-1* and a not edited downstream site.

Ultimately, the editing at the *dpy-10* site was effective but no edits were observed at the *ben-1* locus. Potential explanations for this include issues with the PAM site chosen and sgRNA used with the site chosen unable to support sufficient cutting to stimulate homology-directed repair. However, the guide RNA was designed to end with GG to align with recommendations to yield high editing efficiencies in *C. elegans* (Farboud and Meyer, 2015). An alternative explanation is that no changes were introduced to the guide RNA annealing site using the repair template in our experimental design making it possible for the guide to enable cutting after the edit had already been introduced by integration of the repair template (Paix *et al.*, 2015). It is highly unlikely that the edit at the gene of interest would have not been viable in *C. elegans* as sequencing confirms the presence of the exact sequence in Bristol N2 strain. Finally it is possible that the primers chosen to genotype the worms from jackpot broods were not optimal to identify heterozygous edits.

Utilising a carbendazim-susceptibility phenotype for screening may have yielded better results with unedited ED3005 worms standing out as resistant to the exposure of carbendazim, and worms homozygous for the introduced edit at *ben-1*

becoming fully susceptible to the compound. Additionally, worms with heterozygous edits at the locus of interest could have been either screened as resistant (if no homozygous edits were identified) or sensitive (if homozygous edits were identified), depending on compound concentration used due to the semi-dominance of the trait. I chose the *dpy-10* co-CRISPR strategy so that worms where the injection had been successful in introducing edits in the progeny could be identified with ease, in order to negate my lack of experience with worm injections prior to the experiment.

Overall, an improved alternative screening process could have relied on utilising *dpy-10* co-CRISPR to identify plates where edits have occurred, with progeny on those plates then additionally screened on compound. However, due to time constraints of the project, freedom to test alternative strategies was limited.

5.3.6. SNP linkage highlights regions of Chromosome III and V as important in conferring resistance to carbendazim in AW1783

The EMS induced carbendazim-resistant mutant AW1783 was also used in the SNP linkage mapping experiment to identify the genetic basis of the phenotype recovered from the EMS screen. The results of the experiment suggested linkage to chromosome III and also a segment of chromosome V, as can be seen in Figure 5.7.

The SNP ratio data was filtered, as described in paragraph 5.3.3, including only the CB4856 SNP ratios that were ≥ 0.95 and AW1783 SNP ratios ≤ 0.05 with the running average across 255 of remaining SNP ratios graphed across 4 datasets. Figure 5.7 outlines the running averages of the unique CB4856 SNP ratios of all six chromosomes of CB4856 (grey), AW1783 (yellow), carbendazim-selected cross-progeny (purple), and the no compound selected cross-progeny (green).

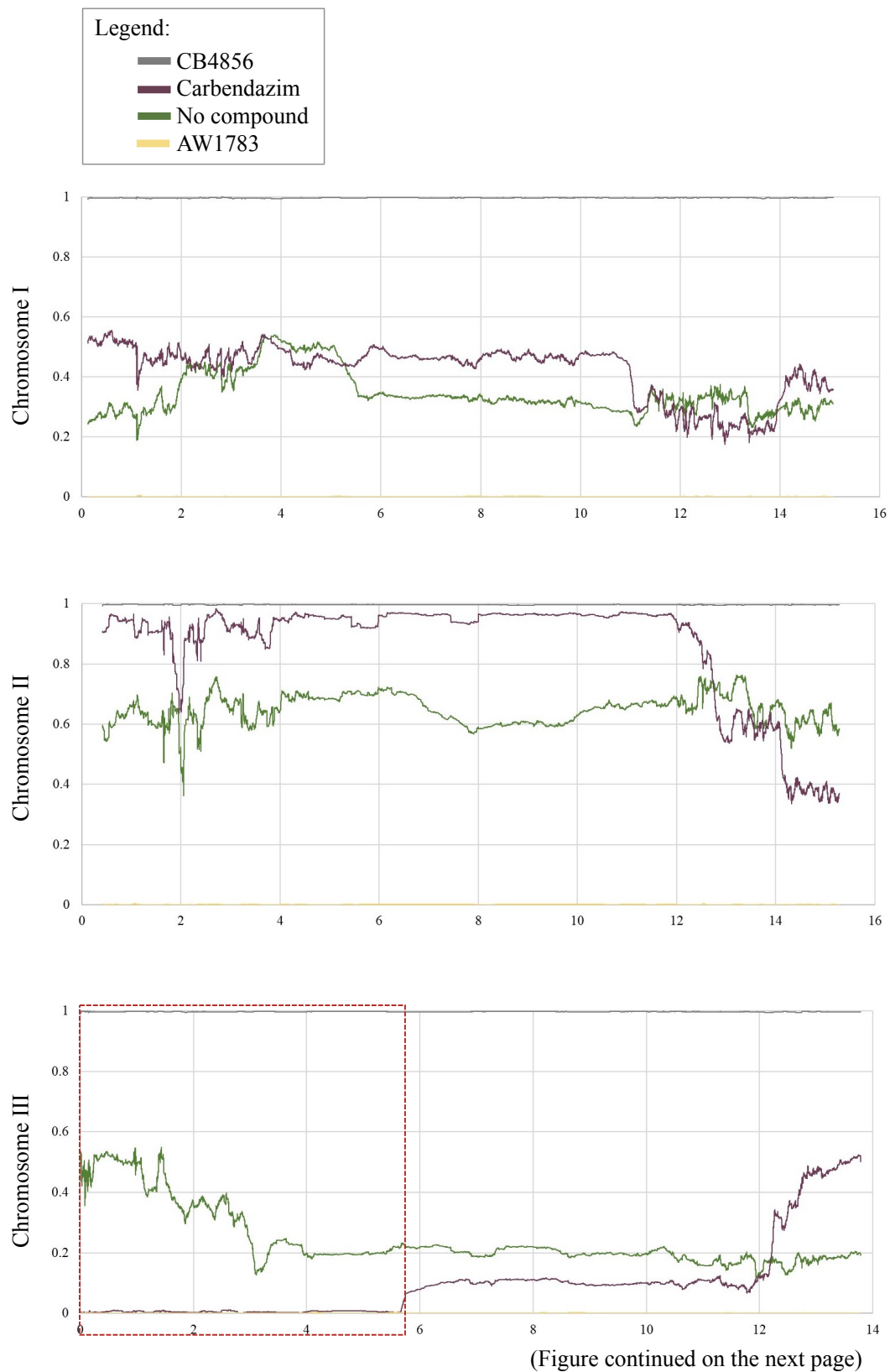
In general, the SNP linkage maps obtained from this experiment are less clean than those obtained using ED3005, with SNP ratios that are not as expected, not around 0.5, in areas other than regions of chromosome III and V that are linked with phenotype. This may be due to a different sample preparation prior to sequencing where the samples were pooled prior to DNA preparation rather than after (as was done in the ED3005 experiment). Pooling samples later ensures that an equal amount of DNA from cross-progeny is included as all samples are prepared separately until the creation of the final mixture, where equal amounts of DNA from all samples is combined. While this method has been reported to work and result in clear SNP linkage maps (Doitsidou, Jarriault and Poole, 2016), it may also result in unequal amounts of DNA from the different cross-progeny being incorporated in the sequencing sample.

The linkage maps reveal two genomic regions that are selected for in carbendazim. These lie on the left arm of chromosome III and right arm of chromosome V. The chromosome III region spans a length of about 6 Mb. It again encompasses the *C. elegans* β -tubulin gene *ben-1*, which has previously been implicated in carbendazim-resistance.

In addition, however, a region of chromosome V, encompassing 12 target genes, also appears to be linked to carbendazim-resistance. None of the targets implicated in the region have been linked to pesticide resistance in the past and the region of chromosome V does not overlap with regions of the chromosome that underlie nematode responses to benzimidazoles (Zamanian *et al.*, 2018) but does, however, overlap with a region highlighted in nematode responses to albendazole (Hahnel *et al.*, 2018). There is also overlap with results on *C. elegans* diverse toxin responses, with the region important in varying susceptibilities to some pesticides,

heavy metals, and chemotherapeutics (Evans *et al.*, 2018), implying that the area may be important in detoxification. I also see highly fluctuating SNP ratios on chromosomes arms consistent with high recombination rates reported in the regions (Rockman and Kruglyak, 2009).

As sequencing of *ben-1* utilising a PCR and Sanger sequencing combination had been successful in identifying the resistance-conferring polymorphism in ED3005, a similar strategy was tried with AW1783. While I was able to isolate a fragment of 200bp using the *ben-1* primers used for ED3005, the Sanger sequencing of the fragment yielded results that did not resemble *ben-1* or any other region of *C. elegans* genome based on Blast searches of nucleotide sequences. This was the result of multiple separate attempts. This resulted in the strategy not being pursued further to identify specific genetic basis of carbendazim resistance in AW1783.



(Figure continued)

