

**The Potential for Evolution of Resistance in
Glasshouse Whitefly (*Trialeurodes
vaporariorum*) to Biocontrol Agents and the
Associated Life History Costs**

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Abstract

Understanding how pests evolve resistance to control is crucial for ensuring food security, yet resistance evolution to biocontrol is overlooked compared to pesticide resistance. In this thesis I investigate co-evolution between pests and their natural enemies in the context of biocontrol to explore how resistance to natural enemies impacts the efficiency and long term effectiveness of sustainable pest control.

In chapter 3 I present the results of a lab experiment quantifying variation in resistance to parasitism in glasshouse whitefly (*Trialeurodes vaporariorum*) and its biocontrol agents *Encarsia formosa*, a parasitoid wasp, and a commercial fungal biopesticide containing *Beauveria bassiana*. I used these results to parameterise a simulation model predicting resistance evolution under different scenarios, varying crop type and biocontrol application regime. I found resistance to biocontrol agents in *T. vaporariorum* to be highly variable, and my simulations suggest resistance can readily evolve, but the speed of evolution depends on environmental context, in particular crop type. In chapter four I confirmed the results of the simulation with a six generation a selection experiment.

Chapter 5 investigated life history trade-offs in *Encarsia* resistant whitefly. I assayed the fitness of *T. vaporariorum* that survived *Encarsia* attack in the lab. Surviving resistant whitefly had reduced fecundity, lower offspring survival, and slower development. These trade-offs imply that while whiteflies can survive parasitism, reduced population growth may constrain resistance evolution.

Chapter 6 explores whether tank-mixing multiple biopesticides may be a viable option for pre-emptive management of resistance evolution and targeting multiple pest species at once. Using *T. vaporariorum* and the tomato leaf miner (*Tuta absoluta*) different biopesticides were applied alone or in combination. Mixing biopesticides showed no antagonistic or synergistic effects on pest mortality, suggesting control efficacy is not compromised by tank mixing and can enhance application efficiency when targeting multiple pest species.

My thesis provides insight into the effectiveness of biological control strategies and the importance of understanding resistance evolution. Although resistance to biocontrol agents is less prevalent than to chemical pesticides, it remains vital for ensuring long-term efficacy.

Ethical approval



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22/11/2022

Dear Mia

Ethics Application Form : [Resistance evolution in whitefly to combat a parasitoid wasp biocontrol agent, 12238](#)

Thank you for your submission of the above ethics application.

The ethical approaches of this project have been approved and you can now proceed with your project.

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Yours sincerely,

Animal Welfare Ethics Review Body

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Chapter 1: Insect pest management in agriculture

1.1 Overview

Insect pests are one of the major biotic constraints in global agriculture causing crop damage and reducing crop yield that can lead to catastrophic economic losses and reduced food security (Savary *et al.*, 2019). Insect pests cause both direct damage to crop through feeding and are vectors for many detrimental bacterial, viral and fungal diseases. Historically the most commonly used insect pest control method (pest control: the process of managing or reducing populations of organisms that are considered harmful to human health, agriculture, ecosystems, or property (Pedigo & Rice, 2009), involved applying synthetic chemical pesticides. However, over reliance on these chemicals has led to the rapid evolution of pesticide resistance under strong selection pressures (Hawkins *et al.*, 2018). This resistance evolution has contributed to global food security issues with problems such as crop loss, reduced crop yield, economic loss and a reduction in the number of effective pest control products for the future (Popp *et al.*, 2013). Not only have chemical pesticides led to an increase in insect pest resistance they have caused many devastating environmental impacts. The overuse has resulted in global exposure, with chemical pesticide run off being found in the water, air and in geological locations (Hayes and Hansen, 2017). This has altered landscapes and ecosystems alongside having detrimental effects on non-target organisms, reducing biodiversity in many ecosystems (Hayes and Hansen, 2017).

Growing awareness of the environmental impacts associated with chemical pesticides has led to an increase in both the number of banned pesticides and the implementation of stricter regulations governing their use (Dara, 2018). This shift reflects a broader movement toward more sustainable pest control methods and integrated pest management (IPM) systems (Dara, 2018). IPM has evolved significantly and is now regarded as a comprehensive approach centred on prevention, monitoring, and control (figure 1; Dara, 2018). The IPM paradigm integrates multiple pest management techniques, emphasising not just reactive measures but proactive strategies that

prevent pest problems before they arise (Dara, 2018). It involves continuous monitoring of pest populations, meticulous planning, and the sharing of knowledge and resources among stakeholders to ensure effective management (Dara, 2018). By adopting these practices, IPM aims to reduce reliance on chemical pesticides, minimise environmental impacts, and promote more sustainable agricultural practices (Angon *et al.*, 2023).

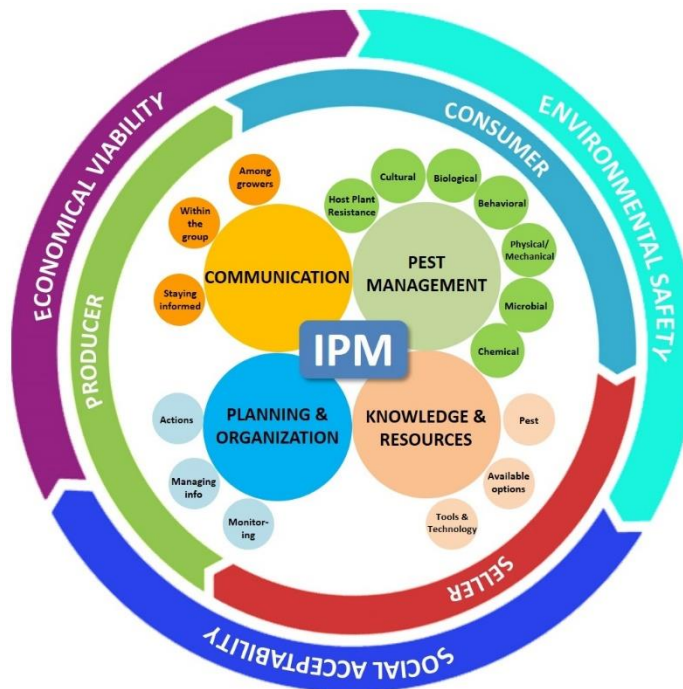


Figure 1: The IPM paradigm showing the key components of integrated pest management approaches whilst acknowledging the business, social, economic and sustainability principles. Reproduced from Dara (2018).

Due to increasingly stringent pesticide regulations and legislation that has banned numerous chemical pesticides, alongside the implementation of the Integrated Pest Management (IPM) directive in 2009, there has been a notable shift in pest management practices (Damalas & Koutroubas, 2018). There had been a large increase in the use of biological control agents (Khan *et al.*, 2023). Biological control agents are living organisms such as predators, parasitoids, or pathogens that control pests through various mechanisms (Khan *et al.*, 2023). This contrasts with nature-based substances, which are non-living naturally derived materials from plants,

microbes, animals, or minerals that can control pests (CABI, 2022). Both biological control agents and nature-based substances can be classified as bioprotection (European Bioprotection Forum, 2023). In 2023 there was a 9% reduction in the sale of traditional chemical pesticides and a 20% increase in the sale of bioprotection agents across the EU compared to 2022 (Eurostat, 2024).

Despite the benefits of bioprotection, there are emerging concerns about the potential for resistance (Paez D & Fleming-Davies, 2020). While the risks associated with resistance to chemical pesticides are well-documented, the potential for resistance to bioprotection agents is less appreciated. Pathogens and parasites used as biocontrol agents can exert significant selective pressure on pest populations, potentially leading to rapid evolutionary changes and the development of resistance (Paez D & Fleming-Davies, 2020). This phenomenon is a growing concern as it could undermine the effectiveness of bio strategies and highlight the need for careful management and monitoring to sustain the efficacy of these natural pest control methods.

1.2 Chemical pesticides, resistance and the environmental impacts

Over the past 50 years, chemical pesticides including organochlorines, cyclodienes, organophosphates, carbamates, pyrethroids and formamidines have been the principal mechanisms for controlling insect pests (Costa, 2015). Insecticides such as DDTs (dichloro-diphenyl-trichloroethane) and neonicotinoids in particular became increasingly popular due to their wide range of target pests and long residual effects, making them one of the most successful pest management strategies across the globe (Costa, 2015).

However, the extensive overuse and often mismanagement of these chemicals has led to severe environmental consequences. Studies have found that up to 99.7% of applied pesticides end up in the wider environment, contributing to pollution of air, soil, and water (Pimentel, 1995). Pesticides with high bioaccumulation and long half-lives can have lingering environmental effects, detectable years after their application (Jayaraj *et al.*, 2017). Moreover, these chemicals adversely affect non-target

organisms, leading to the decline of beneficial insect populations and contributing to the development of insecticide resistance among pest species (Khan *et al.*, 2015).

Not only has the long-term misuse of these chemicals resulted in substantial damage to the environment, but it has also rendered these chemicals less effective at controlling pests which readily evolve to resist their harmful effects. For instance, resistance to neonicotinoids was first discovered in 1996 in *Bemisia tabaci* whitefly, one of the target species for neonicotinoid use (Bass, *et al.*, 2015). Since 1996 neonicotinoid resistance has been reported in many pest species with over 500 publications presenting new cases of neonicotinoid resistance (Bass, *et al.*, 2015).

Resistance evolution refers to the process by which an organism's mortality rate decreases over generations due to a selection pressure, such as pesticide application (Georghiou and Taylor, 1977). Initially, pesticides cause high mortality among pest populations. However, individuals with mutations that provide greater survival advantages under pesticide pressure survive and reproduce. These beneficial mutations are passed on to offspring, rapidly spreading through the population. As a result, resistant genotypes become more common, while non-resistant ones diminish. Insecticide resistance mechanisms can be broadly classified into physiological adaptations, such as changes in enzyme activity or target site alterations, and behavioural adaptations, such as changes in feeding patterns or habitat use (Siddiqui *et al.*, 2022). These adaptive responses enable pests to withstand pesticide exposure, leading to the proliferation of resistant populations and necessitating the development of alternative pest management strategies.

1.3 Behavioural resistance to chemical pesticides

Behavioural resistance in insects can be defined as an evolved behaviour that reduces exposure to fatal environments or inhibits insect death from toxic compounds (Sparks *et al.*, 1989). A common form of behavioural resistance is avoidance, where insects will actively move away and avoid areas that are contaminated or fatal environments. Avoidance is often observed in social insects, for instance, the red imported fire ant, *Solenopsis invicta*, is a significant urban and agricultural pest in South America that

employs unique strategies to mitigate insecticide toxicity (Wen *et al.*, 2022). *Solenopsis invicta* poses significant challenges as its stings cause severe reactions in humans, damage livestock and crops, and compete with native ant species (Wen *et al.*, 2022). These ants are widely controlled using surface treatment insecticides; however, a unique insecticide-resistance strategy has been observed within ant colonies which serves to reduce the toxicity of the insecticides (Wen *et al.*, 2022). *Solenopsis invicta* workers cover the insecticide treated surfaces with soil particles, allowing them to traverse these areas without direct contact with the insecticide (Wen *et al.*, 2022). This behaviour effectively reduces the exposure and toxicity, thereby protecting the colony from the lethal effects of the insecticide (Wen *et al.*, 2022).

Several species of mosquitoes also exhibit behavioural resistance traits against insecticide-treated nets (ITNs) and indoor residual spraying (IRS) (Carrasco *et al.*, 2019). Insecticides are often used indoors to control mosquito populations, and individuals sleep under mosquito nets at night to avoid being bitten. However, avoidance behaviours have been observed in many mosquito species, both temporally and spatially (Carrasco *et al.*, 2019). Temporal behavioural shifts in peak aggressiveness from night to early evening or morning, when most individuals are not sleeping under protective nets, have been seen in species such as *Anopheles farauti* in the Solomon Islands and Papua New Guinea, *Anopheles funestus* in Tanzania, in both *An. funestus* and *Anopheles arabiensis* (Carrasco *et al.*, 2019). Spatially, *Anopheles gambiae* s.l. on Bioko Island and *An. funestus* in Tanzania have been found to favour outdoor host-seeking and biting, which correlates with the increased use of indoor insecticide spraying (Carrasco *et al.*, 2019).

Despite these observations, very few studies conclusively show behavioural avoidance of insecticides as true evidence of *avoidance resistance*. For a behaviour to be considered avoidance resistance, it must be absent in populations that have not been exposed to toxic compounds or environments (Zalucki & Furlong, 2017). This criterion ensures that the observed behaviours are indeed a result of evolutionary adaptation to insecticide exposure rather than inherent behavioural traits. Nonetheless, the observed shifts in mosquito behaviour pose significant challenges to

existing mosquito control strategies and highlight the need for continued research and adaptation in pest management.

1.4 Biological control agents

The use of biological control agents in agricultural pest management has surged in popularity for several reasons, including environmental benefits, reduced non-target mortality, and the rise of pesticide resistance to chemical insecticides (Ehler, 2006). A significant factor driving this increase is the stringent restrictions and reductions in the availability of chemical pesticides due to EU policies and the sustainable use directive. These policies aim to promote more sustainable and eco-friendly pest management practices.

As legislation increasingly bans many chemical pesticides, there is a strong push towards adopting alternatives such as bioprotection agents. This shift suggests that bioprotection agents are viewed as a safer and more sustainable option for future pest management plans (Hillocks, 2012). Bioprotection agents help mitigate the adverse environmental impacts associated with chemical pesticides and contribute to the development of Integrated Pest Management (IPM) strategies (Chandler et al., 2011). This approach aligns with the broader goal of achieving sustainable agricultural practices that protect both crops and the surrounding ecosystem, ensuring global food security. As such, the rise in the use of bioprotection agents represents a pivotal shift towards more responsible and effective pest management in agriculture.

While bioprotection agents can provide environmentally friendly alternatives to chemical pesticides, they also present several challenges and risks. There can be ecological risks of off-target effects, where biocontrol agents may inadvertently harm non-target or beneficial species, potentially disrupting ecosystems and biodiversity (Howarth, 1991). Some biocontrol agents can have limited host specificity, where they have low host specificity, leading to high risks of non-target effects or conversely, they are highly host specific, only able to control limited numbers of pest species (van Lenteren, 2012). Biocontrol agents may not adapt well to varying environmental conditions, reducing their effectiveness across different agroecosystems (van Lenteren, 2012). Biocontrol agents also tend to have a slower effect than chemical pesticides, which may delay pest suppression and affect crop protection (van

Lenteren, 2012). Practical issues such as the complexity and the cost of mass production, storage, and transport of living agents further limit their widespread use (Eilenberg et al., 2001).

There are different biocontrol methods used across global agriculture, these can be broadly divided into four categories: natural, conservation, classical/importation, and augmentative (Van Lenteren *et al.*, 2020). Natural biocontrol relies on cultivating crops in areas where natural enemy populations are already present, allowing them to manage pest populations with minimal human intervention (Van Lenteren *et al.*, 2020). Conservation biocontrol involves managing the landscape to protect and boost the effects of these natural enemies on pest populations (Van Lenteren *et al.*, 2020). Classical (or importation) biocontrol introduces natural enemies bred in controlled conditions into areas with high pest populations where these enemies were previously absent and allowing the natural enemies to establish populations to control insect pests (Van Lenteren *et al.*, 2020). Augmentative biocontrol entails mass-rearing natural enemies and pathogens and continually releasing them into areas with high pest numbers (Van Lenteren *et al.*, 2020).

Augmentative use of biological control agents (chiefly predators and parasitoids) is the favoured pest management strategy in UK agriculture (DEFRA, 2021). This preference is largely due to the high proportion of protected and enclosed growing environments, such as glasshouses and polytunnels, which provide optimal conditions for biocontrol by limiting the dispersal of both parasitic and predatory natural enemy biocontrol agents and pests (Collier and Finch, 2007). In 2023, biological control agents were used in 60% of pest-treated areas of UK protected edible crops. This category included macro-biological control agents such as predatory mites, parasitic wasps and nematodes (Garthwaite *et al.*, 2023). These statistics highlight the critical importance of maintaining the long-term effectiveness of these pest management strategies. Ensuring sustainable use of biocontrol methods is vital for managing pest resistance evolution and supporting long-term agricultural productivity (Garthwaite *et al.*, 2023). By prioritising biocontrol, the UK can reduce reliance on chemical pesticides, mitigate environmental impact, and enhance the resilience of pest management systems.

1.5 Natural enemies used in agricultural biocontrol

1.5.1 Microbial Biopesticides

Biopesticides formulated from live insect pathogens offer control options for insect pests as part of integrated pest management (IPM) programmes that can help reduce reliance on chemical insecticides. Microbial biopesticides contain live parasites and pathogens, such as nematodes, viruses, fungi and bacteria (Lacey *et al.*, 2015). Biopesticides have multiple modes of action, such as, neurotoxicity, altering feeding rates, mating disturbance, desiccation, and suffocation (Abdollahdokht *et al.*, 2022). One major benefit of biopesticides is their target specificity: they generally kill a relatively narrow species range, with few off-target effects for beneficial insects (Oguh *et al.*, 2019). These environmentally sustainable biopesticides can limit the ecological harm associated with some chemical products. However, their specificity can increase the potential for resistance evolution, as the pests are under strong selective pressure and these biopesticides often have a single mode of action, simplifying the evolutionary pathway for pests to develop resistance (Oguh *et al.*, 2019). This specificity is also a practical drawback as multiple biopesticide products may need to be applied to defend a crop against all pest species threatening the crop yield (Oguh *et al.*, 2019).

1.5.1.1 Fungal pathogens as biological control agents

Fungal pathogens are the predominant biological control agents used to control arthropod pests (Kumar, 2019). The large range of host specific fungal pathogens and isolates available and the rise in insecticide resistant species makes fungal pathogens particularly favourable (Kumar, 2019). Fungal pathogens infect their host by directly penetrating the cuticle (see 1.6 (Qu and Wang, 2018).). Fungal spores germinate and enter the insect's cuticle by degradation via enzyme secretion and mechanical pressure. After penetrating the cuticle, motile reproductive spores, called

conidiospores germinate, multiplying in the hosts haemolymph, producing blastospores and mycelium causing host death by filling its body cavity with hypha (Dara, 2017). The hyphae eventually emerge from the insect's cuticle where sporulation then occurs on the host's cadaver increasing spore transmission to other susceptible hosts (Dara, 2017). Host death can also occur through the release of fungal toxins; once the insect host is invaded the fungi produce and release multiple toxins (Dara, 2017). In the case of *Beauveria bassiana*, secondary metabolites are released such as beauvericin, bassianin, bassianolide, beauverolides, tenellin, oosporein, and oxalic (Wang *et al.*, 2021). These toxins help *B. bassiana* to parasitize and kill the hosts as a second mode of action in insect host death (Wang *et al.*, 2021).

1.5.1.2 Viral pathogens as biological control agents

Viral pathogens are still relatively underused as biocontrol agents (Szewczyk *et al.*, 2006). They mainly target lepidopteran pests in agriculture but are known to control Coleoptera, Trichoptera, Hymenoptera, Neuroptera, Diptera, Crustacea and Orthoptera (Szewczyk *et al.*, 2006). Viral pathogens are quite host specific and cause few unwanted ecological effects to non-target organisms and the environment (Marwal *et al.*, 2022). The baculovirus family are the most common and commercially available viral biopesticides, with high specificity to agricultural lepidopteran pests (Hilton, 2008). Two types of baculoviruses are used for controlling pests: nuclear polyhedroviruses (NPVs), viruses with polyhedral-shaped occlusion bodies, and granuloviruses (GVs), a single virion consisting of one nucleocapsid within a single envelope (Hilton, 2008). Baculoviruses target and kill the insect hosts by interfering with their physiology or by releasing insect-specific toxins (Hezakiel *et al.*, 2023). As the polyhydra are ingested by the insect host, toxins are released in the midgut where the virus replicates (Hezakiel *et al.*, 2023). Secondary infection of insect cells occurs and polyhydria accumulate in the nuclei leading to cell death and eventual insect host death (Hezakiel *et al.*, 2023). As with any pathogen there is the potential for hosts to evolve resistance to viral biopesticides, but this has not yet been documented. This may be because uptake of these agents is too low to see a strong selective response, as use of these

agents increases resistance becomes more likely (as is the case with naturally occurring viral infections).

1.5.1.3 Bacterial pathogens and toxins as bioprotection agents

A huge number of bacterial biopesticides already play a vital role in agricultural pest management. The most well-known bacterial biopesticide, biological control agent is produced from the bacterium *Bacillus thuringiensis* or Bt which is a Gram-positive spore-forming bacterium (Schnepf et al., 1998). Many Bt subspecies have evolved to produce crystalised toxins, bioprotection agents, these crystal forms ensure stability and the storage of the toxins virulence factors so that they remain virulent until they are ingested by the insect host, where the crystals dissolve in the high alkaline environments to release the toxin, aiding in host death (Tetreau et al., 2021). Bt crystals have a broad host range and are easily formulated into sprayable bioprotection products (Tetreau et al., 2021). The mode of action is via insect host ingestion; as Bt crystals are ingested by the host, gut enzymes activate the toxins (Singh et al., 2019). Toxins bind to the receptor and leak ions and small molecules leading to septicaemia resulting in insect host death (Singh et al., 2019). Bt is highly flexible in how it can be used as both a biocontrol, the pathogen itself and bioprotection agent from the toxins, in addition to spraying with Bt-based products, it can be integrated into the genome of crops so that the plants themselves contain the toxins and crystals that are harmful to insects, providing the plants with resistance to certain pests (Sanahuja et al., 2011). The use of *Bacillus thuringiensis* (Bt) crops as a pest management strategy has increased in popularity and is highly successful (Sanahuja et al., 2011). Tobacco was the first Bt crop to be produced in 1985, but Bt crops were not commercially available in USA until 1995 where tobacco, cotton, corn and potato were approved to be produced and distributed (Abbas, 2018). In less than 20 years the use Bt crops has risen dramatically. In 2013 76 million hectares of Bt crops were planted globally with the majority in USA, 75% of cotton in USA 90% in China and 95% in India was Bt cotton and 76% of Maize in the USA was Bt maize (Tabashnik and Carrière, 2019).

Although Bt crops have proven to be a successful pest management strategy both as biocontrol and bioprotection products, the rise of insect resistance is a concern (Abbas, 2018). Six years after the initial use of Bt in maize to control the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, the first cases of field evolved resistance were documented (Abbas, 2018). The rise of resistance evolution to Bt crops highlights the need for research that continually monitors the responses of target organisms to biocontrol methods and screens for resistance.

1.5.2 Parasitoids as biological control agents

Parasitoids, mostly belonging to the Hymenoptera or Diptera, play a vital role as biocontrol agents against many agricultural insect pests and are the most used natural enemies across the globe (Colmenarez *et al.*, 2018). Adult parasitoids search for hosts and oviposition occurs either in, on, or near their hosts (Waage and Hassell, 1982). As the larvae develop, they begin feeding on host tissues throughout their developmental stages either within the host, as endoparasitoids, or on the host, as ectoparasitoids (Waage and Hassell, 1982). Most parasitoids used in biocontrol parasitise eggs, while others parasitize larvae, pupae, or even adults (Koller *et al.*, 2023). *Trialeurodes vaporariorum*, the greenhouse whitefly, is one of the most detrimental global crop pests, and also one of the species recorded to be successfully controlled using parasitoid wasps (Pilkington *et al.*, 2010). In the 1930s the Aphelinid parasitoid wasp *Encarsia formosa* was first shown to successfully control *T. vaporariorum* (Pilkington *et al.*, 2010). Despite this early success, it was only when insecticide resistance became problematic in the 1970s that *E. formosa* became the main method used to control greenhouse whitefly (Pilkington *et al.*, 2010). Augmentative biocontrol using *Encarsia formosa* is still the favoured *T. vaporariorum* control method, with wasps released in vast quantities weekly in greenhouses, however, there has been little research investigating the potential for resistance evolution in response to this parasitoid (Hoddle *et al.*, 1998).

1.5.3 Predatory insects as biological control agents

Predatory insects are another major form of augmentative biocontrol; predatory mites, beetles, flies, lacewings, true bugs (Order Hemiptera), and wasps feed on various pest insects and mites (Van Lenteren, 2012). Once released they search and prey upon insect crop pests, managing population numbers and reducing the detrimental damage to crops (Van Lenteren, 2012). Predators are used in both open field and closed glasshouse systems with both generalist and specialists being released (Pijnakker *et al.*, 2020). Predatory mites have been used for over 70 years, however their success as biological control agents was not recognised until the 1980s when phytoseiid mites were shown to control thrip populations (Lindquist, 2011). Predatory mites are now one of the most commonly used biocontrol agents in the glasshouse crop industry (Lindquist, 2011).

1.6 Physiological resistance to pathogens used as biocontrol agents

Insect resistance to pathogens often arises from genetic mutations that enhance their immune responses. Insects possess a highly effective immune system capable of quickly repelling, inactivating, and eliminating pathogens through interconnected cellular and humoral responses (Beckage, 2008). A key component of an insect's defence is its exoskeleton cuticle, primarily composed of proteins and the polysaccharide chitin, which acts as a robust physical barrier against pathogenic invasions (Tajiri *et al.*, 2017).

Pathogens can enter the insect's cuticle through various means, including direct penetration, entry through wounds or abrasions, or by degrading the cuticle with specialized enzymes. Fungal pathogens typically penetrate the cuticle directly, triggering an innate immune response known as the prophenoloxidase cascade in the insect. This response leads to the synthesis of melanin, a crucial defence mechanism. Melanin not only restricts the growth of certain fungi but also inhibits the production of cuticle-degrading enzymes, thereby reducing the likelihood of fungal infections being fatal to the insect (Qu and Wang, 2018).

The effectiveness of these immune responses highlights the evolutionary arms race between insects and their pathogens. As pathogens evolve new methods to overcome insect defences, insects concurrently develop enhanced immune strategies driven by genetic mutations that provide a survival advantage. This dynamic interplay underscores the complexity and efficiency of insect immune systems in their ongoing battle against pathogens.

Once a pathogen has entered an insect's hemocoel or gut, it encounters robust cellular and humoral immune responses (Hillyer, 2015). Cellular responses are primarily mediated by haemocytes, the main cellular components of haemolymph. Haemocytes are vital for conducting cellular immune responses against invading pathogens through mechanisms such as nodulation, encapsulation, and phagocytosis (Manniello *et al.*, 2021). For instance, large pathogens like parasitic eggs are often too big to be engulfed by individual phagocytes and are instead entrapped via encapsulation. This process involves layers of immune cells isolating and neutralizing the pathogen. Conversely, smaller pathogens are typically entrapped and then engulfed and killed by phagocytes (Sideri *et al.*, 2008), see figure 2.

In addition to cellular responses, humoral responses play a crucial role in the insect immune system. These responses involve signalling receptors such as Toll-like Receptors (TLR) and the Immune Deficiency (IMD) pathway, which activate the production of antimicrobial peptides (Sheehan *et al.*, 2020). These peptides help in neutralizing and eliminating the pathogens. The interplay between humoral and cellular immune responses is particularly significant in pathogen recognition and elimination. Surface and humoral receptors recognize foreign pathogens, which then trigger a cascade of intra- and intercellular signalling events. This signalling activates the cellular response, leading to the effective killing of the invading pathogen (Lavine & Strand, 2002), see figure 2. This integrated immune response system highlights the sophisticated and multi-layered defence mechanisms that insects have evolved to protect themselves from a wide array of pathogens. The cooperation between cellular and humoral responses ensures that insects can swiftly and effectively overcome threats, maintaining their survival.

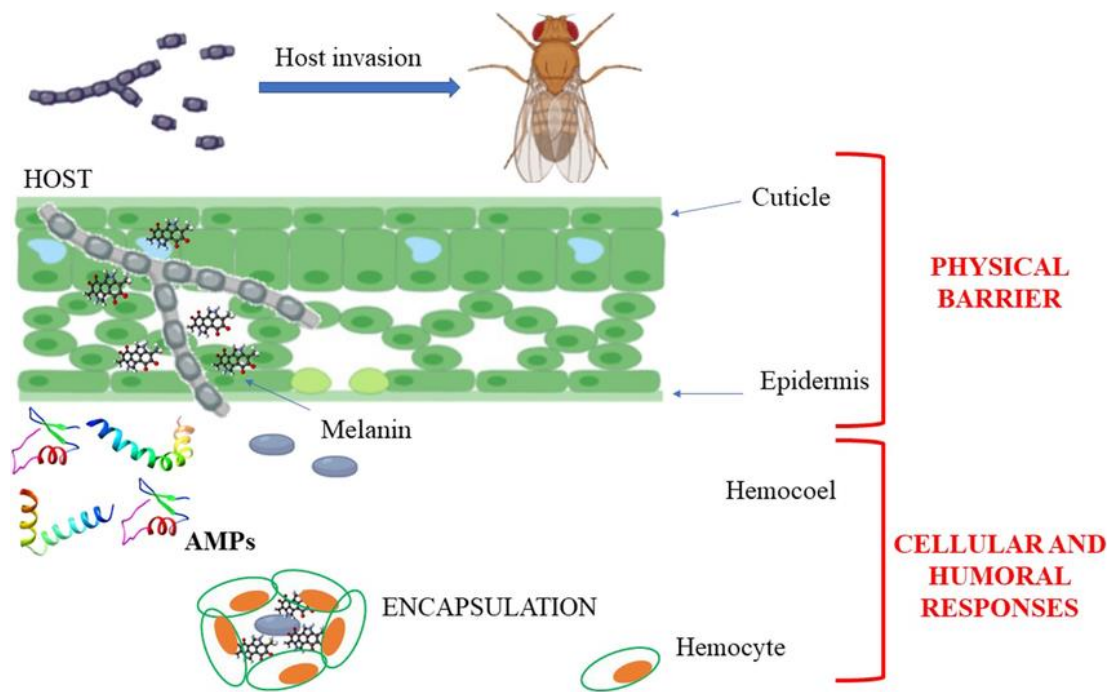


Figure 2: Host immune response to pathogens schematic taken from (Manniello et al., 2021). The schematic shows the hosts defence mechanisms against invasion of a foreign pathogen, indicating the physical, cellular and humoral responses.