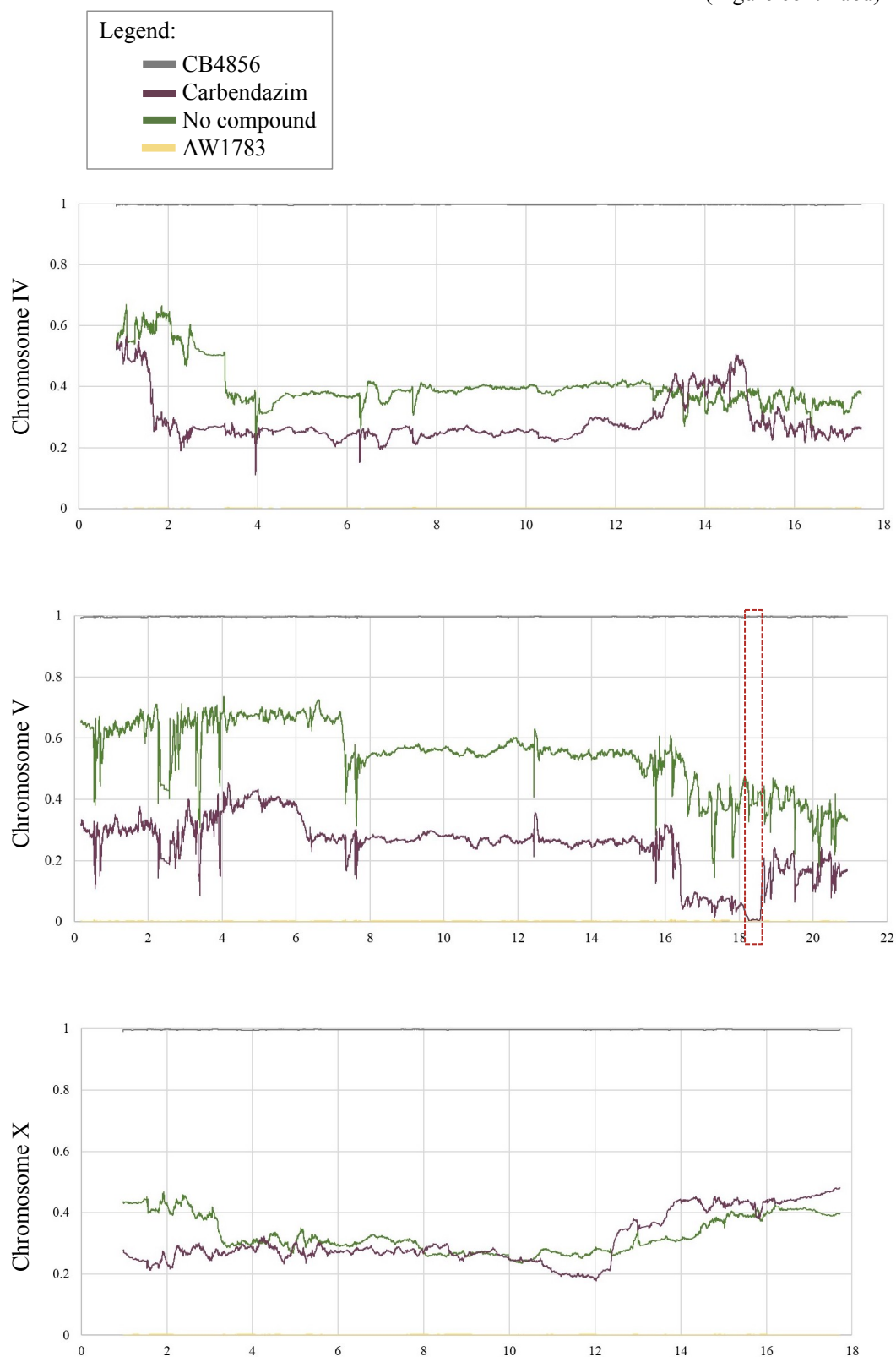


Figure 5.7: SNP linkage map highlighting Chromosome III and Chromosome V as important in carbendazim-resistance in a chemically induced *C. elegans* mutant AW1783 (highlighted with a red dashed line). SNP frequencies are plotted as running averages of every 255 frequencies.

5.3.7. Measuring the rate of pesticide resistance emergence in competition experiments

Competition experiments, a class of evolution experiments, enable the direct comparison of two different genotypes with a limited number of identifiable genetic differences. While competition is implicit in most evolution experiments, competition experiments stand out as the particular parameters (such as starting frequencies and environmental conditions) can be more tightly controlled. Ultimately this means that the experimenter can utilise this set-up to assess the fitness of specific genotypes in a clearly defined context (Teotonio *et al.*, 2017). In regards to studying pesticide resistance and the dynamics of its emergence, this provides an experimental tool to identify the effect of different starting frequencies of resistant individuals and different compound concentrations on the rate of spread of resistance.

5.3.8. Selecting a sensitive strain to assess the competitive fitness of the carbendazim-resistant AW1783

In addition to utilising the chemically mutagenised carbendazim-resistant mutant AW1783 in SNP linkage mapping to identify the genetic basis of the resistance observed I wanted to understand how the strain would fare in different field realistic exposures in comparison to a sensitive strain in a competition experiment. AW1783 was suitable for this study due to its Bristol N2 background, meaning that it could be compared to other strains of the same background to specifically assess the fitness of the carbendazim resistance conferring alleles in a multitude of environments. It would have been more difficult to perform a competition assay utilising a resistant wild isolate as a marker strain of the same background would have

had to be created. As many suitable marker strains with Bristol N2 background already exist, I was able to make a choice between them.

In order to enable visual tracking of population changes once the competition experiment had begun I wanted to identify a GFP-tagged *C. elegans* strain that was sensitive to carbendazim. I chose a strain with a strong pharyngeal GFP signal (*myo-1::gfp*) and a strain with a strong body wall muscle GFP signal (*myo-3::gfp*) to identify whether either would be suitable for the experiment based on their susceptibility to carbendazim.

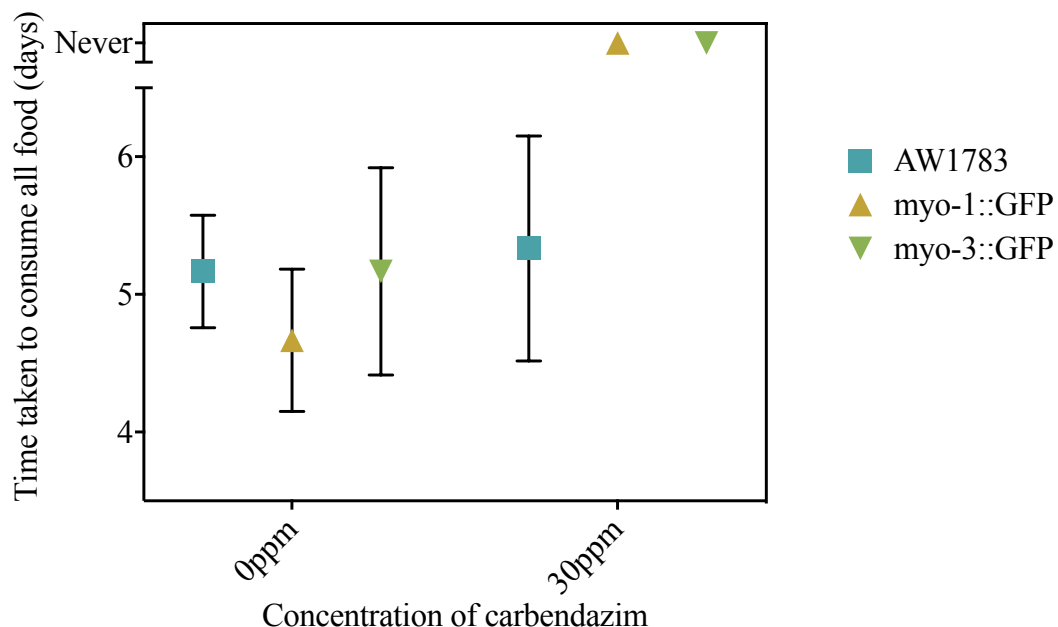


Figure 5.8: Results of a food consumption assay in 0 and 30ppm carbendazim using AW1783 (blue), and *myo-1::GFP* (yellow) and *myo-3::GFP* (green) expressing strains (n=6). The graph indicates similar sensitivity in GFP-tagged strains making either of the GFP-tagged strains suitable for use in a competition experiment against a chemically induced carbendazim-resistant mutant AW1783. One-way ANOVA followed by Tukey's multiple comparisons test does not reveal any significant differences between strains in food consumption in no compound environment. Values are plotted showing the mean of the responses $\pm$ SD.

Results of the food consumption race in 0 and 30ppm carbendazim, shown in Figure 5.8, indicate that both GFP-tagged strains perform similarly in the

benzimidazole environment. No significant differences in food consumption in no compound were discovered in a one-way ANOVA followed by Tukey's multiple comparisons test between any strains. The two GFP-tagged strains were evenly matched based on compound susceptibility and were unable to finish food at 30ppm carbendazim similarly to other N2 strains. I decided to choose the pharyngeal GFP strain (myo-1::GFP) due a clearer fluorescence signal in comparison to the body wall muscle line, which would enable faster worm counting in the competition experiment.

5.3.9. Comparison of the fitness of carbendazim-resistant AW1783 and a sensitive marker strain in carbendazim and no compound

A competition experiment aims to compare two phenotypes in different environments to try to understand the relative fitness of both in response to treatments. In the context of comparing the carbendazim resistance phenotype observed in AW1783 and a high susceptibility phenotype to the same compound in a GFP-tagged line, it enables the investigation of the relative fitness and dynamics of the two strains in no compound and compound environments. Testing different compound exposures can provide an experimental estimate of the potential rate of resistance emergence when different exposures are tested.

5.3.9.1. Compound concentration and exposure affect the rate of spread of cost-free resistance through populations

Firstly, I was interested in the fitness of the two strains when placed in a population at equal proportions under different compound concentrations and different exposures over five generations.

The result of a five-generation exposure to no compound on a 50% resistant and 50% sensitive population indicated that the carbendazim-resistant AW1783 does not perform any worse in no compound than the sensitive GFP-tagged strain, as shown in Figure 5.9. This means that the carbendazim-resistant AW1783 appears to not carry a relatively higher fitness cost in the no compound environment. The finding is another discovery of no cost carbendazim-resistance, with the other identified in the selected lines studied in Chapter 4.

I also wanted to compare the responses of the same starting population to three different compound exposures:

1. constant 10ppm carbendazim;
2. constant 30ppm carbendazim;
3. intermittent 30ppm carbendazim and no compound.

Firstly, I wanted to characterise the changes in 10ppm carbendazim to understand how rapidly the proportion of resistant and sensitive worms can change under compound selection. Secondly, I wanted to observe the effect of increasing the concentration to 30ppm on selection for resistance in a population. Finally, I was interested in the effect of intermittently exposing the population to carbendazim (generations 1, 3, and 5) and no compound (generations 2, and 4) in order to understand how changing compound exposure affects the proportion of resistant individuals in a population across generations, aiming to understand how applying pesticide across generations in the field would affect the spread of resistance alleles.

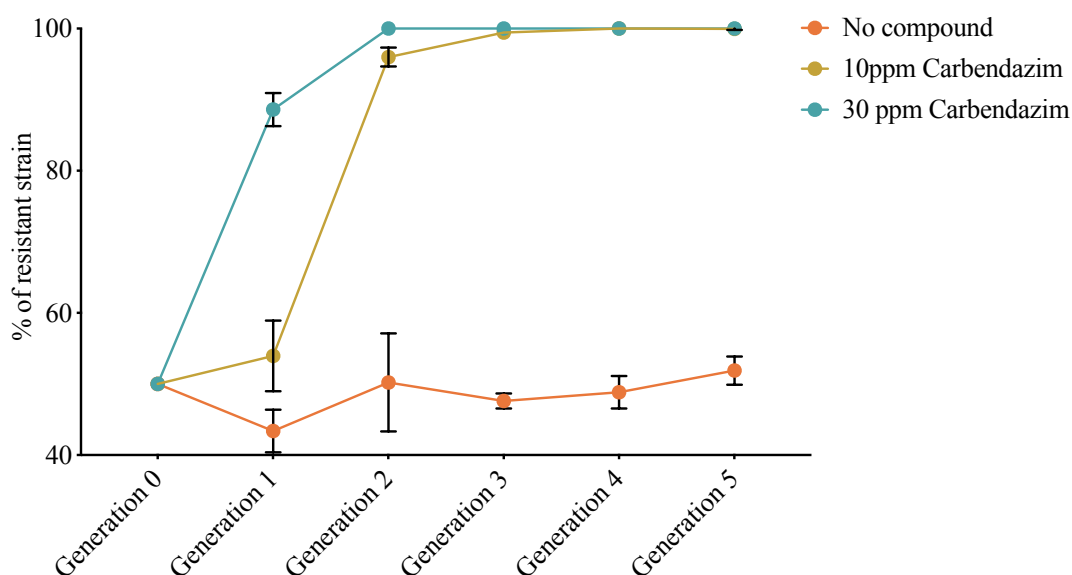


Figure 5.9: Results of the competition experiment, as percentage of resistant strain present, of a 50% resistant and 50% sensitive starting population in three different conditions over 5 generations: no compound (orange), 10ppm carbendazim (yellow), 30ppm carbendazim (blue) (n=18). Values are plotted showing the mean of the responses for all replicate lines $\pm$ SD.