Insect innate immune responses are different for each class of pathogen; for fungal infections many insect cuticles contain hydrophobin compounds that stop or delay fungal conidia (spores) from adhesion and germination. This has been observed in *Dalbulus maidis* plant hoppers, where their cuticle inhibits the conidial germination of *Beauveria bassiana* (Toledo et al., 2011). Some insects also inhibit fungal infection by secreting defensive compounds such as benzoquinones (Duarte et al., 2022). If fungal pathogens penetrate the cuticle the insect initiates an internal line of defence, using antimicrobial peptides to kill the fungal pathogen in the haemolymph. Resistance to the fungal pathogen *Beauveria bassiana* has been found in the Greater wax moth, *Galleria mellonella* (Dubovskiy et al., 2013). In this case, during *B. bassiana* infection, systemic immune defences were suppressed but responses in the cuticle and epidermis of the integument were enhanced, hindering fungal spores entering the host resulting in resistance (Dubovskiy et al., 2013).

Insect defences against viral infection are very different to defences against fungi and involve an RNA interference pathway. This recognises a viruses double stranded RNA and produces small interfering RNAs (siRNAs). These siRNAs bind to complementary viral RNA sequences, guiding the RNA-induced silencing complex (RISC) to degrade the viral RNA, thereby suppressing viral replication and limiting the spread of infection (Rosales, 2016).

Resistance to viral biopesticides has been observed in Lepidoptera the field across the globe (Asser-Kaiser *et al.*, 2011) For instance, the codling moth *Cydia pomonella* has shown widespread resistance to *Cydia pomonella granulovirus* (CpGV), where resistance is due to the virus entering the *C. pomonella* but a systemic block (involving CpRR1 which prevents CpGV viral DNA replication during early stages of the infection), prevents the virus taking hold (Asser-Kaiser *et al.*, 2011). Resistance to parasitoids has also been documented in insect pests, with many aphid species showing resistance to parasitic wasps (Schmid *et al.*, 2011). Many aphid species harbour the bacterial endosymbiont *Hamiltonella defensa* which provides symbiont mediated protection, increasing aphid resistance to parasitoids (Schmid *et al.*, 2011). This resistance has been observed in many aphid species such as the black bean aphid, *Aphis fabae* (Schmid *et al.*, 2011). As resistance has been observed across many biological control agents and global insect pest species, this evolution of resistance could pose a threat to the long-term effectiveness of biological control agents used in agriculture without close monitoring and regulations. Although these mechanisms of resistance can stop host death, there can be life history trade-offs associated with surviving infection, for instance surviving insect hosts can experience reduced fecundity or infertility which can have detrimental effects on the population (Tuomi *et al.*, 1983).

1.7 Physiological resistance to bioprotection agents

While there has been a shift towards the use of biocontrol agents and biopesticides, there is little concern into the potential of resistance despite growing evidence for resistance in many global insect pests to different bioprotection agents (Tabashnik *et al.*, 1994). Resistance to bacterial pathogens has most commonly been observed in Bt toxins, in at least 27 species in laboratory conditions (Tabashnik *et al.*, 1994). This has also been shown in the field in Lepidoptera such as *Plutella xylostella* where the proposed mechanism of resistance had been linked to reduced binding of ICP (*Bacillus thuringiensis* insecticidal crystal proteins) to the brush-border membranes of the mid-gut (Tabashnik *et al.*, 1994). Resistance to Bt crystals can occur at all stages of infection through different mechanisms, for instance degradation of crystals, altering binding membranes and the absence of crystal activation (Siegwart *et al.*, 2015). Resistance to multiple Bt crop toxins has been shown across many insect pests across the globe. Bt resistance has been found in the western corn rootworm, *Diabrotica virgifera virgifera* one of the most devastating corn pests, in North America. *D. virgifera* has shown resistance to many Bt toxins used as a form of biocontrol (Gassmann, 2021). This is particularly problematic as *D. virgifera* have limited adult dispersal, large western rootworm populations occur in localised areas such as continuous maize cultivations, where there is no break in the crop (Gassmann, 2021). As large Western rootworm populations evolve resistance, and, due to limited dispersal, resistant traits spread quickly through the population (Gassmann, 2021).

1.8 Mechanisms of resistance evolution

The evolution of resistance in insect pests to both chemical pesticides and biological control agents often involves complex interactions between genetic mutations, behavioural and physiological adaptations. While there are commonalities in the mechanisms underlying resistance to these two control methods, key differences arise from the distinct nature of chemical and biological agents.

1.9 Common Mechanisms of Resistance Evolution

In many cases, resistance mechanisms are likely to be shared between both chemical and biological and bioprotection agents (Beckage, 2008). This is particularly the case with behavioural mechanisms such as avoidance or broad physiological mechanisms, such as physical and structural barriers or part of the innate immune response (Beckage, 2008). Resistance to both chemical pesticides and biocontrol agents often originates from physiological mutations that confer a survival advantage by altering the insect's physiology or behaviour. Mutations affecting target sites, metabolic pathways, or immune functions can reduce susceptibility to toxic compounds or biological pathogens (Beckage, 2008; Sheehan et al., 2020). Insects also deploy physiological defences such as enhanced immune responses, including the production of antimicrobial peptides, detoxification enzymes, and physical barriers like the cuticle, which act to mitigate the effects of both chemical and biological agents (Lavine & Strand, 2002; Manniello et al., 2021). Additionally, behavioural resistance, such as avoidance of treated areas, is a common evolutionary strategy that reduces exposure and selection pressure from both types of agents (Sparks et al., 1989; Wen et al., 2022).

1.10 Differences in Resistance Mechanisms

Despite these shared mechanisms, resistance to biological control agents generally involves more intricate immune and physiological mechanisms due to the complex nature of living organisms used as biocontrol agents (Cônsoli et al., 2010). Resistance to fungal pathogens often includes enhanced cuticular defences and activation of the prophenoloxidase cascade, leading to melanin synthesis that restricts pathogen growth and inhibits fungal enzyme activity (Qu & Wang, 2018; Dubovskiy et al., 2013). Viral resistance is mediated through the RNA interference pathway, which degrades viral RNA and limits infection, an antiviral defence mechanism, (Rosales, 2016). Resistance to parasitoids can also be facilitated by symbiont-mediated protection, where bacterial endosymbionts enhance the host's immune capacity (Schmid et al., 2011).

In contrast, resistance to chemical pesticides is often on target site insensitivity, metabolic detoxification, and sequestration of toxic compounds (Li et al., 2007). Chemical pesticides typically exert selection pressure on specific molecular targets, leading to mutations that reduce binding affinity or increase metabolic breakdown of insecticides (Tuomi et al., 1983). This mode of resistance is generally less complex than the multi-layered immune responses required to overcome biological agents, alongside biological control agents potential to co-evolve, this often leads to a more rapid evolution of resistance to chemical pesticides (Gassman, 2021).

1.11 Resistance management

The global increase in pesticide resistance has led to the introduction of global regulations and strategic measures to manage insect resistance effectively (Sparks, et al., 2015). In response, the Insecticide Resistance Action Committee (IRAC) was established in 1984 to address these challenges (Sparks, et al., 2015). IRAC's primary role involves the development and maintenance of the Mode of Action (MoA) classification scheme (Sparks, et al., 2015). This scheme provides comprehensive and up-to-date information on the modes of action of both new and existing insecticides and acaricides (Sparks, et al., 2015). By categorizing these compounds, the MoA classification helps in devising effective resistance management strategies, ensuring better crop protection worldwide (Sparks and Nauen, 2015).

Most commonly, integrated pest management (IPM) regimes are advised, which involve a multipronged approach to pest control combining strategies such as pest monitoring and planning alongside the use of multiple control measures such as biological control agents, mechanical barriers, chemical pesticides and cultural control (Ehler, 2006). This approach can aid in the reduction of resistance evolution as it is challenging to evolve resistance to multiple selection pressures simultaneously, potentially causing trade-offs in host resistance to differing modes of action (Ehler, 2006). Although the use of IPM and IRAC strategies have been proven to be beneficial, insect resistance is still prominent as insects can, in theory, evolve resistance to any pest management approach that exerts a strong enough selection pressure (Ehler, 2006). At present there is little research into or concern for the evolution of resistance

to alternative pest management approaches such as biological control agents, leaving the future effectiveness of these approaches unknown (Onstad and Knolhoff, 2023). As resistance to biological control agents has emerged in various insect pest species to different types of biocontrol agents, macrobiologicals, viral, fungal and bacterial pathogens worldwide (Onstad and Knolhoff, 2023) , the evolution of such resistance could pose a significant threat to the long-term effectiveness of biocontrol strategies in agriculture (Onstad and Knolhoff, 2023). Therefore, implementing robust resistance management practices, including close monitoring and strategic use, is essential to slow the development of resistance and preserve the efficacy of biological control agents (Onstad and Knolhoff, 2023).

1.12 Conclusion

As insect pest management is vital for successful sustainable agriculture, the evolution of resistance is an ongoing concern as it could compromise crop protection efficacy leading to economic loss and reduced global food security (Gould et al., 2018). Although evolution of resistance to synthetic insecticides is well researched, threats of pest resistance evolution to the pathogens and parasites used as agricultural biocontrol agents are still underappreciated. The primary aim of this thesis is to bridge this gap and investigate the eco-evolutionary dynamics of resistance evolution in study systems that are of direct relevance to biocontrol. I aim to connect fundamental research on co-evolution between pests and their natural enemies with applied research in pest management. In writing this thesis I aim to highlight the significance of resistance evolution in biocontrol and explore strategies to mitigate it as necessary. The ultimate goal of my thesis is providing empirical data which can be used to ensure the long-term effectiveness of biocontrol and integrated pest management (IPM) systems, vital for protecting the future of sustainable agriculture.

1.13 Aims of thesis

The aim of my thesis is to begin address the gap in biocontrol resistance evolution knowledge by combining fundamental insights into the evolution of resistance with practical applications in biological control. The ultimate goal is that the data generated from my thesis will help inform integrated pest management regimes to increase sustainability and maintain the effectiveness of pest management, reducing crop loss and improving food security

My thesis aims to assess the efficacy and long term effectiveness of biological control agents and the potential for the pests they target to evolve resistance over a short space of time. Chapter 3 aims to explore whether the whitefly *Trialeurodes vaporariorum* can develop resistance to the parasitic wasp *Encarsia formosa* and *Beauveria bassiana* (a fungal biopesticide) through laboratory-based experimental evolution, assessing changes in host susceptibility across generations, with an individual based simulation model written in R to predict risks of resistance evolution under varying application regimes. Chapter 4 complements this by examining resistance development over time in *T. vaporariorum* to *Encarsia formosa* combining empirical assays over six generations. Chapter 5 aims to investigate the potential fitness and life-history costs associated with evolved resistance. Chapter 6 aims to test the efficiency of a strategy (tank mixing of biopesticides) that might mitigate resistance on *T. vaporariorum* and *Tuta absoluta* to determine whether combined applications can enhance pest suppression and reduce the likelihood of resistance. Together, these chapters aim to provide a clearer understanding of the evolutionary interactions between detrimental crop pests and their natural enemies, used as the most common biological control agents contributing to the development of more sustainable and resilient pest management strategies.

Chapter 2. General methods and study systems

Throughout chapters three-six the following methodologies are used in preparation for the use of *Trialeurodes vaporariorum* (glasshouse whitefly) and *Tuta absoluta* (also known as tomato leaf miner) in experiments. These methodologies include the use of controlled environments, selecting appropriate plants for testing host fecundity and survival and ensuring effective and consistent rearing of the insect populations. These methodologies are used in experiments to test the potential for resistance to evolve in pests to biocontrol agents (chapter 3 and 4), the potential life history trade-offs that could limit resistance evolution (chapter 5) and the potential methods to mitigate resistance and improve biocontrol efficiency in the field (chapter 6).

2.1 Study system

Trialeurodes vaporariorum is the model species used in chapters 3, 4, 5 and 6 for testing evolution of resistance to biocontrol agents in this thesis - sections 2.1-2.4 below outline the ecology of this species as an agricultural pest and review the common biocontrol methods used to control it. *Tuta absoluta* is the second model species used in chapter 6 for testing whether tank mixing of four different biopesticides has the potential to reduce the likelihood of resistance to biocontrol evolving while maximising the efficiency of using biocontrol agents in the field to control multiple insect pests. Section 2.5-2.6 outlines the ecology of this species as an agricultural pest and the common management strategies to control it. In sections 2.7-2.8 I have outlined the methods used throughout all experiments to rear both of these non-model insect pest species and their biological control agents.

2.1.1 Ecology of glasshouse whitefly *Trialeurodes vaporariorum* as a pest species

The glasshouse whitefly, *T. vaporariorum*, (hereafter referred to as 'whitefly') is a polyphagous hemipteran pest widely distributed across the globe and is one of the most economically important pests in horticulture (Kapantaidaki *et al.*, 2015). Individuals feed and reproduce on over 275 host plant species both in open fields and protected environments such as environmentally controlled glasshouses (Pym *et al.*, 2019). Both adult whitefly and nymphs can cause direct and indirect damage to crops. Through feeding on phloem, whitefly leave a honeydew residue on the foliage, which in turn promotes the growth of fungal sooty mould, damaging the plant (Johnson *et al.*, 1992). Indirectly whitefly can carry and transmit many plant viruses that cause crop loss and have significant consequences for crop yield, such as tomato yellow leaf curl virus, which stunts plant growth, curls leaves and inhibits flowers from developing leading to severe reductions in crop yields each season (Duffus *et al.*, 1996).

Whitefly are hemimetabolous insects with six developmental stages: egg, four instar nymphal stages, and adult. Whitefly are an arrhenotokous haplodiploid species haploid males are produced from unfertilised eggs, and females from fertilised eggs. Females have the capacity to store sperm and skew the sex ratio in favour of diploid females (Las, 1980). Adult females oviposit in a circular pattern on the underside of leaves, where they can lay up to 500 eggs over their lifespan. Eggs usually hatch within six days depending on environmental conditions (Las, 1980). The first instar nymphs are initially mobile, able to move a few millimetres but become immobile when they insert their stylet to begin feeding on foliage from the second instar onwards (Las, 1980). When they emerge as adults they have white waxy coated wings that lie flat over the abdomen, they mate and disperse by flight within one hour of emergence (Perring *et al.*, 2018). The whitefly developmental period varies, dependent upon environmental conditions, but usually takes between 22-30 days from egg to adult (Las, 1980). Egg laying is continuous throughout the adult female's lifespan of up to 40 days, depending on environmental conditions; thus, this developmental variation allows whitefly generations to overlap (Las, 1980). This generational overlap causes large influxes of adults within a short time frame, and this, combined with polyphagy,

are the main reasons that whitefly is a highly successful, and thus highly problematic, agricultural pest (Capinera, 2008).

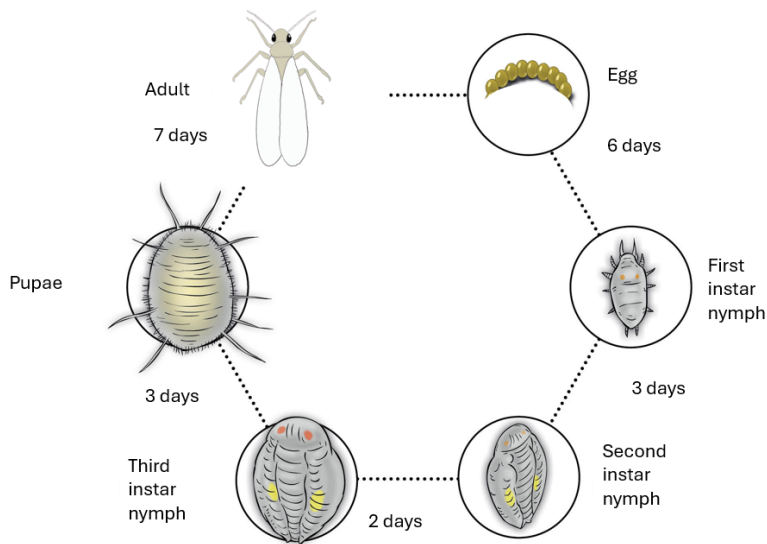


Figure 3: Lifecycle of *Trialeurodes vaporariorum* reared at 26°C from egg to adult, adapted from Syngenta Ornamentals UK graphic.

Whitefly resistance to pest management regimes, either to traditional chemical pesticides or to biological control agents, is likely to evolve in populations of whitefly when there is standing genetic variation for resistance traits (Ewens, 2013). When this genetic variation is heritable, there will be high variation in survival across individuals within a population when pest management regimes are applied to control and kill whitefly pests (Ewens, 2013). When the control agent which induces resistance is applied, selection reduces this variation in survival because individuals that have inherited genetic traits for resistance will have high survival and reproduce passing on genetic traits to offspring, while those with low inherited resistance fail to reproduce and are killed (Ewens, 2013). Although there is a large body of research on genetic diversity of a closely related species of whitefly, *Bemisia tabaci*, very little is known in regard to *T. vaporariorum* (Kareen *et al.*, 2018). When comparing phylogeographical structuring of the two whitefly species based on mtCOI haplotypes, the genetic variants found in the mtCOI gene, it has been found that *B. tabaci* is a species complex with more than 47 relevant mtCOI haplotypes whereas in *T. vaporariorum*, only 16 mtCOI haplotypes have been detected (Kareen *et al.*, 2018). This suggests that as *T.*

vaporariorum populations have lower genetic variability, the potential for resistant traits to spread through a population is high, if resistance is present in the population, however, resistance is less likely to evolve due to reduced genetic diversity (Kareen *et al.*, 2018). Whereas in populations with high genetic diversity, resistance is more likely to evolve but the spread of resistance through the population is likely to be slower (Barboza *et al.*, 2019). Understanding the genetic diversity of *T. vaporariorum* is vital for understanding if and how traits underlying resistance evolve, and how selection pressures, behaviours, geography and host differences, impact their ability to evolve resistance or transmit disease (Kareen *et al.*, 2018). Though this literature begins to provide an insight into genetic variation in *T. vaporariorum* populations, more comprehensive sampling, and sampling within closed systems over time is required.

2.1.1.2 *Trialeurodes vaporariorum* resistance to insecticides

Whitefly have been found to be resistant to numerous synthetic chemical insecticides traditionally used in whitefly pest management regimes. One of these insecticides is DDT (Dichloro-diphenyl-trichloroethane), which by the 1950s was the most well-known and used insecticide globally. The widespread use of DDT eventually resulted in many pests evolving resistance to various synthetic pesticides (Berry-Caban, 2011). This indicates that the problem of pesticide resistance was identified relatively soon after DDT began to be used extensively. The emergence of resistance highlighted the challenges associated with relying heavily on synthetic chemicals for pest control and the need for alternative strategies (Berry-Caban, 2011). In 1976, a study revealed increased resistance among populations of *Trialeurodes vaporariorum* in South East England. Specifically, seven out of 17 sampled populations demonstrated resistance to DDT, and 15 out of 17 populations showed resistance to malathion, an organophosphate insecticide commonly used to control a variety of insect pests (Wardlow *et al.*, 1976). This early evidence of strong resistance emphasised the growing challenge in managing pest populations using conventional chemical pesticides.

More recently, resistance to neonicotinoids, a modern class of pesticides, has been identified in *T. vaporariorum* populations (Karatos et al., 2012). The adoption of neonicotinoids increased after *T. vaporariorum* populations reduced mortality rates when exposed to older classes of synthetic pesticides, such as organophosphates and pyrethroids, drove the need for new effective control agents (Karatos et al., 2012). Resistance to neonicotinoids has now been reported globally for *T. vaporariorum* and the related whitefly *B. tabaci*. (Karatos et al., 2012). The metabolic mechanisms underlying resistance in these two whitefly species appear to be similar, involving overexpression of P450-dependent monooxygenases which detoxify the insecticide (Karatos et al., 2012). The rapid evolution of resistance in *T. vaporariorum* to a broad range of synthetic pesticides poses a significant threat to effective pest management. The dwindling number of effective chemical options due to resistance highlights the importance of preventative resistance mitigation research and continuous monitoring under integrated pest management. Managing and mitigating the impacts and evolution of resistance more effectively can ensure the long-term efficacy and efficiency of pest control measures, protecting crop yields from these destructive agricultural pests.

2.1.2 Ecology of *Encarsia formosa* as a parasitoid of *Trialeurodes vaporariorum*.

Encarsia formosa is a Chalcidoid parasitoid wasp in the family Aphelinidae and is an effective natural enemy for controlling approximately 15 whitefly species across the globe (Hoddle et al., 1998). *E. formosa* is mostly uniparental, exhibiting thelytokous parthenogenesis producing mainly females, this parthenogenesis can also be induced by the bacterial endosymbiont, *Wolbachia* (Hoddle et al., 1998).

E. Formosa males are occasionally produced under three different circumstances: (i) when *E. formosa* are reproducing in cooler climates; (ii) when *E. formosa* abundance is high and (iii) under hyperparasitism. Hyperparasitism occurs when a parasitoid develops on another parasitoid. In the case of *E. formosa*, male larvae develop as parasitoids on female *E. formosa* larvae that are already parasitising whitefly nymphs (Gerling, 1966).

Encarsia formosa has a lifecycle of approximately 28 days from egg to adult, with adults emerging between 14-22 days post-parasitism dependent on the environmental conditions and the hosts developmental stage (Milenovic *et al.*, 2023). *Encarsia formosa* development time can depend on the *Trialeurodes vaporariorum* hosts instar phase they parasitise; the younger the whitefly nymphal instar stage the longer the *E. formosa* developmental time (Hu *et al.*, 2002).

E. formosa has six development stages: egg, first, second and third instar larval stages, pupae and adult (Hu *et al.*, 2002). *Encarsia formosa* larvae are visible from the 1st instar stage where they are approximately 0.15mm long with a cylindrical body shape with a long tail (Hu *et al.*, 2002). Second instar larvae are approximately 0.78mm long and develop a comma body shape with a prominent yellow gut in their hosts (Hu *et al.*, 2002). During late instar development, from the third instar and pupation onwards, melanisation of the whitefly nymphal host occurs, and wasp larvae are no longer visible inside the whitefly nymph (Hu *et al.*, 2002). Once the wasp has pupated, the whitefly nymph is dead and no longer provides nutrients for *E. formosa*, the adult *E. formosa* wasps emerge by chewing a hole in the fourth instar whitefly dorsal surface and crawling out (Hoddle, 2008). From emergence as adults *E. formosa* being searching for *T. vaporariorum* hosts to feed on and parasitise.

Encarsia formosa locates *T. vaporariorum* hosts by detecting honeydew secretions from the whitefly adult and nymphs, whitefly host density and host size determines the likelihood of successfully locating hosts for parasitism, with larger, denser colonies being more easily detectable (Hoddle *et al.*, 1998). Once a whitefly nymph is successfully located, an adult female *E. formosa* will either oviposit or feed on the nymph. For oviposition the *E. formosa* female pierces the nymph with its ovipositor and lays a single egg inside the whitefly nymph's haemolymph; within 24 hours the oviposition site melanises on the whitefly nymphal casing leaving a visible dark scar on the host (Hoddle *et al.*, 1998). Studies have found that *E. formosa* can lay in all nymphal stages but will not pupate until the *T. vaporariorum* host is in the fourth instar phase or above, leading to the variation in developmental time described above (Hoddle *et al.*, 1998).

Alongside parasitising hosts, *E. formosa* females also feed on the whitefly nymphs, killing them in the process providing two biocontrol mechanisms (parasitism and predation) (Liu *et al.*, 2015). They feed on all whitefly host developmental nymphal stages excluding eggs but have a preference for older instars (Liu *et al.*, 2015). Whitefly hosts used for feeding are killed during feeding and so will never be parasitised. To feed *E. formosa* pierce the nymph with their ovipositor opening the nymph and feeding on the haemocoel (Nell *et al.*, 1976). *Encarsia formosa* wasps also feed on honeydew excreted by the whitefly, providing essential nutrients and carbohydrates that can increase wasp fecundity as well as egg hatch rates and developmental success (Liu *et al.*, 2015). Host feeding is more beneficial for parasitoid reproductive success than feeding on honeydew however; when *E. formosa* were provided with honeydew and given the option to feed on hosts, those that fed on hosts laid more eggs in total (Burger *et al.*, 2005).

Encarsia formosa can be used to control effectively whitefly due to the high levels of mortality they cause. A single female *E. formosa* can parasitise *and* oviposit up to five eggs per day, ovipositing an average of 59 eggs during its lifetime. *Encarsia formosa* will also host feed on up to three nymphs per day, killing on average a total of 95 nymphs over a 12- day life expectancy, making them one of the most successful whitefly control strategies (Deeksha *et al.*, 2023). Despite *E. formosa*'s success and widespread use it is still unknown whether whitefly can evolve resistance to parasitism. However, there is increasing evidence for the evolution of resistance to parasitoids in similar insect pests, alongside anecdotal evidence on the failure of *E. formosa* as a biocontrol agent increasing over a crop growing season, suggesting the evolution of resistance could be the cause. If the reduced success of *E. Formosa* is related to resistance many mechanisms could be the cause, through evolution of the whitefly nuclear genome or the selective sweep of certain bacterial endosymbionts in a whitefly population that offer protection against parasitism.

2.1.3 Fungal pathogens as a control method for *Trialeurodes vaporariorum*

Whitefly are also commonly controlled in protected edible cropping systems using fungal biopesticides, particularly those formulated from the entomopathogenic fungus *Beauveria bassiana* in commercially produced suspensions (Quesada-Moraga *et al.*, 2006). Although this is a widely accepted and well used biocontrol method, there is speculation that the fungal biopesticide products lose their effectiveness over time (Quesada-Moraga *et al.*, 2006). This reduction in effectiveness could be due to the evolution of resistance in the whitefly, which has not been studied before in *T. vaporariorum*, but evidence exists in other insect species, such as the greater wax moth, *Galleria mellonella*. When *G. mellonella* were placed under high selection pressure through exposure to high doses of *B. bassiana*, *G. mellonella* larval mortality significantly decreased over 25 generations, likely due to resistance evolution (Dubovskiy *et al.*, 2013). Other than this, few studies have assessed the potential evolution of resistance to fungal biocontrol agents, as such, further research is sorely needed across different pest species and fungal pathogens to understand the mechanisms and manage the risk.

2.1.4 Ecology of *Tuta absoluta* as a pest species

Tuta absoluta (hereafter referred to as ‘tomato leaf miner’) is a leaf miner moth native to South America. It is a major threat to agricultural crops, in particular tomato crops in approximately 60% of tomato growing regions across the world (Santana Jr *et al.*, 2019). Although favouring solanaceous crops, they also feed and develop on Amaranthaceae, Convolvulaceae, Fabaceae, and Malvaceae (Desneux *et al.*, 2021). Tomato leaf miner are holometabolous insects with four developmental stages: egg, larvae, pupae and adult (Jamwal *et al.*, 2022). Their lifecycle is approximately 21-30 days from egg to adult depending on environmental conditions, and adults have a lifespan of around 17 days, with females able to oviposit for their entire adult life (Jamwal *et al.*, 2022). Adult females use plant volatiles to locate and move towards a

host plant where they lay approximately 15 yellow eggs per day (260 eggs during their lifespan) on the underside of leaves on the upper part of the plants. Once the eggs hatch, the larvae feed by mining the leaf mesophyll, burying inside and feeding upon leaf tissue producing thin mines, when the leaf is destroyed, they move to a neighbouring leaf for the same process (Desneux *et al.*, 2021). *Tuta absoluta* are capable of mining into all parts of the crop and can cause plant death by removing leaf material disrupting photosynthesis and preventing fruit and flower development before killing off the plant entirely (Jamwal *et al.*, 2022). Once the larvae have pupated, they drop off the leaf producing cocoons in the soil, where they remain until they emerge as adults (Urbaneja *et al.*, 2009). The pupal stage is approximately 9-11 days depending on conditions and *T. absoluta* can also overwinter as eggs, pupae or adults. Adults are sexually active as soon as they emerge and find mates using volatile sex pheromones (Biondi *et al.*, 2018). Tomato leaf miner are one of the most devastating agricultural pests across the globe due to their high reproductive rates, short generation time and generational overlap, rapid offspring development and high survival rates, the cryptic nature of larvae, and adult and larvae resistance to many insecticides and pest management regimes (Urbaneja *et al.*, 2009).