In response to 10ppm carbendazim the 50% resistant and 50% sensitive starting population showed the fixation of resistance by generation 3, indicating the very rapid spread of resistance through the population. The results at this concentration, shown in Figure 5.9, enabled us to observe a sigmoidal increase in the proportion of resistant worms in the population.

Using a higher concentration of 30ppm resulted in the fixation of the resistant AW1783 in the population by generation 2, as shown by Figure 5.9. This indicates a dose response with the higher concentration imposing stronger selection for the resistant strain as shown by a reduction in the time taken for the resistant strain to become fixed.

The slowest disappearance of sensitive animals from the population was observed when the population was intermittently exposed to 30ppm carbendazim and no compound. The sensitive worms disappeared from the population by generation 4

under those experimental conditions, as indicated in Figure 5.10. In addition, the results again imply that resistance is not costly, as loss of resistance is not observed when the selection pressure of compound is lost at generations 2 and 4.

The results indicate that the resistant proportion increases in the population rapidly and the speed with which this happens depends on compound concentration, with higher concentration increasing speed, and duration of exposure, with breaks in exposure resulting in a slower rate. However, the differences in time taken between different exposures are subtle. This may be due to the high proportion of resistant worms already present in the starting population.

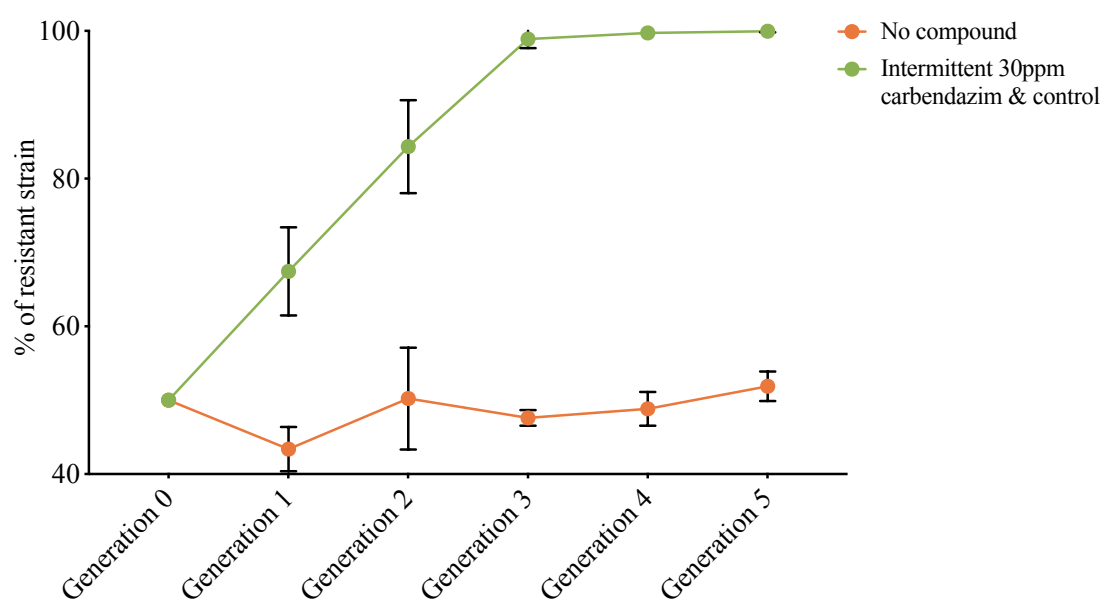


Figure 5.10: Population changes, as percentage of resistant strain present, over five generations in a competition experiment of a 50% resistant and 50% sensitive starting population in no compound (orange) and intermittent 30ppm carbendazim and no compound (green) (n=18). Values are plotted showing the mean of the responses for all replicate lines $\pm$ SD.

5.3.9.2. *Even a high proportion of sensitivity in the starting population completely disappears within five generations of compound exposure*

As the prior results only focused on changes in a population with an already high proportion of resistance, I was also interested in how quickly an overall sensitive population could become resistant if only 1% of the initial population was already resistant. By exposing a population that consisted of 99% of sensitive GFP-tagged worms and 1% of resistant AW1783 to 30ppm carbendazim, I discovered that the sensitive worms completely disappeared from the population within 5 generations of compound exposure, as shown by Figure 5.11. During the final two generations the rate at which sensitive worms were disappearing from the population appeared to be slowing down in comparison to the first three generations.

Exposing the same starting population to no compound resulted in no significant changes over five generations. The resistant worms were maintained at low frequencies in the population suggesting that comparatively, in no compound, AW1783 did not have a fitness advantage.

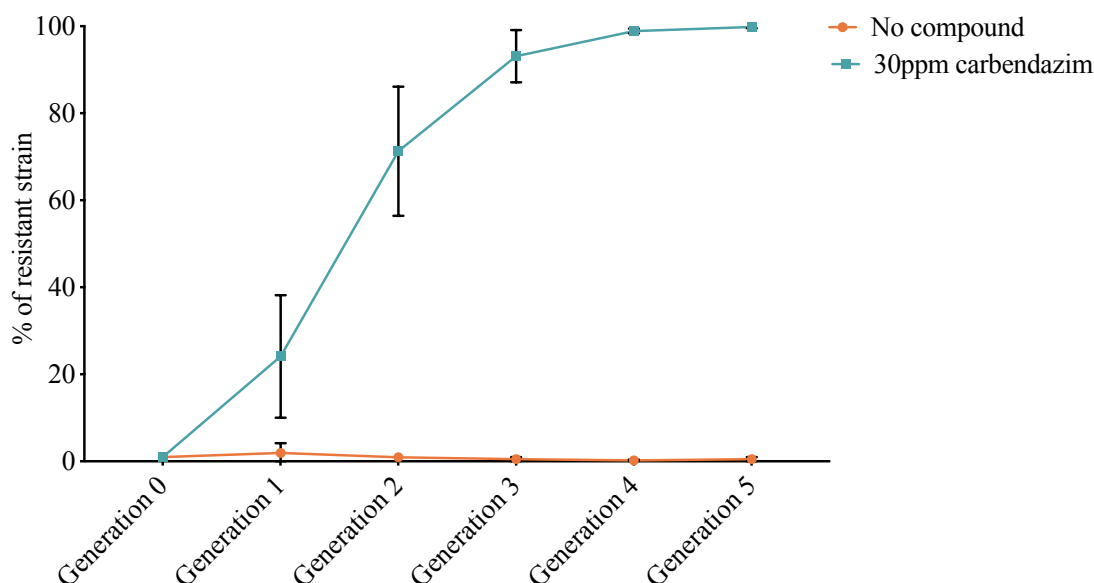


Figure 5.11: Results of a competition experiment of a 1% resistant and 99% sensitive population in carbendazim and no compound indicating the change in % of resistant strain (AW1783) across 5 generations (n=18). Population changes are shown as percentage of resistant strain present. Population changes in no compound are shown as an orange line and changes in 30ppm carbendazim are shown as a blue line. Values are plotted showing the mean of the responses for all replicate lines $\pm$ SD.

5.3.10. The molecular basis of evolved resistance in the polymorphic

Portuguese strain

Chapter 4 outlined the results of a selection experiment where a modified highly heterogeneous *C. elegans* strain was exposed to different compound environments for 15 generations, as a result of which, in 30ppm carbendazim, the strain adapted to the new environment. While I investigated the fitness cost of this adaptation, questions relating to the genetic basis of this resistance remained unanswered. As outlined in Chapter 4, I was interested in identifying whether the resistance emergence observed arose from standing genetic variation within the highly polymorphic *C. elegans* strain or from *de novo* mutations.

Given my findings in this chapter have strongly linked *ben-1* polymorphisms to resistance and, importantly, cost-free resistance in *C. elegans*, as also observed in

the experimentally selected lines, I wanted to identify whether *ben-1* alleles might explain carbendazim resistance observed in the selected lines and whether that resistance arose from standing natural genetic variation present in the population.

First I identified, by using CeNDR (Cook *et al.*, 2017), that two out of the 16 *C. elegans* wild isolates used in the original derivation of the polymorphic Portuguese strain, MY16 and JU346, had a naturally occurring *ben-1* mutation. The location and effect of these polymorphisms are illustrated in Figure 5.12. MY16 has a naturally occurring substitution resulting in a single change in amino acid sequence from alanine to proline while JU346 contains a missense mutation that results in a premature stop codon. These wild isolates were not tested for carbendazim sensitivity in the original chemical susceptibility screen (Chapter 3).

I decided to amplify the *ben-1* region in all six lines selected on carbendazim for 15 generations, using worm lysis of 50 adult worms from generation 15 lines of the selection experiment as template DNA, and sequenced the amplified product to identify polymorphisms in the region. I was able to confirm that all carbendazim-selected lines had the MY16-like *ben-1* polymorphism resulting in an Ala185Pro substitution, suggesting that the emergence of resistance during the selection experiment could be explained by standing natural variation in the population rather than the introduction of new allelic combinations or mutations in the population. The sequence of the MY16-like *ben-1* is shown in Appendix II.

It is interesting that no JU346-like polymorphisms were identified suggesting either that the polymorphism ended up not being incorporated into the final population during the creation of the Portuguese strain (discussed in Chapter 4 introduction) or that selection for the MY16 polymorphism was possibly advantageous by providing a noticeably higher advantage in the compound

environment relative to the JU346 SNP. As around 70-80% of variation was determined to be of parental origin with not all polymorphisms incorporated during the funnel cross of EEV-A₀ derivation (Teotonio *et al.*, 2012), it seems likely that the JU346 *ben-1* allele was not present during the selection experiment.

I additionally used the same protocol to amplify *ben-1* in pools of 50 worms from generations 10, 5, and 0 of the selection experiment and was able to see the MY16 polymorphism in the generation 10 lines but no *ben-1* polymorphisms in the generation 5 or 0 lines. This suggests that, as a significant level of resistance was observed at generations 5 and 10, while generation 0 was sensitive to the compound, a slight increase in the proportion of worms carrying the resistance-conferring allele in the population results in a strong phenotype change and that generation 5 from the selection experiment contained a mixture of carbendazim-sensitive and resistant worms, but below the level of detection using PCR on pools of animals. This finding fits with the results of the competition experiment using carbendazim resistant AW1783 and a sensitive GFP-marker strain, which indicated rapid fixation of resistance in the population under compound selection. As these results only give a rough idea of the changes occurring in allele frequencies in populations, the results could be improved by using methods that would quantify these fluctuations.

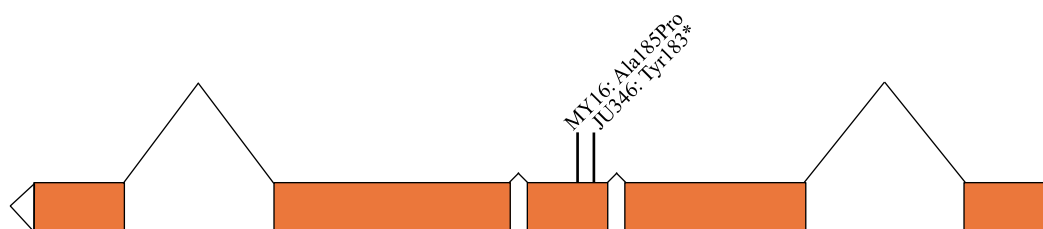


Figure 5.12: Schematic of coding sequence of *ben-1* with mutations conferring carbendazim-resistance in wild isolates used in the funnel cross to create EEV-A₀ (Portuguese strain), MY16, and JU346, highlighted. 5' and 3' UTR are shown as white rectangle and triangle respectively. Five exons are shown as orange blocks, and introns as thin lines between blocks.

5.3.11. Summary of the genetic basis of benzimidazole resistance from different biological events

Overall, I have identified the genetic basis of carbendazim-resistance in wild isolates ED3005, ED3012, JU363 as well as in the lines selected on carbendazim for 15 generations, with polymorphisms in a *C. elegans* β -tubulin gene *ben-1* implicated in resistance in all strains. These are all illustrated in Figure 5.13. While I was unable to specify the genetic basis of resistance in AW1783, a chemically induced carbendazim-resistant mutant, a SNP linkage experiment again implicated *ben-1* as well as a region of chromosome V associated with detoxifying mechanisms to the mechanism of this no cost resistance.

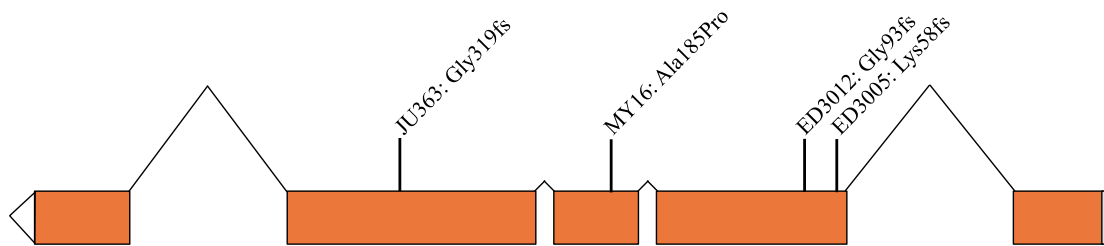


Figure 5.13: A schematic of coding sequence of *ben-1* highlighting polymorphisms conferring carbendazim-resistance in wild isolates JU363, ED3012, ED3005 and showing the location of the MY16 *ben-1* variant that confers carbendazim-resistance in the experimentally selected lines. 5' and 3' UTR are shown as white rectangle and triangle respectively. Five exons are shown as orange blocks, and introns as thin lines between blocks.

5.4. Discussion and future work

5.4.1. Carbendazim-resistance from three separate biological events converges on the same genomic location

This experimental work characterised the genetic basis of carbendazim-resistance in strains from three separate biological events. I found that the phenotype converged on the *ben-1* locus in all cases and importantly no fitness cost of carrying the resistance-conferring mutation was detected.

In the case of *C. elegans* wild isolates ED3005, ED3012, and JU363, naturally occurring biologically balanced polymorphisms result in frameshift mutations and introduction of premature stop codons within the *ben-1* sequence. In the experimentally selected carbendazim-resistant lines a MY16-originating *ben-1* polymorphism that results in a single amino acid substitution confers strong carbendazim-resistance. Finally, in the chemically mutagenised resistant variant AW1783, region of chromosome III containing *ben-1*, as well as a region of chromosome V implicated in detoxifying responses (Evans *et al.*, 2018) and benzimidazole-resistance (Hahnel *et al.*, 2018), is linked to the phenotype.

It is striking that the genetic cause of resistance in these three separate experimental set-ups converges on the same genetic basis. A possible explanation for this is the particularly low fitness cost of these mutations. In all instances the genome alterations that confer resistance inflict no changes in performance in a no compound environment.

5.4.2. Explaining the emergence of carbendazim-resistant wild isolates

Wild isolates ED3005, ED3012, JU363 are naturally resistant to benzimidazoles or carbendazim despite not being the target organism for their use and therefore expected to not have been exposed to the pesticide. This implies that the naturally occurring polymorphisms have spread through the population in very minimal compound exposure environments, implying that the fitness cost of carrying these mutations conferring carbendazim resistance is very low. The presence of such low cost resistance in natural populations is of course an issue as it highlights a problematic assumption of high cost of resistance alleles in resistance management strategies that rely on compound rotation (Ffrench-Constant, 2006). Where compound rotation is used, the proportion of resistance alleles is expected to decrease to pre-use levels when the selective pressure of the compound is removed due to the high fitness cost of resistance in the no compound environment.

Low-cost resistance would behave differently under these conditions. The proportion of resistance alleles would no longer decrease in the population once the compound selection is removed but would remain at a stable level until the same compound is utilised for pest control again, effectively resulting in accumulating resistance every time the compound is used. Pest populations with low cost resistance could be controlled instead by using alternative compounds, making sure that no cross-resistance is present.

In addition, these polymorphisms appear to have arisen and spread completely through population in three separate cases. This finding is in line with a recent report of extreme allelic heterogeneity at the *ben-1* site in *C. elegans* (Hahnel *et al.*, 2018). The emergence of these naturally resistant wild isolates has been suggested to be due to local selective pressures (Hahnel *et al.*, 2018). The results of the selection

experiment support this idea, that is, in general, difficult to test as no data is available on compound applications in any particular areas that *C. elegans* wild isolates are collected from. As I identified that the selection experiment resulted in selection for a resistance-conferring allele (MY16-like *ben-1*) present in the population within 10 generations, it is possible to see how a mutation in *ben-1* can rapidly spread through a population and remain balanced within said population upon loss of selective pressure. This notion is also supported by the results of the competition experiment, where resistance alleles were maintained in the population in the absence of compound in the environment but the spread of resistance through the experimental population was correlated with the presence of compound. The results of the evolution experiments can therefore be very useful to inform and experimentally support *in silico* modelling of resistance.

5.4.3. Characterisation of the benzimidazole binding pocket suggests how various polymorphisms in β -tubulin result in reduced susceptibility

It is important to note that not all *ben-1* polymorphisms confer resistance to carbendazim as demonstrated by the susceptible CB4856 that carries a missense mutation in exon five of the protein coding sequence (Hahnel *et al.*, 2018). A recent study, which for the first time characterised the active site of benzimidazoles using the fungi *Podosphaera xanthii* and molecular docking modelling, found that resistance-conferring mutations lie outside of the predicted binding pocket of the compound, as shown by Figure 5.14. Resistance is instead conferred by mutations that lie in structurally important locations and result indirectly in a conformational change at the

binding site (Vela-Corcía *et al.*, 2018). This explains the sensitivity observed in CB4856 despite the presence of a polymorphism in the gene sequence.

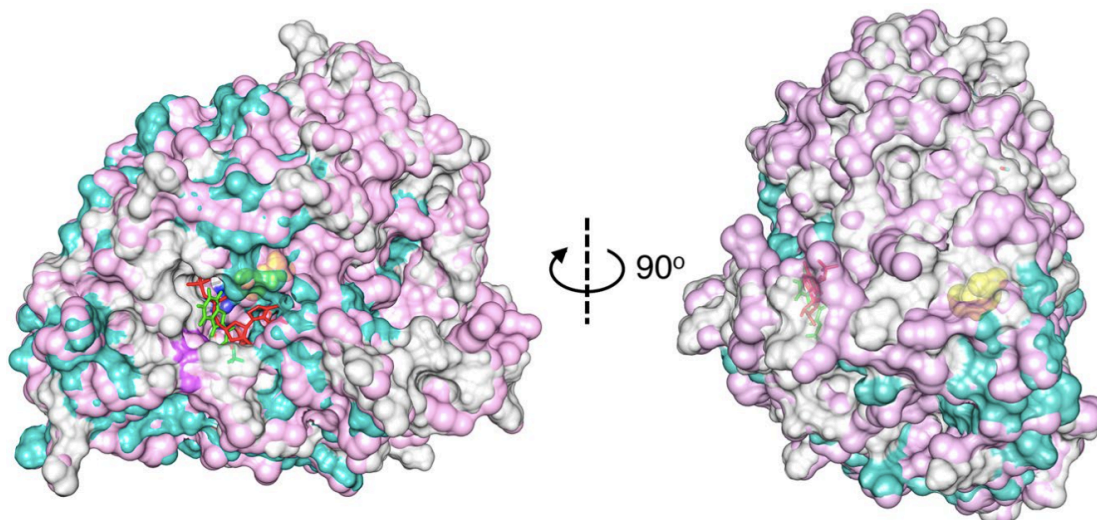


Figure 5.14: Superimposed surface models of mammalian β -tubulin model (cyan; PDB ID: 1JFF) in complex with GTP (red) and the *P. xanthii* β -tubulin model (pink) with carbendazim (green) docked in the proposed benzimidazole fungicide binding site. The alignment reveals a high level of coverage between models (grey) and reveals the proximity of the putative benzimidazole and GTP binding sites. Upon 90° rotation of the model, the residue 198 (yellow) that is involved in benzimidazole resistance in *P. xanthii*, as the E198A mutation, is shown to locate inside of the protein at a position that is separate from the proposed binding site. Figure from (Vela-Corcía *et al.*, 2018).