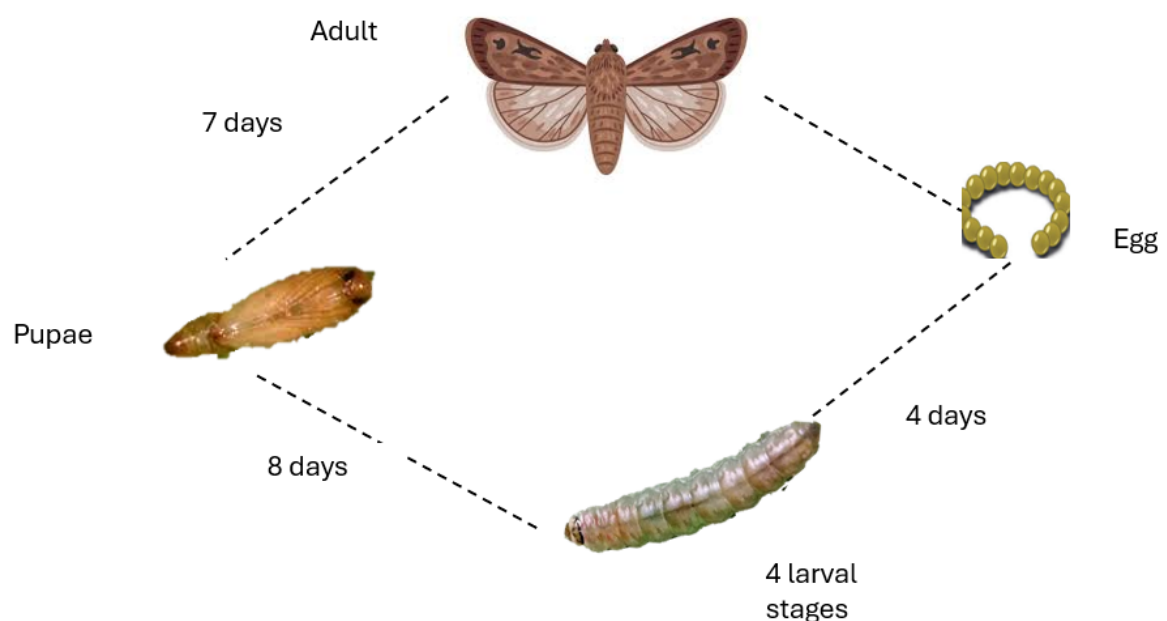


Figure 4: Lifecycle of *Tuta absoluta* reared at 24⁰C from egg to adult.

2.1.4.1 *Tuta absoluta* resistance to insecticides

Like the majority of horticultural pests, tomato leaf miner has developed resistance to many chemical synthetic insecticides due to overexposure and strong selection pressure increasing selection for genotypes with high survival (Desneux et al., 2010). Insecticide resistance evolves quickly in this species in some cases due to genetic variation in target binding sites for toxins. Changes in these binding sites alter the ability of the insecticide toxins to bind to the insect tissues (Desneux et al., 2010). Other mechanisms of resistance involve the enhanced detoxification of the toxin when it enters the host haemolymph (Guedes *et al.*, 2019). In another case, tomato leaf miner exhibit high levels of resistance to the diamide chemical insecticide Chlorantraniliprole through an additional mechanism. This resistance mechanism was not due to target site alterations or detoxification, but overexpression of a gene encoding a UDP-glycosyltransferase (UGT). How this mechanism confers resistance remains to be tested, but the diversity of resistance mechanisms which have evolved in this species suggest that tomato leaf miner are evolutionarily labile with high genetic variation and adaptive potential for resistance evolution (Grant *et al.*, 2023).

2.1.4.2 *Tuta absoluta* biological control agents

Due to the rising abundance of tomato leaf miner and increased resistance to chemical insecticides, natural enemies and biopesticides have been integrated into pest management strategies for controlling tomato leaf miner (Desneux et al., 2010). Integrated Pest Management (IPM) regimes now frequently and successfully employ *Trichogramma* egg parasitoids and hemipteran larval predators, such as *Nesidiocoris tenuis* and *Macrolophus caliginosus*, to combat this pest (Urbaneja et al., 2009). These biological control agents have been widely adopted and have demonstrated effectiveness globally, offering a sustainable and environmentally friendly alternative to traditional chemical methods (Ferracini *et al.*, 2019). (see Chapter 1.8.2)

Trichogramma egg parasitoids, specifically *Trichogramma achaeae* and *Trichogramma chilonis*, are parasitoids of *T. absoluta*, (Schöller *et al.*, 2006). Female wasps locate host eggs by following plant volatiles emitted by the host plants and host eggs, once located the female oviposits into the host egg and lays a single egg (Schöller *et al.*, 2006). The *Trichogramma* larvae then develop within the host egg, consuming its contents and preventing the host insect from developing. The *Trichogramma* larvae pupate and emerge as adult wasps in approximately 7–14 days (Schöller *et al.*, 2006). Adult parasitoids mate shortly after emergence, after which females seek fresh hosts to oviposit on, parasitizing up to 50 eggs in her adult life span of 3–14 days (Schöller *et al.*, 2006). This parasitic relationship helps control pest populations naturally and is widely used in integrated pest management programs.

Most commonly biopesticides produced from *Bacillus thuringiensis* (Bt) have also been found to rapidly and significantly reduced damage to tomato crops by controlling tomato leaf miner populations. Bt produces proteins (Cry and Cyt toxins) that are toxic to specific insect pests when ingested Biopesticide formulations are sprayed on the crop, where contaminated foliage is ingested by the tomato leaf miner larvae (Ferré & Van Rie, 2002; González-Cabrera *et al.*, 2011). There are many different control options used to control tomato leaf miner, however, there is limited research on the potential to evolve resistance to non-chemical means of pest control. Given the tendency of this pest to rapidly evolve resistance in response to chemical insecticides, regular testing and careful management is required to assess and manage the risk of resistance to ensure the long term effectiveness and efficiency of biological control options (Silva *et al.*, 2015).

2.2 Rearing and maintenance

2.2.1 Host plant growth

Whitefly were reared on organic *Solanum melongena* black beauty aubergine plants grown from seed (seed provided by Tamar Organics). Host plants were chosen due to their commonality in protected edible and horticultural environments alongside their suitability for laboratory experiments. Aubergine plants were selected due to being well produced in glasshouses for edible food production and often favoured by the whitefly. Aubergine also produced a high proportion of foliage, compared to plants such as tomato, providing more leaves and surfaces for a higher rate of oviposition for the pest species at approximately 500 eggs per individual whitefly. Aubergine plants were grown in a plant-growth room with constant environmental conditions at 24° C, 70% RH, with a 16:8hr light-dark cycle. Aubergine seeds were sown in a mixed peat sowing compost, in seed trays, when seedlings were approximately four weeks old, they were re-potted into fertiliser enriched compost in individual two litre plant pots. Plants were watered every two days, and dead leaves and flowers removed for optimal foliage growth. All plants were fertilised fortnightly with commercial recommended dosage of Tomorite liquid fertiliser. Plants were used for colony cages and in experiments when they were between 10-14 weeks from sowing, by which time aubergine plants had at least six large healthy leaves.

2.2.2 *Trialeurodes vaporariorum* (Glasshouse whitefly) rearing

Whitefly used in all experiments originated from a colony established in 2021 from Bioline AgroSciences stock, provided as approximately 500 pupae on tobacco leaves. Tobacco leaves were placed in a bugdorm-6M620 cage and monitored for whitefly emergence. Once adults had emerged, they were collected by a mechanical pooter and a colony was established and maintained in a bugdorm-6M620 cage in a controlled environment room at constant environmental conditions; 26° C, 70% RH,

with a 16:8hr light-dark cycle, consistent with experimental conditions. A large whitefly colony with at least 4000 adults was established, with whitefly reared on Black Beauty aubergine plants and maintained throughout the entirety of the experiments which make up this thesis. In 2023, the initial colony was divided between two bugdorm-6M620 rearing cages to increase the size of the colonies and reduce risk of insect predators, pests and pathogen infection wiping out the entire colony. Fortnightly at least one new plant was added to each cage and every four weeks the colony cages were completely refreshed. This involved collecting approximately 4000 adult whitefly from each of the current colony cages (1 & 2) using an electric pooter and placing them into new cages, keeping the two colonies separate, with four fresh aubergine plants that were approximately 10 weeks old. Cages were labelled with their cage ID and date of set up. The old colony cages were then removed, plant material disposed of, and cages disinfected. Plants in the colony cages were watered every two days and fertilised weekly with liquid commercial recommended dosage of Tomorite liquid fertiliser. Colonies were inspected weekly and dead leaves and plants removed, if unwanted pests, predators, insects or pathogens were present, the cage was removed and placed in an isolation control room, where adult whitefly were collected via a mechanical pooter and a new colony cage was established.

2.2.3 Experimental set up of *T. vaporariorum*

To obtain whitefly nymphs for experimental work, adult whitefly were transferred to uninfested aubergine plants placed in Watkins and Doncaster 40cm flower pot cages in a controlled environment plant growth chamber. Approximately 75-100 *T. vaporariorum* adults were collected from the laboratory colony using a mechanical pooter and placed in each cage. The number of cages set up was dependent on the experiment and required number of replicates (details given in chapters three, four, five and six). Caged plants were placed in large plant trays, and the trays were filled with water every two days, to avoid removing plant pot cages from chambers. Adult *T. vaporariorum* were left in the plant pot cages for four days to lay eggs before being removed from the cages. Due to the *T. vaporariorum* native status in the UK, adults were considered safe to remove from cages outside. To remove the adults the plants

were taken outside, cages opened, and plants were shaken. Any remaining whitefly were removed using a pooter and euthanised by freezing (the immobile eggs/nymphs remained on the plant leaves). Once adults were removed plants were left for a further 10 days in the chamber before being used in experiments, by which time the majority of the nymphs had reached second instar life stage.

2.2.4 *Encarsia Formosa*

Encarsia formosa parasitic wasps (hereafter referred to as 'wasps') were provided by Bioline AgroSciences for use in experiments in chapters three, four and five. Wasps were delivered as pupae, in melanised glasshouse whitefly nymphal cases in sealed filtered petri dishes two days before use in experiments. Wasp pupae were kept isolated in a separate controlled environment cabinet from all whitefly to avoid unexpected parasitism. The controlled environment facility rooms were kept at a constant 26° C, 70% RH, with a 16:8hr light-dark cycle until emergence as adults. Once adult wasps had emerged, they were used in experiments, wasps that were not used were euthanised by freezing and disposed of.

2.2.5 Host parasitism

For experiments in chapters three and four that included wasps as a treatment group to parasitise whitefly, wasps were added during the experiments. Second instar whitefly nymphs on aubergine plants in flowerpot cages were roughly counted and adult wasps were added to the plant cages in a 1:5 ratio (1 wasp per every five nymphs). After 48 hours, wasps were removed by taking the plant out of the cage shaking vigorously outside and replacing the mesh cage (immobile nymphs remained attached to the plant when they were shaken). Any wasps not removed via shaking were removed via a mechanical pooter, euthanized by freezing and disposed of. Whitefly nymphs were left for a further 10 days to develop until whitefly adults eclosed or parasitism was evident through complete melanisation and mummification of the whitefly nymphal case, resulting in a black appearance (see figure 3A/B). In some

cases, during experiments, parasitism events were identified and recorded at earlier stages; a positive parasitism event was classified as a black melanisation mark on the nymphal case 48 hours after exposure (figure 3C), where oviposition has taken place.

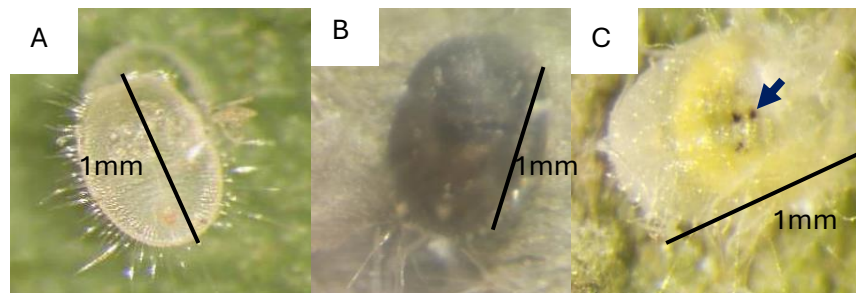


Figure 5: A) An unparasitised whitefly pupae, B) A melanised whitefly nymph. C) A whitefly nymph with three melanisation marks from positive parasitation by *E. formosa*, showing wasp larvae development. Scale bars indicate whitefly pupae are 1mm in length.

2.2.6 *Tuta absoluta* rearing and experimental set-up

Tuta absoluta used in chapter six were supplied as pupae from Andermatt Biocontrol AG. Pupae were supplied a maximum of five days before use in the experiment. All *T. absoluta* rearing and experiments took place in separate environmental chambers at 24° C, 70% RH, 16:8hr L:D. Upon arrival pupae were sorted and placed into small plastic tubs with filtered mesh lid; a maximum of five pupae were placed in each tub which were monitored daily for emergence. Once emerged, the tubs containing adults were placed in the fridge for a maximum of 15 minutes to sedate adult moths, ensuring they did not escape. Adult moths were removed and placed into fresh small plastic containers with mesh lids containing a 40% sucrose solution on soaked cotton wool balls. A maximum of two adults were placed in each tub and kept for up to 24 hours whilst being fed, the cotton wall balls containing a sucrose solution were replaced every 12 hours, before transfer to experimental tomato plants.

During the experiment 25 to 30 adult *T. absoluta* were placed into two large mesh cages containing nine tomato plants in each cage. Cages were placed in a controlled environment chamber for the duration of the experiment. Adult *T. absoluta* were given three days to mate and lay eggs on tomato leaves before being removed. Adults were removed using an electric pooter or captured by hand in a tube. Experimental tomato plants were left for four days and were inspected closely for *T. absoluta* larvae to develop to the first larval stage, the commercial recommendation for biopesticide use. Black sticky traps were hung around the outside of the cages to reduce the risk of adult and larval *T. absoluta* escaping. Once larvae had reached the first instar life stage the plants were exposed to biopesticide treatments which I outline in detail in chapter 6. Once experiments were finished all infested plant material was frozen and autoclaved for disposal and the controlled environment chambers were heat blasted and disinfected to ensure any experimental material or insects were killed.

Chapter 3: The potential for resistance evolution to biological control agents.