Based on our findings of widespread natural variants resistant to benzimidazoles, the future of the use of these compounds in the field appears uncertain. However, a potential solution is offered again by the characterisation of the binding site of benzimidazoles (Vela-Corcía *et al.*, 2018). As suggested by my results of colchicine-sensitivity in the lines selected on carbendazim, adaptation to carbendazim did not result in resistance to colchicine, indicating that colchicine has an alternative mechanism of action despite also targeting β -tubulin. This proposes the presence of an additional binding site on the benzimidazole resistance conferring β -tubulin or possibly another β -tubulin, where multiple β -tubulin genes are present and transcribed as in *C. elegans*. This hypothesis was confirmed in a comparison of the

binding sites of carbendazim and diethofencarb, a member of N-phenyl carbamates compound group in *P. xanthii* where resistance to carbendazim resulted in increased sensitivity to diethofencarb (Vela-Corcía *et al.*, 2018). This finding has been previously also reported in *Botrytis cinerea* and *Neurospora crassa* (Fujimura *et al.*, 1992; Leroux *et al.*, 1999) suggesting an effective method to delay new benzimidazole resistance by utilising a mixture of benzimidazoles and phenylcarbamates. This method was introduced in France as an anti-resistance measure in *Botryotinia fuckeliana*, but resistance to both compounds in mixture quickly evolved (Leroux *et al.*, 1999) suggesting that the conformation changes that result in benzimidazole resistance may delay the emergence of resistance to non-benzimidazole tubulin disruptors but do not fully prevent it.

It is unclear whether the binding sites of benzimidazole and colchicine or diethofencarb are located on the same β -tubulin proteins in *C. elegans* as the species has six β -tubulin genes: *ben-1*, *mec-7*, *tbb-1*, *tbb-2*, *tbb-4*, *tbb-6* (Hurd, 2018). While only mutations in *ben-1* have been shown to result in decreased susceptibility to benzimidazoles in *C. elegans*, other β -tubulins may confer sensitivity to alternative tubulin assembly disrupting agents, such as colchicine, in this species.

5.4.4. Importance of *ben-1* in carbendazim resistance of *C. elegans*

Our findings suggest that in most cases *ben-1* explains carbendazim-resistance in *C. elegans* but that other genomic regions may also be linked to the trait. The mapping results of the AW1783, EMS allele, highlighted linkage to a region of chromosome V previously also identified as a region contributing to benzimidazole-resistance traits in *C. elegans* (Hahnel *et al.*, 2018). Reports suggest that not all benzimidazole or carbendazim resistance observed is explained by *ben-1* variants but

does explain as much as 73.8% of phenotypic variation observed in susceptibility to the compound (Hahnel *et al.*, 2018) and our findings are very much in agreement with that. Results of a food consumption race comparing ED3005 and AW1783 neatly highlighted the additive effect of the chromosome V region in the resistance phenotype in the chemically mutagenised strain.

5.4.5. Replaying the tape of life

It is in general suggested that a limited number of high-fitness solutions are available in most environments with natural selection identifying these solutions to the particular challenges of the environment in a deterministic way. However, the result of adaptation also depends on the specific events that occur before and during adaptation and that are likely to differ from case to case, suggesting that an outcome would be difficult to predict with any certainty.

Our findings of convergence in the mechanism of resistance to carbendazim in *C. elegans* offer an interesting insight to the debate between the role of determinism and contingency in evolution by offering an experimental example of evolutionary repeatability of carbendazim (and benzimidazole) resistance. As the experiences that make evolution challenging to predict, such as the order in which particular mutations appear (or the timing of rare environmental changes outside the lab), are difficult, if not impossible, to duplicate across different incidents of adaptation by natural selection. Therefore, observing the emergence of carbendazim resistance conferred by the same genetic locus, *ben-1*, in three separate and independently derived resistant populations suggests high repeatability of evolution of carbendazim resistance.

It appears that in this instance the most common high-fitness solution to the presence of carbendazim in the environment is the alteration of *ben-1*. Additionally,

fitness benefits can be provided by metabolic resistance, resulting from alterations of the bio-transformation mechanisms of benzimidazole that are, in comparison to *ben-1* mutations, less common. This paints a picture of the landscape of benzimidazole resistance emergence where fitness increase can result independently from β -tubulin target site resistance or metabolic resistance with the, less common, option of combining the two for even higher fitness benefits in the compound environment.

Additional research into the landscape of resistance emergence and adaptation will hopefully enable us to grasp the interplay of parameters resulting in rapid resistance emergence with more clarity.

5.4.6. Future work

The findings of this chapter have indicated the importance of *ben-1* in carbendazim resistance but the contribution of an additional locus on chromosome V, identified in the strain AW1783, has remained unclear. While previous work suggests that this locus confers resistance by a detoxifying mechanism, it would be interesting to confirm that, as metabolic resistance was not detected in any other resistant strain characterised. As there were difficulties sequencing the *ben-1* of AW1783, it would also be important to determine what in particular confers resistance on the left arm of chromosome III. Together, a better characterisation of the genetic basis of resistance in the strain AW1783 would aid in improving the understanding of the interplay of target site and metabolic resistance to benzimidazoles.

Additionally, while the gene editing of the ED3005 *ben-1* to N2-like *ben-1* was not successful, a further investigation of contribution of the *ben-1* polymorphisms to benzimidazole resistance in the *C. elegans* wild isolates could illuminate the natural occurrence of alternative mechanism of resistance (e.g. metabolic resistance) to

benzimidazoles in cases where *ben-1* polymorphisms do not explain the entire resistance phenotype observed. So far, only the additive effect of *ben-1* mutations has been studied by introducing the F200Y polymorphism to the *ben-1* or deleting the gene altogether in N2 background (Hahnel *et al.*, 2018). A better characterisation of the genetic basis could be utilised to understand the different paths to natural resistance of benzimidazoles.

Chapter 6: General Discussion

6.1. Summary of results

The body of work described in this thesis is the first record of utilising a multicellular animal to study natural prevalence, dynamics of evolution, and genetic basis of pesticide resistance in a holistic manner. The main findings of Chapters 3, 4, and 5 are highlighted below.

Chapter 3:

- Chemical susceptibility screen of genetically diverse *C. elegans* wild isolates can be used to quantify the prevalence of naturally occurring resistance alleles prior to field studies.
- Variation in chemical susceptibility to agrochemicals is widespread in a genetically diverse panel of *C. elegans* wild isolates, with resistant and sensitive isolates identified in 8 out of the 28 compounds tested. No particular general physiological targets (muscle and nerve, growth and development, respiration) appear to be correlated with higher variation in susceptibility.
- The variation in chemical susceptibility observed across the panel of *C. elegans* wild isolates is not caused by a universal mechanism (e.g. cuticle permeability).
- Wild isolates ED3005, ED3012, JU363 were found to be resistant to carbendazim, while five wild isolates (CB4857, CB4858, ED3005, JU363, JU1652) were identified as less susceptible to aldicarb.

Chapter 4:

- *C. elegans* can be utilised in multi-generational selection experiments to compare the adaptation to compounds with high and low variation in chemical susceptibility in wild isolates.
- Multi-generational selection experiment resulted in rapid emergence of resistance to carbendazim that appears to carry no fitness cost in no compound under lab conditions, while no adaptation to aldicarb or dieldrin was observed.
- Resistance to carbendazim results in cross-resistance to other benzimidazoles but not to non-benzimidazole tubulin disruptors suggesting a benzimidazole-specific mechanism of resistance.

Chapter 5:

- Analyses of the genetic basis of carbendazim resistance from three biological events (wild isolates, experimentally selected lines, chemically induced mutant) all reveal the contribution of different *ben-1* mutations (*ben-1* encodes β -tubulin in *C. elegans*) in reduced carbendazim susceptibility.
- The findings of competition experiments suggest no fitness cost of carbendazim resistance in no compound, with the spread of resistance through populations dependent on the presence of compound selection.

6.2. Perspectives on using *C. elegans* in strategies to mitigate pesticide resistance

6.2.1. How widespread is agrochemical resistance in wild populations?

The findings presented in Chapter 3 outline the first study of variation in chemical susceptibility among the genetically distinct populations of *C. elegans* on this scale. Screening for natural occurrence of resistance among *C. elegans* in the manner presented in Chapter 3 suggests that approaches such as this have the ability to uncover the extent and the underlying genetic basis of the variation observed in agrochemical resistance. This is important as the prevalence of resistance alleles in populations before compound treatments is a parameter that affects the speed at which resistance emerges. These results indicate that, in our model of natural variation in wild populations, alleles resulting in altered susceptibility to agrochemicals are widespread. The findings of Hahnel *et al.* (2018) support the identification of wild isolates resistant to carbendazim (and, more widely, to benzimidazoles) independently.

While *C. elegans* is a non-target species, the results nevertheless have implications for resistance management strategies. The potential presence of resistance in wild populations of pests, prior to the introduction of agrochemicals, would mean that resistance could emerge faster, without necessity for beneficial *de novo* mutations to occur. Furthermore, resistance alleles that have arisen in no compound environments would not necessarily carry a high fitness cost and would be able to increase rapidly in their frequency under the selection pressure of the compound and remain in the population at stable proportions upon the loss of the selection pressure. Overall, this suggests that the presence of resistance alleles limits

the number of suitable resistance management strategies as a number of them assume resistance emerging from *de novo* mutations.

Additionally, as highlighted in Chapter 3, reports of resistance already exist for the majority of the compounds used in the chemical susceptibility screen. Therefore, in terms of compounds that are already in use in agriculture, no correlation between the amount of variation seen in this screen and resistance reported in the field is observed. However, utilising a check of natural resistance in a model of natural variation, such as a panel of *C. elegans* wild isolates, would better ensure the longevity of the pesticide in the field. Identification of a large amount of variation in susceptibility would enable the suggestion of best resistance mitigation strategies.

6.2.1.1. *Limitations of the chemical susceptibility screen*

The screen assessed variation in susceptibility to each compound by scoring the overall lethality, movement, and fecundity following chronic exposure. Later experiments were required to quantify these differences and, in general, food consumption assays were used for this. Thrashing was utilised in the case of aldicarb, suggesting that the chemical susceptibility screen was suitable to assess susceptibility across a wider range of compounds than the food consumption assay. In the case of the chemical susceptibility screen and food consumption assays the overall effect of the compound was measured, meaning that the combined effect of the compound on mortality, movement, and fecundity affected the category it was placed in. While this enabled me to observe the diverse effects of the compounds used, it meant that these specific measures (that can indicate differences in specific susceptibility to the compound) were not quantified.

An alternative option to screen for the effects of the agrochemicals would have been to quantify mortality across a range of compound concentrations in the panel of wild isolates. Measuring the dose required to kill 50% of the population, would have enabled more direct comparison with published work. At the same time it would have disregarded the multi-generational effects of the compound by not providing any information on eggs laid or movement of the animals. Arguably, this would have weighed similarly cases where the same concentration results in equal mortality but vastly different effects on fecundity. This would have failed to establish differences in susceptibility in a context relevant to the field, where multi-generational effects are important. For example where the dose was lethal to half of the worms in both cases but the remaining worms on one plate had severely reduced egg laying, while the fecundity of the worms on the other plate was not affected.

6.2.1.2. *Practical suggestions for improving screens of chemical susceptibility*

In a more labour-intensive improvement of the chemical susceptibility screen, all three parameters mentioned above could be quantified. A set-up, which would not rely on later quantification of variation, would have to enable the collection of diverse data on lethality, fecundity, and movement in order to establish variation in responses to chronic exposure and across generations. It is likely that this would be a technologically more advanced screen that relies on the presence of specialised equipment (worm sorter (Burns *et al.*, 2006; Hahnel *et al.*, 2018), cameras to record multi-well plates (Yemini *et al.*, 2013; Gomez-Marin, Stephens and Brown, 2016; Javer, Ripoll-Sánchez and Brown, 2018) etc.).

Despite the limitation of the chemical susceptibility screen in not providing quantitative data, in the context of the aims set, it enabled the collection of informative data to identify wild isolates resistant to carbendazim and aldicarb. Therefore, I think that a screen of similar function would be highly useful in pesticide research and development to gauge the presence of natural variation to the effects of agrochemicals in the future. While the occurrence of resistance alleles in the field must also be identified before the release of an agrochemical, this earlier laboratory assessment would enable to assess this faster and cheaper prior to more expensive and slower field studies. This would be particularly insightful in cases with a track record of high level of conservation of MOA of the pesticide between *C. elegans* and pest species. An example of this would be spirotetramat resistance that, as outlined in the introduction, has a similar mechanism in *C. elegans* and whiteflies with a homologous mutation conferring reduced susceptibility in both cases (personal communications Anthony Flemming).

6.2.2. How fast does pesticide resistance emerge?

The results from Chapter 4 and 5, respectively, outline the findings of a multi-generational selection experiment and a competition experiment that, when taken together, provide insights on utilising *C. elegans* to study the dynamics of pesticide resistance emergence. The findings also offer experimental evidence on the interplay and roles of multiple variables, such as dose (discussed in 6.2.2.1) and fitness cost of resistance (discussed in 6.2.3), in the emergence of resistance. Briefly, the results discussed suggest that high pesticide doses result in faster spread of resistance alleles and that fitness cost of pesticide resistance influences changes in the proportion of resistance alleles in a population upon the loss of selection pressure.

6.2.2.1. *Importance of dose in the rate of benzimidazole resistance emergence*

The competition experiment, results of which are covered in Chapter 5, investigated the dynamics of emergence of carbendazim resistance specifically by utilising a chemically induced carbendazim-resistant mutant. The findings indicated that carbendazim resistance carries no fitness cost in the absence of compound and that the speed by which resistance spreads in a population is correlated with compound concentration, with a higher concentration resulting in a faster rate of resistance fixation. In general, pesticide resistance has been shown to emerge faster if higher doses are used, but a lack of experimental studies on the effect has limited the application of this rule in policy and the development of resistance management strategies so far (Van den Bosch *et al.*, 2011).

The role of the dose rate of pesticides in the emergence of resistance has been a subject of some debate. Evolution of resistance can be viewed as having three key phases: emergence (resistant strain arises for the first time in the population), selection (frequency of resistance increases in the population due to selection pressure of the compound), adjustment (resistance has spread through population and new strategies are required to control the pest population) (Van den Bosch *et al.*, 2011). The effect of the dose primarily concerns the emergence and selection phases of resistance.

The competition experiment serves as an experimental model of the selection phase of resistance, where resistant individuals are already present, and indicates that a higher concentration indeed results in faster fixation of resistance in the population. This is in line with the findings of other competition experiments using different triazole and Q₀I (quinone outside inhibitors) in the lab and in the field (Van den

Bosch *et al.*, 2011) as well as another using a benzimidazole. In the latter, competition experiments in 1.25 μ M albendazole between Bristol N2 and two different lines with *ben-1* mutations (a deletion strain and an F200Y mutant) result in an increase of resistance alleles across seven generations. Additionally, the considerably lower dose of albendazole used (in comparison to the lowest dose of carbendazim (1.25 μ M in comparison to about 50 μ M or 10ppm)) reiterates the effect of the concentration as resistance alleles do not become fully fixed under the low selection pressure of albendazole (Hahnel *et al.*, 2018).

Additionally, based on my competition experiments, carbendazim resistance only spreads under the selective pressure of the compound rather than by positive selection for carbendazim resistance in no compound. Together, the observation of rapid spread of cost-free resistance in the selection experiment and competition experiment provide a strong case for the importance of characterising the prevalence of naturally occurring resistance alleles. Where the presence of these alleles in the field is confirmed, their rapid fixation could result from high application doses or the use of compounds with particularly long half times under environmental conditions.

The isolation of *C. elegans* wild isolates resistant to carbendazim is interesting in this respect, as some benzimidazoles have particularly long half times under environmental conditions, with carbendazim persisting in soil for 1-12 months (International Programme on Chemical Safety (IPCS), no date). It is therefore likely (but difficult to confirm) that local selective pressures, as suggested by Hahnel *et al.*, 2018, have driven the emergence of benzimidazole-resistant wild isolates.

6.2.3. Evaluating the fitness cost of resistance

The multi-generational selection experiment used a modified *C. elegans* strain to compare the adaptation to diverse compound environments as well as a no compound environment. This, together with the findings of Chapter 3, enabled me to connect the prevalence of natural variation in chemical susceptibility to rapid emergence of resistance in a selection experiment in the case of carbendazim. In general, a connection between high prevalence of naturally occurring resistance and rapid emergence of resistance was not identified as multi-generational selection on aldicarb, another compound where resistant wild isolates were characterised, failed to result in noticeable adaptation to the compound. This provided a point of comparison of emergence of resistance to the two compounds.

Multiple aldicarb-resistant strains have been identified in *C. elegans* (Nguyen *et al.*, 1995) and mutants resistant to aldicarb have emerged in the field (Mota-Sanchez and Wise, 2019). Similarly, resistance to benzimidazoles has been widely reported in *C. elegans* and in the field (Kwa *et al.*, 1993; Leroux *et al.*, 1999; Liu *et al.*, 2014; Krücken *et al.*, 2017). Fitness cost of resistance may be offered to explain the differences observed in resistance emergence.

6.2.3.1. Aldicarb

Aldicarb-resistant mutants are likely to carry a high fitness cost in no compound environments, suggesting that resistant alleles are more likely to emerge under the selection pressure of the compound. The characterisation of development of aldicarb-resistant *C. elegans* mutants has revealed that these worms either have a slower growth rate or impaired movement, with many of the strains being significantly smaller as adults (Nguyen *et al.*, 1995).

The toxic effect of aldicarb is the cellular inhibition of AChE, which results in the build-up of ACh and thus the overstimulation of ACh receptors. The resistant strains are able to reduce the accumulation of ACh by defective aspects of ACh transmission (e.g. synthesis, transport). However, defective transmission of the major excitatory neurotransmitter ACh further affects locomotion, egg laying, feeding, and male mating, resulting in a reduction of fitness in comparison to wild type worms (Rand, 2007). Therefore the emergence of mutations resulting in resistance to aldicarb and the spread of resistance through a population can only occur under compound selection where these mutations that confer a fitness cost in no compound are beneficial in comparison to wild type.

The identification of aldicarb-resistant wild isolates in Chapter 3 suggested that alleles conferring decreased susceptibility could spread through populations with no recorded aldicarb exposure. While these naturally occurring alleles are not expected to carry a fitness cost, this was explicitly not quantified and would have been difficult to do so due to different genetic backgrounds of the wild isolates. It is possible that an initial fitness cost in no compound, imposed by the particular polymorphisms conferring resistance, has in aldicarb-resistant wild populations been modified by additional background changes that result in no fitness cost in a compound-free environment. A mechanism of resistance with modified fitness cost has been observed in resistance to AChE inhibitors in *T. urticae* (Kwon *et al.*, 2012). Further study of the genetic basis of resistance to aldicarb in wild isolates would be required to determine the answer.

6.2.3.2. *Carbendazim*

The findings of low fitness cost of benzimidazole resistance in a no compound environment in Chapters 4 and 5 have highlighted misconceptions about this important parameter in resistance mitigation. While fitness cost is included in the majority of models of resistance emergence, it is regularly expected to be high, conferring considerable disadvantage in a no compound environment (Bourguet *et al.*, 2010). This concept underlies commonly used resistance management strategies, such as compound alternation. Utilising compound alternation, which relies on the reduction of resistance frequency to pre-application levels upon the loss of selective pressure of compound, in a situation where cost-free resistance has emerged, such as resistance to benzimidazoles, would result in rapid compound failure due to resistance.

In broad terms, the fitness cost of resistance in no compound explains why natural selection does not fix genes conferring resistance throughout animal populations (Gemmell and Read, 1998). However, in the context of pre-existing resistance conferring variation, resistance alleles that have spread through populations prior to the introduction of pesticides, this observation does not apply. Therefore, real-world resistance emergence cannot always be assumed to arise from novel mutations that arise under compound selection pressure and may, as a result, indeed not carry a fitness cost. Instead the assumptions regarding fitness costs in no compound should be grounded on experimental findings and take into account the possibility of cost-free and cost-modification events that require a deeper understanding of the underlying biology (Ffrench-Constant and Bass, 2017). As resistance can arise particularly rapidly from standing genetic variation, the parameter must be carefully studied and

suitable alternative resistance management strategies utilised where no cost is identified.

It is possible that in some ways benzimidazole resistance is an exception to the rule and a large majority of resistance cases do indeed carry a fitness cost in no compound. However, the presence of a case in *C. elegans*, where the loss-of-function of a conserved gene, *ben-1*, is tolerated without any cost, enables us to gather information on the general parameters that enable cost-free resistance. Further characterisation of the parameter may perhaps in the future aid in predictions of cost of resistance, which, for now, remains difficult to do with any accuracy. The capabilities of utilising *C. elegans* to experimentally inform mathematical models, as showcased by the findings reported in this work, will surely find increased use in the future.