Abstract

Understanding pest resistance to pest management regimes is crucial for global food security and the effectiveness of pest management strategies. Attention has historically been focused on the longevity and potential resistance to chemical synthetic pesticides. In this study, the potential evolution of resistance to biological control agents was studied in glasshouse whitefly (*Trialeurodes vaporariorum*), a detrimental pest on global crops, particularly protected edible and soft fruits. This study was conducted via a lab-based experiment assessing variation in survival to *Encarsia formosa* parasitoid wasps and a commercial fungal biopesticide containing *Beauveria bassiana*. A simulation model was also designed to compare whitefly survival rate over time in the fungal treatment, *E. formosa* and a treatment alternating between the two biocontrol agents each generation on both tomato and aubergine crops, common protected edible crops. I found that there is significant variation in survival between whitefly lines in both the fungal and wasp treatment groups, with higher survival rates in the fungal treatment. In the simulation, survival rates significantly increased in all treatment groups over time but differed depending on the crop. Whitefly had the highest survival in the aubergine crop compared to tomato in all simulated scenarios. Survival was highest in the alternating treatment in the tomato crop but highest in the wasp treatment in the aubergine crop, suggesting crop has an effect on survival rates. This suggests that in general whitefly do have the potential to evolve resistance to biological control agents and survival rates could be dependent on crop.

3.1 Introduction

Insect pests can cause significant crop damage and economic losses, with arthropods destroying an estimated 18–20% of the annual crop production worldwide (Sharma *et al.*, 2017). If insect pests are not controlled, they can have detrimental environmental effects such as biodiversity loss and can pose a potential risk to human health via the spread of disease (Fortuna *et al.*, 2022). Traditionally insect pests were mainly controlled using chemical synthetic pesticides. However, due to increasing knowledge of the negative effects of these chemicals on the environment, human health and the rise in pesticide resistance, many of these pesticides have become ineffective or banned under government legislations (Donley, 2019). This has led to a large shift to adopt more sustainable pest management practices, such as integrated pest management (IPM) systems (Deguine *et al.*, 2021). With a rise in the use of biological control agents IPM founded in 1970s is now seen as a paradigm based on prevention monitoring and control (Dara, 2018). IPM involves the use of several pest management techniques, alongside continual monitoring and planning, sharing knowledge and resources (Angon *et al.*, 2023). However, there are currently few resistance management techniques for insects pests controlled with biological control agents (Siegwart *et al.*, 2015).

Agricultural insect pest control is threatened due to the evolution of pesticide resistance. Over 500 insect and mite species are now resistant to over 400 pesticide compounds across the globe (Siddiqui *et al.*, 2023). Most cases of insect pesticide resistance evolution are through physiological mechanisms due to high genetic variation and continual exposure to high selection pressures. However, behaviour plasticity has also been shown to play a role in the evolution of resistance (Siegwart *et al.*, 2015).

Where there is standing genetic variation for resistance in a pest population, latent resistant traits are already present in the insect population before the selection occurs, meaning that the population has a variation in susceptibility prior to application of pesticides. When insect pest populations are exposed to high selection pressures when pesticides are overused and applied in large quantities, individuals with the resistant traits thrive and survive producing resistant offspring leading to pest populations evolving resistance to the pesticide quickly (Hawkins *et al.*, 2018). In a

study on pesticide resistance in two spotted spider mite populations, it was found that standing genetic variation is thought to be the cause for the evolution of resistance to the chemical pesticide bifenazate used to control the mite populations (Shi *et al.*, 2019). Prior to the application of bifenazate, in spider mite populations the frequency of the mutated G126S in the mitochondrial cytochrome b gene was low with 1 in 288 individuals carrying the gene. Five years after continuous bifenazate applications exposing the mites to high selection pressures, it was found that all of four Beijing two spotted spider mites resistant populations carry the mutated gene. This suggests standing genetic variation for bifenazate resistance exists in mite populations (Shi *et al.*, 2019). Where individuals in a pest population carry *de novo* mutations in the target site or major metabolic resistance genes, resistance mutations occur once the pesticide has been applied, leading to resistance as the frequency of the mutations increases. When pesticides are applied, individuals within the population that carry resistant traits through physiological or behavioural mechanisms such as target site mutations, metabolic detoxification, and behavioural adaptations survive (Chareonviriyaphap *et al.*, 2013). These individuals may pass these resistant traits on to offspring. Thus, selective pressure leads to rapid evolution of resistance within pest populations, resulting in the pesticides becoming less effective or ineffective over potentially a relatively short time frame, such as an agricultural growing season of up to nine months, increasing the number of resistant pests and ineffective compounds (Hawkins *et al.*, 2018).

In tobacco whitefly *Bemisia tabaci* multiple biotypes have been identified. Biotypes are populations within a species that are genetically similar but differ in important phenotypic traits such as host preference or insecticide resistance.

Two of the main biotypes, biotype B (MEAM1) and Q (MED) showed varying susceptibility to neonicotinoid pesticides. In the MED biotype, at least two novel P450s, (CYP303 and CYP6CX3) were found as detoxification mechanisms of neonicotinoid resistance in *B. tabaci*, suggesting the cause of resistance to be *de novo* mutations (Ilias *et al.*, 2015). P450s are a family of enzymes which play a vital role in the metabolism of various substances within organisms. Due to this increase in resistance, reduced efficiency of the pesticides and detrimental environmental effects, there has been a rise in the use of integrated pest management, particularly the use of biological control agents.

The use of biological control agents in agriculture pre-dates the use of synthetic modern pesticides. However, due to the effectiveness of chemical pesticides the use of biocontrol agents was overshadowed until the 20th century when the benefits of biological control agents were more widely acknowledged (Bale *et al.*, 2007). Biocontrol agents are naturally occurring organisms that are used to control pests and disease in agriculture. As many biocontrol agents have small target host range, and as they are naturally occurring, they are a sustainable and environmentally friendly approach to minimise the use and reliance on chemical pesticides (Ram *et al.*, 2018). The major categories of using biocontrol agents, particularly in agriculture, are augmentative control, conservation control and classic biocontrol. Augmentative biocontrol can be split into inoculative biocontrol, where natural enemies are released in small numbers over an extended period of time to establish and maintain a natural enemy population, that can suppress pest populations over time. Inundative control, where there is a mass release of natural enemies; repeatedly released for an immediate control of insect pests (Hajek and Eilenberg, 2018). Conservation biocontrol is the creation or maintenance of an environment that supports and grows naturally occurring natural enemy populations. However, this can remove economically viable land from agriculture (Perez-Alvarez *et al.*, 2019). Classic biocontrol introduces natural enemies from the pest's native habitat to control invasive pest species in a new environment where the pest lacks effective natural enemies, this method is thought to be a long term solution as long as the natural enemy released does not become invasive itself (Vänninen, 2005).

For all types of biocontrol, there are multiple categories of biocontrol agents. They can be predators: insect, nematodes or mite predators that directly consume insect pests. One of the most successful predators used in augmentative biocontrol is the predatory mite. *Amblyseius swirskii*, *Phytoseiulus persimilis*, *Neoseiulus cucumeris* and *Neoseiulus californicus* are some of the most successful mites controlling two-thirds of arthropod pests (Knapp *et al.*, 2018). A second biocontrol natural enemy are parasitoids that lay their eggs on or within the bodies of host pests, leading to the death of the host as the parasitoid larvae develop. *Encarsia formosa* is one of the first and most successful biological control agents. It is a known endoparasitoid for many whitefly species across the globe (Hoddle *et al.*, 1998). Pathogens, including bacteria, fungi, viruses, and nematodes, infect and kill pests, often through the release of toxins

or via the disruption of essential physiological processes. (Lacey *et al.*, 2015). Biocontrol agents have much evidence for their success within agriculture, as biocontrol is now used readily across the globe with little concern for the potential of insect pests evolving resistance. However, there is evidence to suggest that insect pests can vary in their susceptibility to biocontrol agents, and some pest species have evolved resistance (Bardin and Siegwart, 2022).

As biocontrol agents are naturally occurring organisms, in the early stages of biocontrol use there was a misconception that insect pests would not evolve resistance in a similar way to chemical pesticides (Schulz *et al.*, 2019). It was assumed that the biocontrol agents naturally co-evolve with the pest, suggesting that the relationship between the pest and biocontrol agent is maintained, and the biocontrol agents remain a long-term effective pest management option (Holt and Hochberg, 1997). As many biocontrol agents are host specific, there was a hypothesis that biocontrol agents have often evolved specific mechanisms to infect and kill the pest host, which could reduce the likelihood of resistance evolution (Antonovics *et al.*, 2013). However, over the last few decades there has been a large increase in evidence to show insect pests have evolved resistance to many major biocontrol agents threatening the lifespan and effectiveness of biocontrol (Bardin and Siegwart, 2022).

One of the most well-known cases of viral biocontrol resistance is in the codling moth (*Cydia pomonella*), a pest of European apple orchards. Granulovirus based insecticides were used to control the codling moth in early 1990s. However, by mid 2000s there was widespread resistance requiring the development of new viral biocontrol agents to control the codling moth (Mangan *et al.*, 2023). Many aphid species have developed mechanisms to resist parasitoid attack. The pea aphid, *Acyrtosiphon pisum*, has shown parasitoid resistance against the parasitic wasp, *Aphidius ervi*, when infected with the bacterial symbiont *Hamiltonella defensa* and its associated bacteriophage APSE (*Acyrtosiphon pisum* secondary endosymbiont) (Oliver *et al.*, 2012). Parasitoid resistance has also been observed in *Listronotus bonariensis*, the Argentine stem weevil, when exposed to the parasitoid *Microctonus hyperodae*. It was found that the efficiency of the parasitoid and successful attacks had significantly declined over 25 years (Tomasetto *et al.*, 2018). When resistance to fungal biopesticides was explored, significant resistance was observed in the Greater wax moth, *Galleria mellonella*, when it was exposed to high selective pressures from

the pathogenic fungus *Beauveria bassiana*, one of the most common active ingredients in many commercial fungal biopesticides (Dubovskiy, *et al.*, 2014). This evolution of resistance is seen throughout agricultural systems. However, in intensified agriculture systems or protected horticulture where there is reduced availability of refuge areas and diversity of enemy species, biocontrol resistance is more likely to occur (Tomasetto *et al.*, 2011).

Insect pests are a major issue for crop health and yield in protected horticulture, where crops are cultivated within controlled environments such as glasshouses or tunnels. These enclosed spaces provide favourable conditions for pest proliferation, with constant temperatures, light and humidity levels (Hussey and Bravenboer, 1971). As an insect pest population in closed environments is likely introduced into the environment via human carrier, vents, soil or founding crops, pest populations are established from a small number of individuals. This causes the established population to carry only a subset of the genetic diversity, contributing to the low genetic diversity within the population (Hawkins *et al.*, 2019). As many glasshouses and tunnels are well controlled closed systems, once a pest population is established within, there is limited dispersal of individuals in or out of the environment (Messelink *et al.*, 2021). This limited dispersal can lead to reduced genetic diversity within the insect population (Ovčarenko *et al.*, 2014). As mating out with the population is constrained, gene flow between populations is restricted resulting in a reduction in genetic variation (Diekötter *et al.*, 2010). This limited dispersal also increases an individual's chance to mate with closely related individuals. This can lead to increased levels of inbreeding, which further reduces genetic diversity by increasing the prevalence of homozygosity (Calleri *et al.*, 2006). In these isolated populations, genetic drift can have a significant impact on reducing the genetic diversity through random changes in allele frequencies there are less random changes in allele frequencies in the population passed over generations (Pélissié *et al.*, 2018).

This limited genetic diversity due to dispersal has been observed in many insect pests such as the glasshouse whitefly *Trialeurodes vaporariorum*. A study was undertaken on the effects of genetic diversity and dispersal of the glasshouse whitefly *Trialeurodes vaporariorum*. Population samples had been taken from 12 glasshouse locations throughout the UK. 91 out of the 96 UK individual whitefly populations had a mitochondrial COI sequence belonging to haplotype mtH1, suggesting low level of

genetic variation in the overall population. The population structure suggested that glasshouse agroecosystems restricted gene flow between glasshouse whitefly populations (Kareen *et al.*, 2019). If pests evolve resistance to pesticides or biological control agents within a closed system, the reduced gene flow could increase the rate resistant selective sweeps occur and resistance could become fixed within the population, quickly resulting in a large resistant population with significant detrimental effects on pest management strategies (Calla *et al.*, 2020).

In this study, I investigated the potential for the evolution of resistance by assessing variation in susceptibility in one of the most detrimental crop pests. I exposed the glasshouse whitefly *Trialeurodes vaporariorum* to commonly used commercially available biological control agents, the fungal biopesticide BotaniGard®, which contains *Beauveria bassiana*. These fungal pathogen spores adhere to the insect cuticle, germinate, penetrate the cuticle, and then grow inside the insect body to cause death. The second treatment was the parasitoid wasp *Encarsia formosa* supplied by Bioline. *Encarsia formosa* is an endoparasitoid, laying a single egg inside a whitefly nymph, killing the whitefly as the larvae develops, and as a second mode of action, they feed on unparasitised nymphs.

I exposed 35 whitefly lines reared on organic aubergine plants in a controlled environment facility to three treatments: (1) control- no biological control agent, (2) fungal biopesticide BotaniGard® and (3) *E. Formosa* parasitoid wasps at the commercial recommendations. All 35 lines were represented in all three treatments, and I assessed the variation in survival between the lines within a treatment and compared survival in each line across all treatments. If there is variation in survival between lines within a treatment, this could suggest that populations have the potential to evolve resistance over time. If survival per line is similar across all treatments, this could suggest there is cross resistance. Cross-resistance occurs when an organisms resistance to one, in this case insecticide confers resistance to another insecticide, even where the insect has not been exposed to the latter product (IRAC, 2024).

Cross resistance could be detrimental for pest management strategies of combining or applying biocontrol agents sequentially to control a higher proportion of pests. The mortality rate would not increase, but high selection pressures would remain thereby increasing the resistant population growth and having detrimental impacts on crop

health and yield. However, if a line has a higher survival in one treatment and low survival in another this would suggest a trade off in resistance. A trade off occurs when an organism evolves resistance, but this resistance comes at a cost to the organisms fitness in other areas. In this case, if the whitefly are resistant to one biocontrol, they are more susceptible to the second. The pest was chosen for this study as it is one of the most detrimental horticultural pests, and the biological control agents were chosen because they are the most commonly used agents to control whitefly in protected edible horticultural systems across the UK. Based on this empirical data set, I wrote an individual-based model (written from scratch in R) to simulate and forecast the potential resistance evolution of *T. vaporariorum* populations. Individual-based models (IBMs) are simulation models that represent the behaviour and interactions of individual organisms within a population. These models capture the heterogeneity and stochasticity of individuals to predict and forecast emerging traits in a population over time. I simulated resistance evolution of *T. vaporariorum* populations in commercial glasshouses to the above biological control agents, if these agents were applied every generation over 200 generations, I added in a fourth treatment alternating between the two control agents to begin to investigate the potential resistance management strategies.