6.2.4. Uncovering the genetic basis of resistance: lessons from the *ben-1* locus

I identified the genetic basis of carbendazim resistance in wild isolates, selected lines, and a chemically induced mutant. While the importance of β -tubulin genes and detoxifying mechanisms in resistance to benzimidazoles has been described in *C. elegans* and pest species before (Driscoll *et al.*, 1989; Furtado, de Paiva Bello and Rabelo, 2016; Krücken *et al.*, 2017; Hahnel *et al.*, 2018), a comparison of resistance mechanisms from different events in strains of the same species has not been presented. As such, the result of convergence on the *ben-1* locus presents a novel insight on an established relationship between the gene and benzimidazole-resistance. The findings suggest that by far the most likely mechanism of resistance to carbendazim is a modification of the β -tubulin encoding gene in *C. elegans*.

It is interesting that the mutations of *ben-1*, which are likely to result either in loss of function or a conformation change, confer no fitness cost in no compound for *C. elegans* wild isolates, selected lines or chemically mutagenised strain. While it is possible that tests in field would establish a cost that cannot be identified by lab studies (Ffrench-Constant and Bass, 2017), the isolation of carbendazim-resistant wild isolates suggests that, as these variants have been able to persist at high frequencies in field conditions and as the variants seem to have arisen independently multiple times, indeed there is no cost to losing a fully functional copy of *ben-1*. This raises the question of how worms can seemingly tolerate the absence of this β -tubulin?

6.2.4.1. *Understanding the highly polymorphic nature of the ben-1 locus*

C. elegans has in total six β -tubulin genes: *tbb-1*, *tbb-2*, *ben-1*, *tbb-4*, *mec-7*, *tbb-6*. *tbb-1*, *tbb-2*, and *ben-1* are all broadly expressed and *tbb-1* and *tbb-2* function in a partially redundant way under lab conditions. The additional β -tubulin genes *mec-7*, and *tbb-4* are only expressed in different subsets of neurons and *tbb-6* appears to be a highly diverged β -tubulin that holds an unclear role (Hurd, 2018) but has been reported to be highly expressed during the unfolded protein response (Munkácsy *et al.*, 2016).

This suggests that *tbb-1* and *tbb-2*, which are suggested to not be sensitive to benzimidazoles due to a lack of phenylalanine at position 200 (Chambers *et al.*, 2010), compensate for the lack of a functional BEN-1 in carbendazim-resistant strains. The β -tubulin genes *tbb-4*, *mec-7*, and *tbb-6* have so far not been linked with responses to benzimidazoles despite having a phenylalanine at position 200 and thus predicted being able to bind the drugs. It is suggested that their more specialised

functions means that the disruption of microtubules containing these β -tubulins would have much more subtle effects (Saunders *et al.*, 2013). Additionally they could likely not compensate for the lack of BEN-1 or therefore explain the lack of fitness cost observed in the tested environment.

While a number of benzimidazole-resistant variants have been identified in the field, with current records kept by FRAC indicating the presence of 131 plant pathogen species resistant to benzimidazoles (FRAC, 2018), resistance in wild populations has so far been primarily associated with three alterations of the β -tubulin protein: F200Y, E198A, and F167Y (Kotze *et al.*, 2014). This suggests a considerably lower level of allelic variation in pest species relative to the observations in *C. elegans* where the functionally redundant *tbb-1* and *tbb-2* appear to compensate the predicted loss-of-function of *ben-1* that happen through a variety of mutations.

An interesting insight is obtained from the comparison of *C. elegans* and *Haemonchus contortus* (a parasitic nematode) β -tubulin families. An illustration of a proposed model of the evolution of nematode β -tubulins, including a comparison of *C. elegans* and *H. contortus*, is shown in Figure 6.1. *H. contortus* has only four β -tubulin genes with the isoforms 1 (*tbb-iso-1*) and 2 (*tbb-iso-2*) being closely related, like in *C. elegans*, and isoforms 3 (*tbb-iso-3*) and 4 (*tbb-iso-4*) having separate, more diverged roles (Saunders *et al.*, 2013), again, similarly to the *C. elegans* counterparts.

However, *C. elegans* seems to have two additional β -tubulins, *ben-1* and *tbb-6*, which are also the only β -tubulins where polymorphisms with high predicted functional effects have been identified in wild isolates (Hahnel *et al.*, 2018), which suggests a non-essential role of these genes in the worms. Relative to other nematodes, two additional gene duplication events in *C. elegans* (Figure 6.1, events ‘G’ and ‘I’) have resulted in two additional β -tubulin genes. In the case of the

predicted loss-of-function mutations of *ben-1* this explains the cost-free nature of the resistance to benzimidazoles in *C. elegans* as the *ben-1* paralogues, *tbb-1* and *tbb-2*, compensate for the loss and are not sensitive to benzimidazoles. Therefore, in *C. elegans* the three genes appear to carry similar roles, with the only apparently specific role of *ben-1* being sensitivity to benzimidazoles.

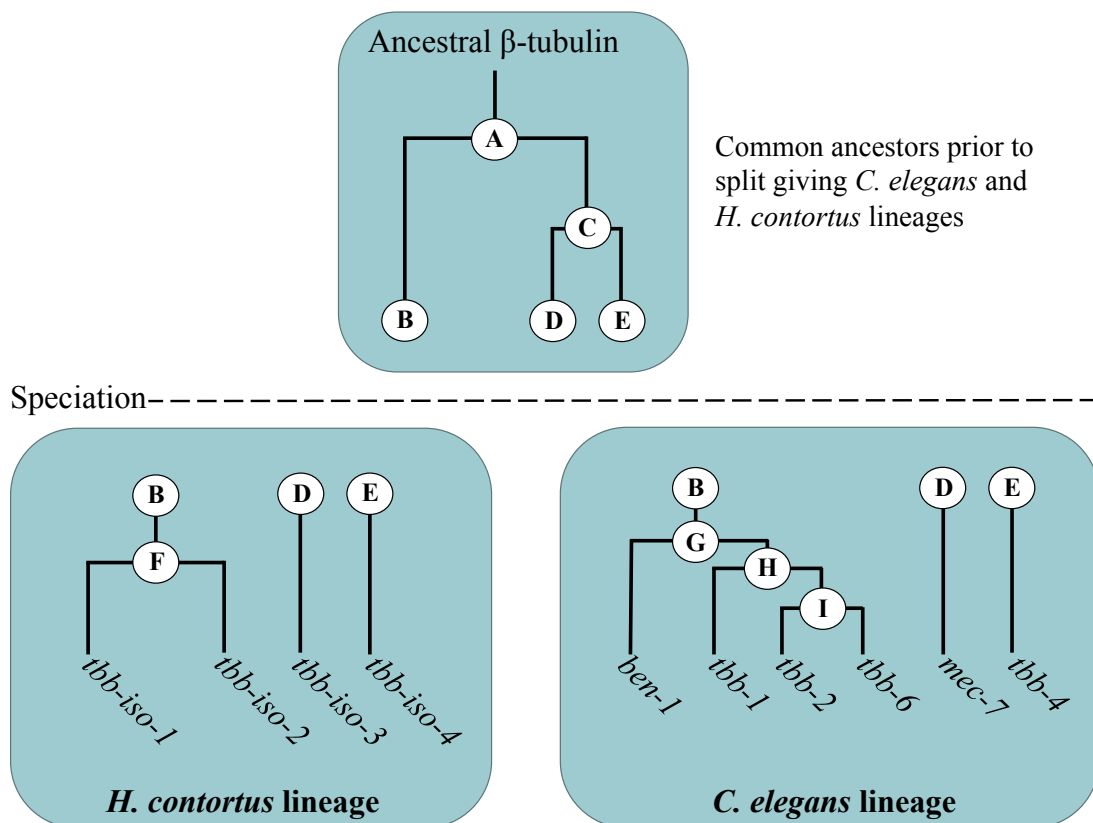


Figure 6.1: A proposed model of the evolution of β -tubulins in nematodes. For simplicity, a single β -tubulin gene in the ancestral nematode is assumed to exist. Gene duplication events are indicated by labelled nodes. The speciation event separating the *C. elegans* and *H. contortus* occurs sometime after the duplication event 'C' and is marked with a dotted line. The figure is adapted from (Saunders *et al.*, 2013).

The evolutionary relationship between *C. elegans ben-1* and parasitic nematode β -tubulin isoforms 1 and 2, provides an explanation to the involvement of both isoforms in benzimidazole resistance of parasitic nematodes and also the lower level of allelic variation observed in pests. It can be suggested that only mutations that

alter the binding site of benzimidazoles indirectly (Vela-Corcía *et al.*, 2018) while the β -tubulin remains functional are tolerated.

6.2.5. Replaying the tape of life: understanding the genetic and evolutionary basis of pesticide resistance

The identification of the genetic basis of benzimidazole resistance indicated convergence of the mechanism of resistance on one gene locus, *ben-1*, in three sets of experimentally derived *C. elegans* strains. The *ben-1* polymorphisms identified in the wild isolates and the experimentally selected resistant lines were all different, with the same locus selected for across the lines that adapted to carbendazim. Additionally, the results of the genetic basis of carbendazim resistance in the chemically induced mutant indicated the presence of both target site (β -tubulin) and metabolic resistance (detoxifying mechanism) mechanisms that resulted in an increased resistance phenotype.

Similar trends of parallelism have been spotted in evolution experiments studying the emergence of antimicrobial resistance. In particular, resistance to trimethoprim has been shown to evolve in a stepwise manner, through mutations restricted to a gene encoding the enzyme dihydrofolate reductase, which plays a role in DNA precursor synthesis. Parallel populations were found to evolve resistance by similar mutations occurring in a similar order. On the converse, resistance to other antibiotics used evolved through a diverse combination of mutations in genes involving translation, transcription, and transport (Toprak *et al.*, 2012), indicating that mutations are not always limited to a single locus. In general, the study of antimicrobial resistance has found that parallelism in adaptation can exist on the level of functional groups, such as genes or pathways, and the changes can be effected

through a number of different mutations, while some particularly important mutations may exist (Lobkovsky and Koonin, 2012; Blount, Lenski and Losos, 2018).

The work described in this thesis suggests that resistance to agrochemicals, in the example of benzimidazoles, carries a similar element of predictability. The mental visualisation of benzimidazole resistance emergence as a fitness landscape, based on these findings, suggests the presence of a limited number of paths by which resistance to this class of compounds can be reached. This landscape appears to be shaped by characteristics of the pesticide target like the rate of mutations at the locus as well as the effect of the mutations, with a fitness benefit in compound environment paving the way to resistance and no fitness cost in no compound enabling the allele to be maintained in populations beyond the use of the compound. This is in agreement with the conditions which are thought to control the degree of repeatability of evolutionary trajectories: the number of loci involved in adaptation, the existence of alternative adaptation pathways, and the environmental conditions (Lobkovsky and Koonin, 2012).

While the quantification of predictability of evolution is still an emerging field, experimental studies like this can provide invaluable insight. An improved understanding of the parameters, which lead to the emergence of agrochemical resistance, can help in mitigating and predicting resistance. In an age of increased expectations for food production but slowing discovery of new compounds, ensuring the continued effectiveness of the chemical pest control measures is essential. Thus, global food security can be improved through efforts focussing on the development of new pest control measures and, additionally, to keep up with natural selection, leveraging the power of experimental systems in understanding resistance biology.

Appendix I: Permutation test

Code of the permutation test used to determine the statistical differences of wild isolates in a food consumption race at 50ppm carbendazim reported under heading 3.3.10.2.

Code for the test was provided by L.D. Lord:

```
%permutation test by L.D. Lord July 2019
% ld.lord13@gmail.com

% this script compares the observed difference of the sample means with the
difference
%obtained by two randomly formed group of equivalent size from the same
%data over 100,000 permutations. it computes the % of the time the real
%difference pf the means exceeds the difference between the randomly formed
%groups (random_difference). the p-value is derived from this ratio.

load('N2.mat')
load('CX11307.mat')
load('ED3012.mat')
load('ED3005.mat')

%%%%%choose group of interest for comparison with N2%%%
group2 = ED3012

sumvect = 0

for x = 1:100000;

real_difference = mean(N2) - mean(group2);

%create random sample
concatenated_data = vertcat(N2, group2);

for i = 1:3
    mycell1{i} = concatenated_data(randi(6,1),1);
    mycell2{i} = concatenated_data(randi(6,1),1);
end
mymat1 = cell2mat(mycell1);
mymat2 = cell2mat(mycell2);

random_mean1 = mean(mymat1);
random_mean2 = mean(mymat2);
random_difference = abs(random_mean1 - random_mean2);
```

```
%check real vs random difference of the means
```

```
if real_difference > random_difference;
```

```
    sumvect = sumvect + 1;
```

```
else
```

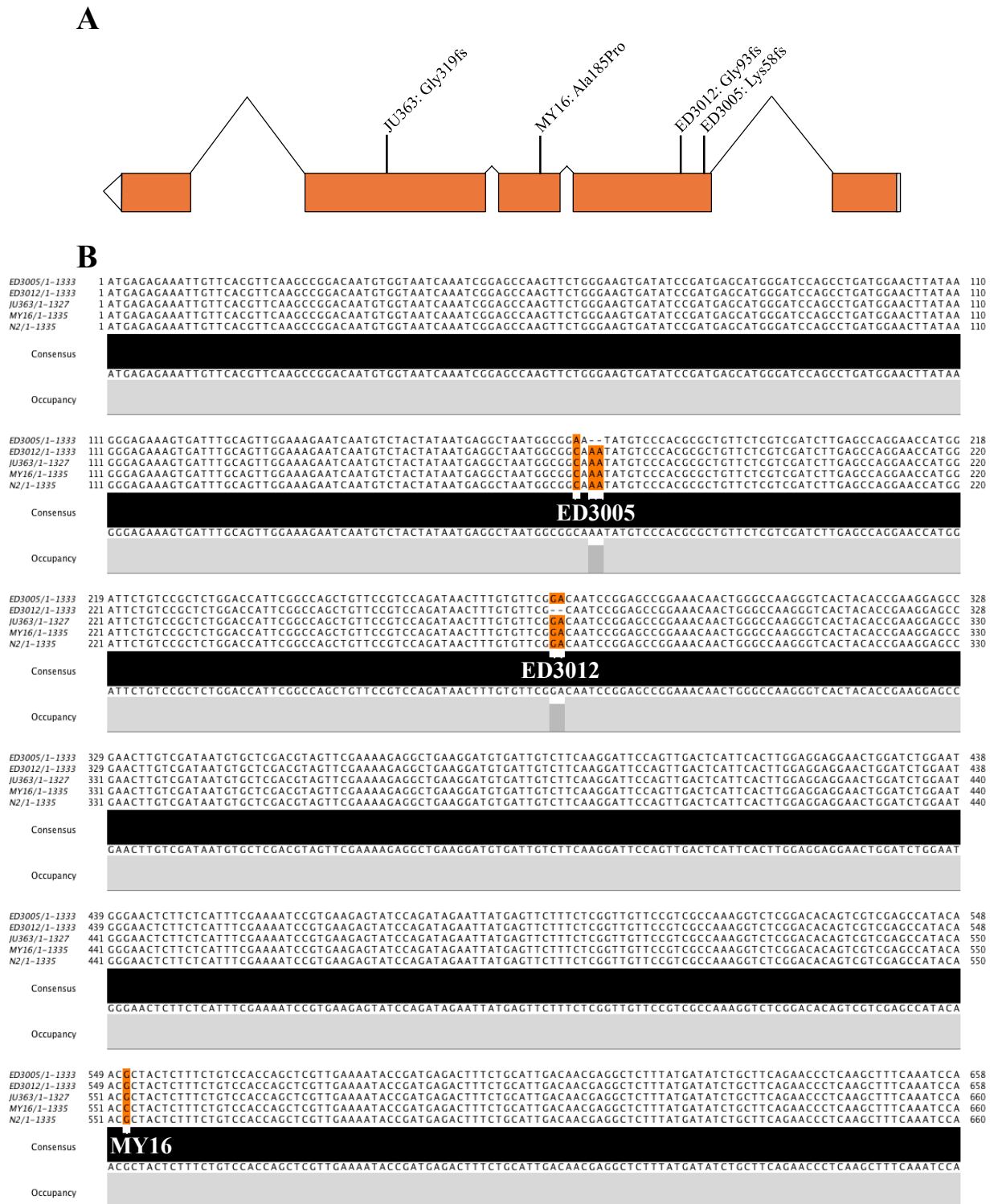
```
    sumvect = sumvect + 0;
```

```
end
```

```
end
```

```
final_pval = 1-(sumvect/x);
```

Appendix II: Sequences of *C. elegans ben-1*



(Figure continued on the next page)

(Figure continued)

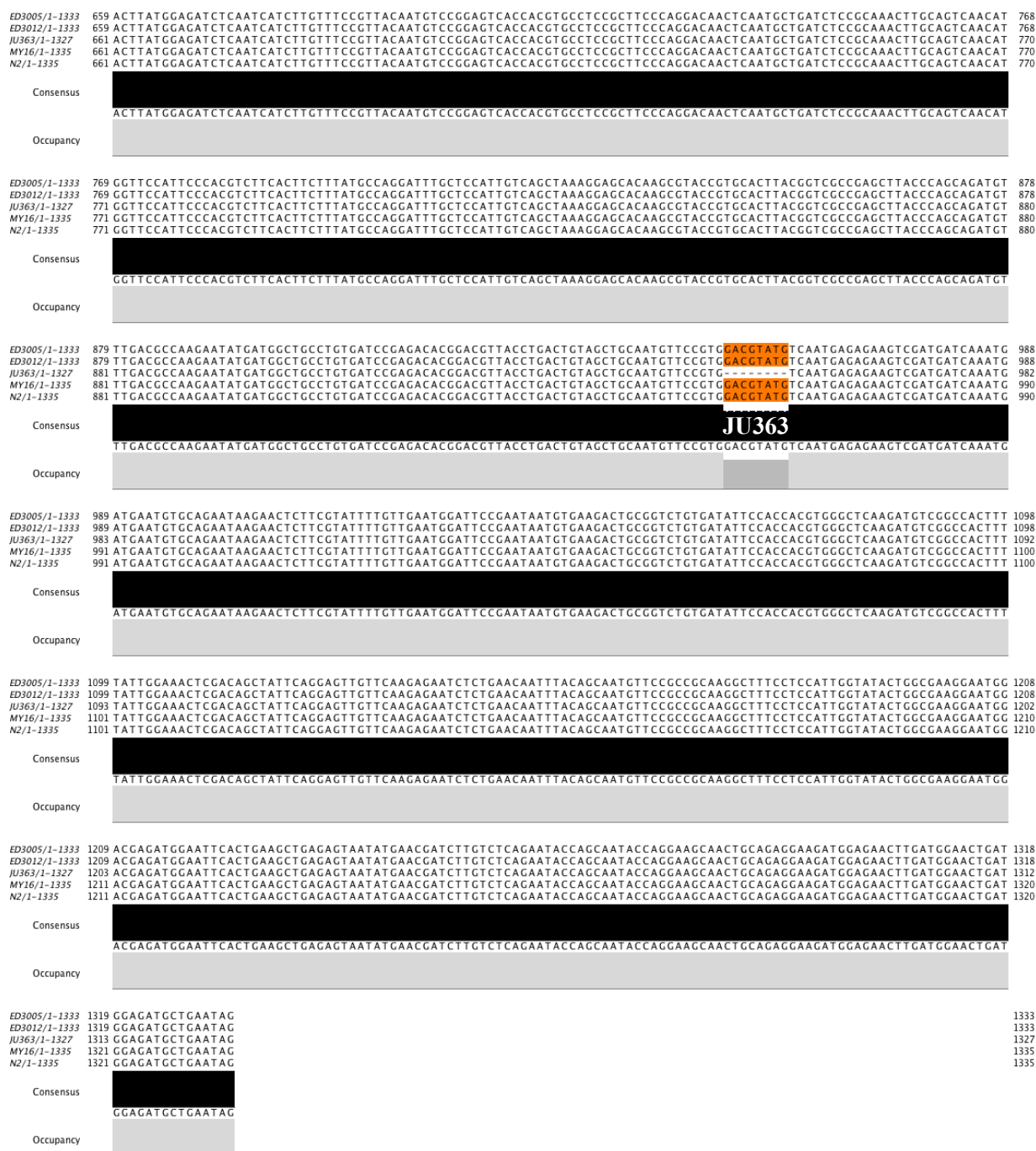


Figure: (A) Schematic of coding sequence of *ben-1* highlighting polymorphisms conferring carbendazim-resistance in wild isolates JU363, ED3012, ED3005 and showing the location of the MY16 *ben-1* variant that confers carbendazim-resistance in the experimentally selected lines. 5' and 3' UTR are shown as white rectangle and triangle respectively. Five exons are shown as orange blocks, and introns as thin lines between blocks. (B) Alignment of *ben-1* sequences of ED3005, ED3012, JU363, MY16, and N2 with orange highlights of specific polymorphisms in all wild isolates. Wild isolate indicated underneath the highlighted region.

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