3.2 Methodology

3.2.1 Experimental methodology

3.2.1.1 Host Plants

Aubergine plants used in this experiment followed the same growing procedures set out in Chapter two general methods.

3.2.1.2 Experimental set up

Trialeurodes vaporariorum were collected from the established colony, which was set up and maintained as described in Chapter 2.71-2.73 general methods.

To isolate virgin *Trialeurodes vaporariorum* adults for the founding generation in this experiment, two aubergine plants containing over 200 *T. vaporariorum* pupae were removed from the colony BugDorm 6M620 and placed in a separate BugDorm-6M610 in the controlled environment facility at constant 26°C, 70% rH and a 16:8 light dark cycle. To ensure adults were virgins, they needed to be collected within one hour of emergence. Pupae were monitored every hour from lights on for eight hours for emergence. *T. vaporariorum* adults that had emerged after the eight-hour observation window were discarded. Virgin adults were collected by aspiration, anaesthetised using CO₂ and sexed; one newly emerged adult female and one newly emerged adult male were placed in a clip cage on the underside of an aubergine leaf (see appendix 8.1, fig 36 for clip cage design). If an uneven number of females and males were present, the adults not used in the experiment were placed into the colony cage. Each pair were given four days to lay an egg clutch, before being moved in the clip cage onto a new leaf. If an egg clutch was laid, the leaf was numbered and tagged, and a black organza bag was placed over the leaf. Each pairing was moved to a new leaf until death or failure to lay. The number of eggs, date laid, and number of clutches were recorded for each pairing. Clutches were left for F1 offspring to emerge. Deceased adults were collected, sexed and frozen in ethanol for DNA extraction and PCR amplification.

Newly emerged F1 adults were used to generate F2 nymphs which would be exposed to different treatments and assayed for resistance. F1 adults were paired in a reciprocal crossing scheme (see fig 4) following the methodology above ensuring virginity. One F1 male and one F1 female from two different clutches were placed in a clip cage on the underside of an aubergine leaf. These pairings were classified as one line; three replicates of each pairing were set up by using 3 F1 brothers mated to 3 F1 sisters from different clutches. For each cross the reciprocal was also established (fig 4), i.e. if 3 F1 brothers from line A mated with 3 F1 sisters from line B, 3 F1 brothers from line B would also be paired with 3 F1 sisters from line A. In total, 35 lines with a minimum of three replicate pairings were established (135 pairings/ lines in the total experiment), each line was labelled A-AL (three lines did not contain enough replicates and were discarded). The 3 replicated lines were set up on different plants to allow for all lines to be represented in each treatment, as an entire plant was a separate treatment group. The control, fungal and wasp treatments contained 20, 15 and 12

plants respectively. Multiple lines were placed on individual plants within 24 hours to allow for synchronicity in F2 instar development. Every 24 hours, a new batch of plants was used. Clip cages were moved every four days onto a fresh plant until death or failure to lay. Every four days the egg clutches were counted and tagged and left to develop to 2nd instar nymphs. Once F2 2nd instars were observed in all line clutches on the plant, they were exposed to treatments. In the control treatment, there was a total of 647 individual nymphs with an average of 18 per line. In the fungal treatment, there was a total of 913 nymphs with an average of 26 individuals per line. In the wasp treatment, there was a total of 750 nymphs with an average of 21 individuals per line.

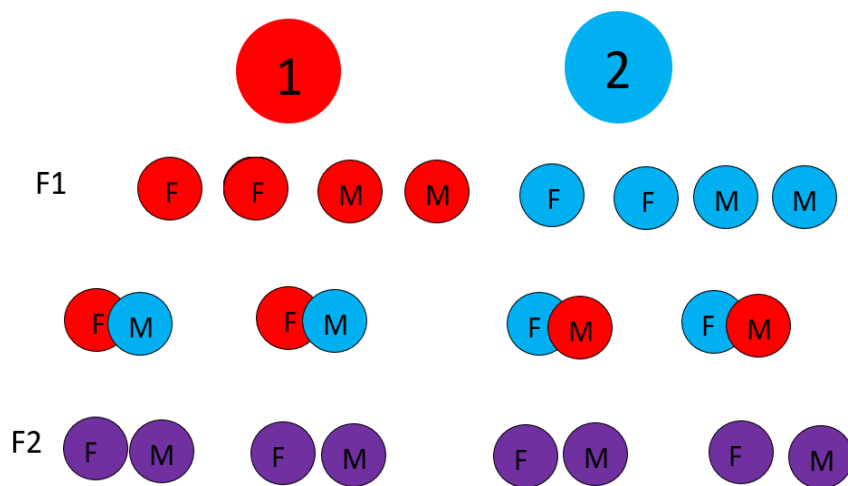


Figure 6 The reciprocal crossing scheme used to establish the 35 whitefly lines for the experiment crossed offspring of two independent whitefly lines, crossing females and males in both directions. Red circles indicate one line, the blue circles a second whitefly line. F1 and F2 indicate the first and second generations of offspring. Females from line one are mated with males of line two and vice versa. The purple circles show the offspring as a result of breeding line 1 with line 2. This scheme occurred for all 35 lines.

3.2.1.3 Treatment exposure protocol

Each plant was subjected to either a control treatment, (sprayed with distilled water), a Fungal biopesticide treatment, (sprayed with a commercially available product BotaniGard© containing *Beauveria bassiana* spores), or *Encarsia formosa*, (parasitoid wasps). The control, fungal and wasp treatments included 20, 15 and 12 plants respectively.

Individual plants were exposed to one treatment group to avoid contamination, with each treatment group containing approximately the same number of replicates. Leaves were treated when 2nd instar nymphs were present, approximately 10 days after laying. The fungal biopesticide treatment group was a *B. bassiana* suspension sprayed no longer than 30 minutes after mixing. The fungal suspension was mixed to the commercial concentration recommendations using 0.0625g of BotaniGard©, a wettable powder containing 220 g/kg *Beauveria bassiana* GHA present as 4.4×10^{10} CFU/g, mixed into 100ml distilled water.

A sparmax dh-12s airbrush was attached to a compressor to spray the control and fungal treatment groups, to replicate commercial spraying. The airbrush was cleaned before and after each treatment spraying ethanol through the airbrush attachment. All spraying was conducted in a fume hood, with a plastic guard placed behind each leaf to be sprayed to reduce double exposure to other leaves. Undersides of leaves were sprayed individually to the manufacturers' guidelines of spraying until runoff. Once sprayed, the plants were left for 15 minutes to dry off and placed in individual plant cages to avoid exposure to other plants. The cages were placed back into the controlled environment facility for 10 days.

Encarsia formosa were released following procedures outlined in general methods chapter 2.74-2.75. Each line on the plant was exposed separately, and *E. formosa* were placed in individual leaf bags with the *T. vaporariorum* nymphs for each line and placed in a bugdorm 6M620 placed in a separate controlled environment cabinet to

ensure parasitisation of the treatment groups and colony does not occur. They were left for 48 hours before *E. formosa* were removed.

Nymphs from each treatment group were checked under the microscope for 10 days after treatment exposure to determine if they were alive, parasitised, dead or emerged as adults. All adults collected were placed in ethanol or frozen. All plant material containing nymphs was removed from the plant and frozen for 24 hours before disposal.

3.2.2 Simulation methods

3.2.2.1 Model description

The individual-based model of pest resistance simulates a homogeneous environment of horticultural protected edible crops of either aubergine or tomato plants in a glasshouse. The individual based model was coded by myself and is explained below. Insects, in this case whitefly, enter into the environment at the start of their lifecycle and can move around but remain inside this closed system according to their life cycle as it is a closed environment, spatial dispersal is not included in the model. Individual whitefly are born, mate, reproduce and die according to pre-set rules. If the whitefly are controlled, either a fungal biopesticide spray, a parasitoid wasp, or alternating between the fungal and wasp treatment is applied every generation. Whitefly with different genotype lines are not equally susceptible to the biocontrol agents. The model progresses on a whitefly generation time step and simulations are run over a hundred generations. By varying crop, and treatment regimens and repeating the simulations, there is potential to investigate which traits have most influence on the risk of resistance evolution.

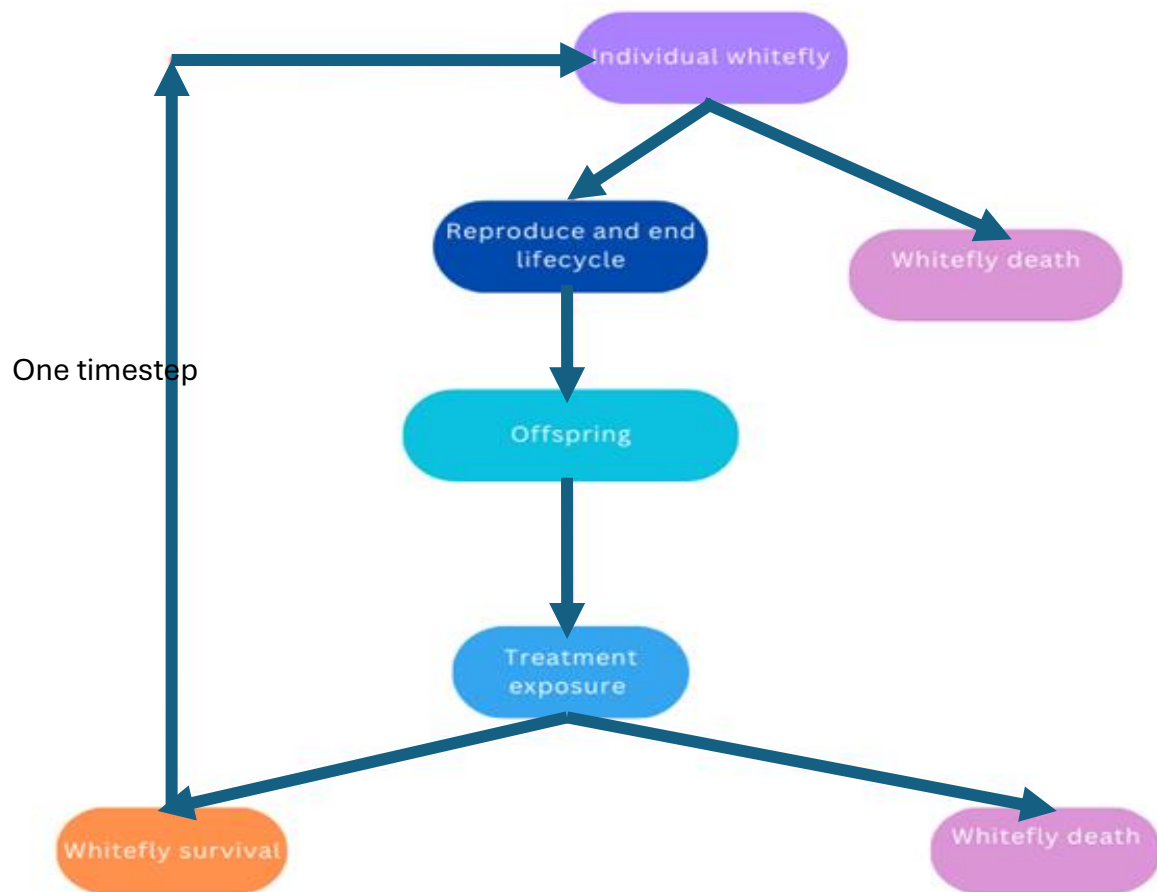


Figure 7: A schematic flow chart of an individual in the individual based model according to the model pre-set rules, for one timestep.

3.2.2.2 Model initialisation and parameters

At model initialisation, an adult population of 1000 individual whitefly were added to the simulated glasshouse environment. *Trialeurodes vaporariorum* (hereafter referred to as whitefly) have temperature dependant developmental times. These model parameters are based on 26°C, where one complete generation takes approximately 30 days from egg to death. Below I describe the rationale behind key steps in the model, quoting relevant code where appropriate. Full code can be found on Git hub at:

<https://github.com/MiaGraham24/Resistance-evolution-3/blob/main/3.simulation%20analysis.R>

The founding adult population is allocated characteristics:

```
# Function to initialise whitefly population in a simulated glasshouse
```

```
# Conditions based on Trialeurodes vaporariorum at 26°C
```

```
initialiseaub <- function(N = 1000) {
```

```
# Create matrix for individual data, with 15 allocated characteristics
```

```
inds <- array(data = 0, dim = c(N, 15))
```

```
# Assign IDs from 1 to N
```

```
inds[, 1] <- 1:N
```

Individuals are also assigned a resistance trait value, defined as the probability of mortality if exposed to a pathogen. For this model, these trait values are assigned by binomial distributions of any probability based on the average mortality from the empirical data set for the following treatments: Control, 0.2, Fungal biopesticide 0.65, *Encarsia formosa* parasitoid wasp 0.69 and the alternating treatment each generation mortality alternates between the fungal treatment and wasp treatment value.

```
inds[, 2] <- rbinom(n = N, size = 1, prob = 0.02) # Control mortality
```

```
inds[, 3] <- rbinom(n = N, size = 1, prob = 0.65) # Fungal mortality
```

```
inds[, 4] <- rbinom(n = N, size = 1, prob = 0.69) # Wasp mortality
```

```
# Initial mating status: none have mated yet
```

```
inds[, 5] <- 0
```

Sex - as the sex ratio is even, sex is randomly assigned by a binomial distribution with a probability of 0.5.

```
inds[, 6] <- rbinom(n = N, size = 1, prob = 0.5)
```

Offspring - the number of offspring is assigned with a poisson distribution on the average fecundity of the crop type. On the two most common protected edible crops, aubergine and tomato at 26°C, over a generation whitefly fecundity is 500 eggs and 200 eggs respectively.

```
inds[, 7] <- abs(round(rpois(n = N, lambda = X), digits = 0))
```

```
# All individuals start alive (0 = alive)
```

```
inds[, 8] <- 0
```

```
# Set mate and parental ID to 0
```

```
inds[, 9] <- 0 # Mate ID
```

```
inds[, 10] <- 0 # Mum ID
```

```
inds[, 11] <- 0 # Dad ID
```

Assign spatial x/y coordinates for whitefly movement around a glasshouse

```
inds[, 12] <- sample(1:5, size = N, replace = TRUE)
```

```
inds[, 13] <- sample(1:5, size = N, replace = TRUE)
```

Wing length: average wing length in mm assigned by a normal distribution, this value is not affected by inherited traits.

```
inds[, 14] <- abs(round(rnorm(n = N, mean = 23.25, sd = 2.89), digits = 0))
```

Treatment application (0 = no treatment yet applied)

```
inds[, 15] <- 0
```

For mate allocation, female individuals are assigned a male mate. Females only mate once, and their male partner ID is chosen randomly, females are then assigned to mate.

First, individuals are split into male and female subpopulations based on their assigned sex (**males <- subset(inds, inds[,6]==1)** and **females <- subset(inds, inds[,6]==0)**). Each female is checked for a mating status: if her *Mate ID* field (inds[,9]) is 0 (indicating she hasn't mated yet), she is assigned a mate using random sampling from the pool of available males:

```
females[i, 9] <- sample(males[,1], size=1, replace=TRUE)
```

This ensures all females are paired with at least one male. A mating count is also incremented (**females[i, 5] <- females[i, 5] + 1**) to track the number of times mating was attempted (though under current assumptions, females only mate once).

After mating occurs, all male individuals are assumed to have contributed and are updated to produce no offspring (**males[i, 7] <- 0**), reflecting that only females lay eggs in this model. The updated male and female populations are recombined using `rbind()` to return a unified, post-mating population matrix:

```
inds <- rbind(males, females)
```

Mated females in the population are assigned a number of offspring drawn from a Poisson distribution (`rpois(n = 1, lambda = 500)`), where the average fecundity (lambda) can vary depending on crop type. Each offspring inherits traits from both parents, calculated as the mean of the parents' values for each resistance trait (columns 2–4 in the matrix), plus normally distributed noise (**`rnorm(n = 1, mean = 0, sd = 0.01)`**) to simulate mutation. A new individual is also assigned a randomly determined sex (**`rbinom(n = 1, size = 1, prob = 0.5)`**), an unmated **status** (**`new_inds[, 5] <- 0`**), a unique ID, the spatial coordinates of its mother, and both parental IDs (**`new_inds[,10] <- mum_ID, new_inds[,11] <- dad_ID`**). The offspring's mortality value for each treatment is computed as the average of its parents' mortality

probabilities, consistent with an infinite alleles genetic model. If any of these treatment-specific mortality values equal 1, the individual is considered nonviable and not added to the population. If the number of new individuals exceeds 100

(if(dim(new_inds)[1] > 100)),

a random sample of 100 is selected (**sample(..., size = 100)**),

and only those are added to the existing population (**rbind(inds, new_inds)**).

Once reproduction is complete, all mated adults are marked as dead (**inds[,8] <- 1**) and are subsequently removed from the population.

For simulations, the response variable was whether population evolved resistance when exposed to one of four treatments on either aubergine or tomato crops, no biological control agent, fungal biopesticide applications every generation, *Encarsia Formosa* applications every generation or alternating between the two biocontrol agents every second generation.

Control, *E. formosa* and fungal treatments functions were modelled based on the probability of mortality set within the initialisation of the model.

The simulation ran for 140 generations with a burn in of 20 generations for 20 replicate generations per treatment. Treatment exposure occurred from generation 21, and at generation 41 the population numbers stabilised.

The simulation begins by initialising the population based on the selected crop—either aubergine or tomato—using the functions **initialiseaub(N = N_init)** or **initialisetom(N = N_init)**. The core simulation loop runs for a specified number of generations (**time_steps**), during which several life cycle processes occur sequentially at each time step. These include mate allocation (**inds <- mate(inds)**), offspring addition (**inds <- add_offspring(inds)**), and removal of old individuals (**inds <- old_die(inds)**). After these biological events, background mortality is applied uniformly across the population using **inds <- cont_mort(inds)**.

Depending on the treatment regime and after a designated burn-in period (`burn_in`), additional mortality due to biological control agents is imposed. For example, if the treatment is set to “encarsia” and the current generation exceeds the burn-in (`if(treatment == "encarsia" & ts > burn_in)`), parasitoid-induced mortality is applied via `inds <- use_parasitoids(inds)`. Similarly, the “fungal” treatment invokes fungal biopesticide mortality (`inds <- use_fung(inds)`), while the “alternating” treatment switches between the two agents every generation post burn-in using a modulus condition:

```
if ((ts %% 2) == 0) {

  inds <- use_parasitoids(inds)

} else {

  inds <- use_fung(inds)

}
```

The loop counter `ts` is incremented each cycle (`ts <- ts + 1`), and the population state at each time step is stored for later analysis (`inds_hist[[ts]] <- inds`). To incorporate stochastic variability, the entire simulation is repeated multiple times via the `simulations` function, which calls `run_sim` iteratively over the number of replicates.

Table 1 The IBM treatment protocol and the number of replicate simulations per biocontrol treatment group for each crop type for 140 generations.

Biocontrol agent	Crop	Number of replicates
No biocontrol application	Aubergine	20
Fungal biopesticide	Aubergine	20
<i>Encarsia Formosa</i>	Aubergine	20
Alternating treatments	Aubergine	20
No biocontrol application	Tomato	20
Fungal biopesticide	Tomato	20
<i>Encarsia formosa</i>	Tomato	20
Alternating treatments	Tomato	20

3.2.3. Data analysis

Empirical and simulation data were analysed in R version 4.1.3. Survival variation between treatments and lines was assessed using a generalised linear mixed model (GLMM) with a negative binomial distribution to handle overdispersion and included random effects of plant using the glmer function in the lme4 package (Bates *et al.*, 2015). Proportional survival was modelled using the cbind function (number dead, number alive) as the response variable. Cross resistance was analysed using the Pearson correlation coefficient. The simulation data were analysed using beta regression models comparing treatment group probability of survival at generations 41 and 140 and the effect of crop type (Cribari-Neto *et al.*, 2010). Proportional survival was calculated at 1-predicted mortality value that was provided from the simulation model as the response variable, by binomial probability distributions. Cross resistance was analysed using the Pearson correlation coefficient. Model diagnostics, including residual simulations with the DHARMA package, (Hartig ,2022). were employed to validate model assumptions and ensure the robustness of my results.

3.3 Results

3.3.1 Empirical data results

3.3.1.1 Whitefly average survival to two biological control treatments.

The effects of BotaniGard© *Beauveria bassiana* fungal biopesticide, (hereafter known as 'fungal treatment') and *Encarsia formosa* parasitoid wasp (hereafter known as 'wasp treatment') on whitefly survival 10 days after exposure was assessed. Whitefly survival to two biological control agents was assessed over 35 whitefly lines. In the control treatment, there was a total of 647 individual nymphs with an average of 18 per line. In the fungal treatment, there was a total of 913 nymphs with an average of 26 individuals per line. In the wasp treatment, there was a total of 750 nymphs with an average of 21 individuals per line. The control, fungal and wasp treatments contained

20, 15 and 12 plants respectively. Plants in the control, fungal and wasp treatments had an average of 54, 46 and 50 nymphs respectively.

There was a significant effect of treatment on survival ($X^2_2 = 249.58$, $P < 0.001$), whitefly survival was significantly higher in the control treatment (97.97%) than in the fungal (35.35% $z = 15.49$, $p < 0.001$) and wasp (30.66% $z = 16.62$, $p < 0.001$). Survival was also significantly lower in the wasp treatment compared to the fungal treatment ($z = 3.63$, $p < 0.001$), suggesting the 35 whitefly lines are more susceptible to the *E. formosa* treatment (Figure 8).

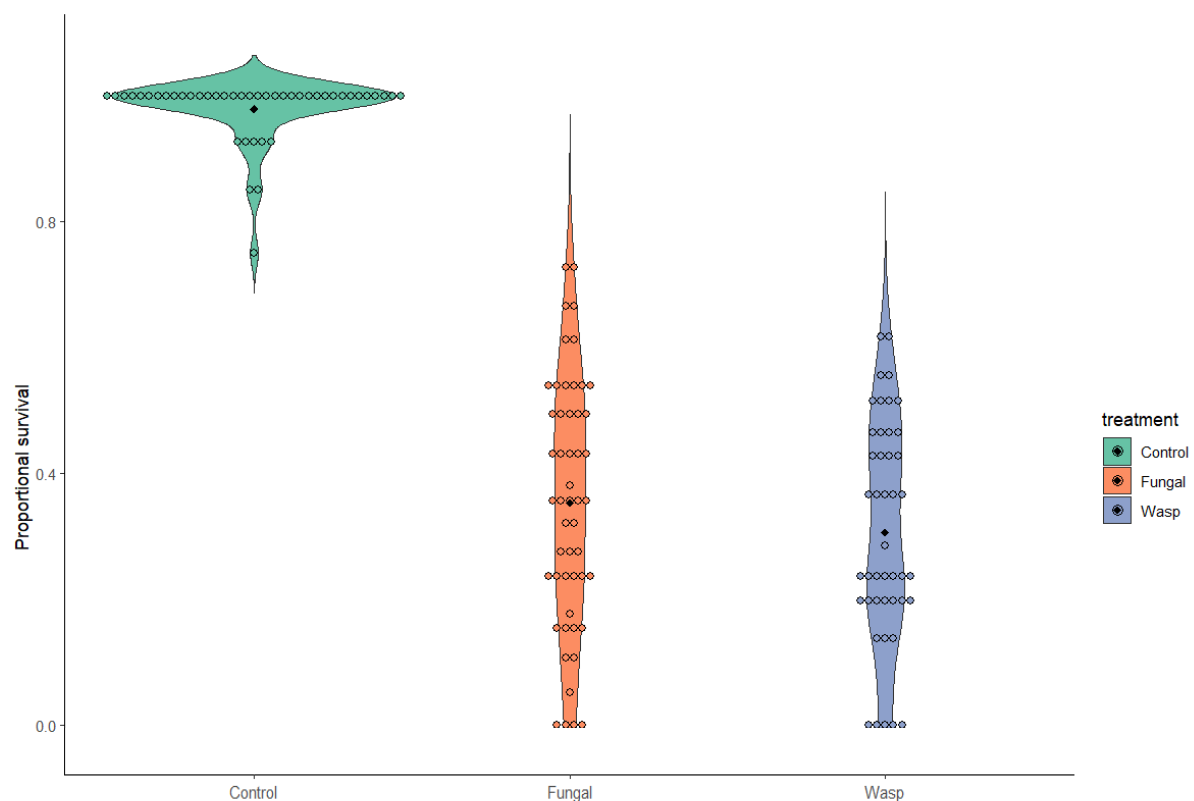


Figure 8: The proportional survival of 35 lines in a control, fungal and wasp treatment 10 days after exposure. Violin plots show the average survival in each treatment. the control treatment in green, the fungal biopesticide BotaniGard© *Beauveria bassiana* treatment in orange and the *E. formosa* parasitoid wasp treatment in purple. The mean proportional survival for all lines is plotted as a black points, individual survival is plotted as open circles.

3.3.1.2 whitefly survival between lines

There was a significant effect of line on whitefly survival ($X^2_{34} = 112.34$, $P < 0.001$). There was no significant interaction effect between line and treatment, indicating that there was significant variation in survival due to line. In the control treatment across lines. There was substantial variation in the proportion of nymphs that survived in each replicate, this was 75% -100% in the control, 0-72.7% in the fungal and 0-62.5% in the wasp treatment. (Figure 9).

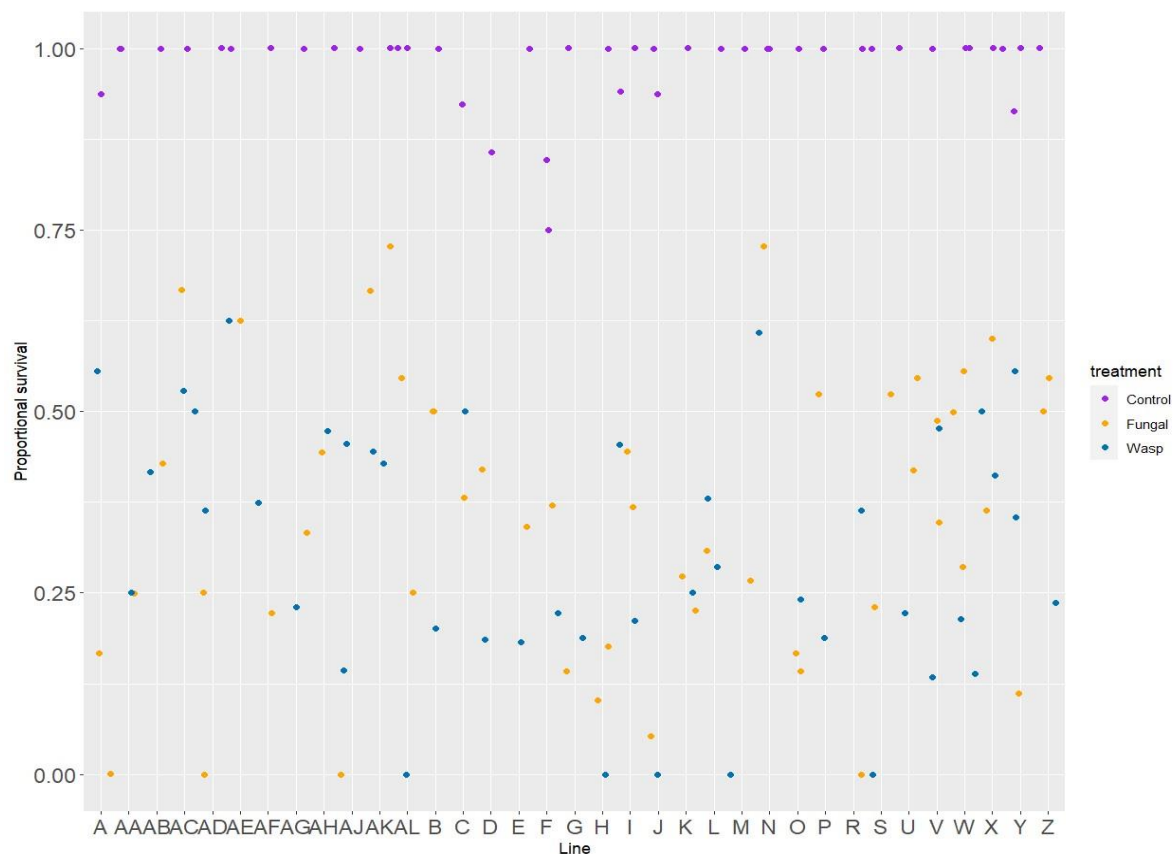


Figure 9 The proportional survival for 35 whitefly replicate lines, ordered by proportional survival. Represented by points 10 days after exposure to the control treatment in purple, the fungal biopesticide BotaniGard© *Beauveria bassiana* treatment in orange and the *E. formosa* parasitoid wasp treatment in blue.

3.3.1.3 The potential for trade-offs in whitefly resistance to biocontrol

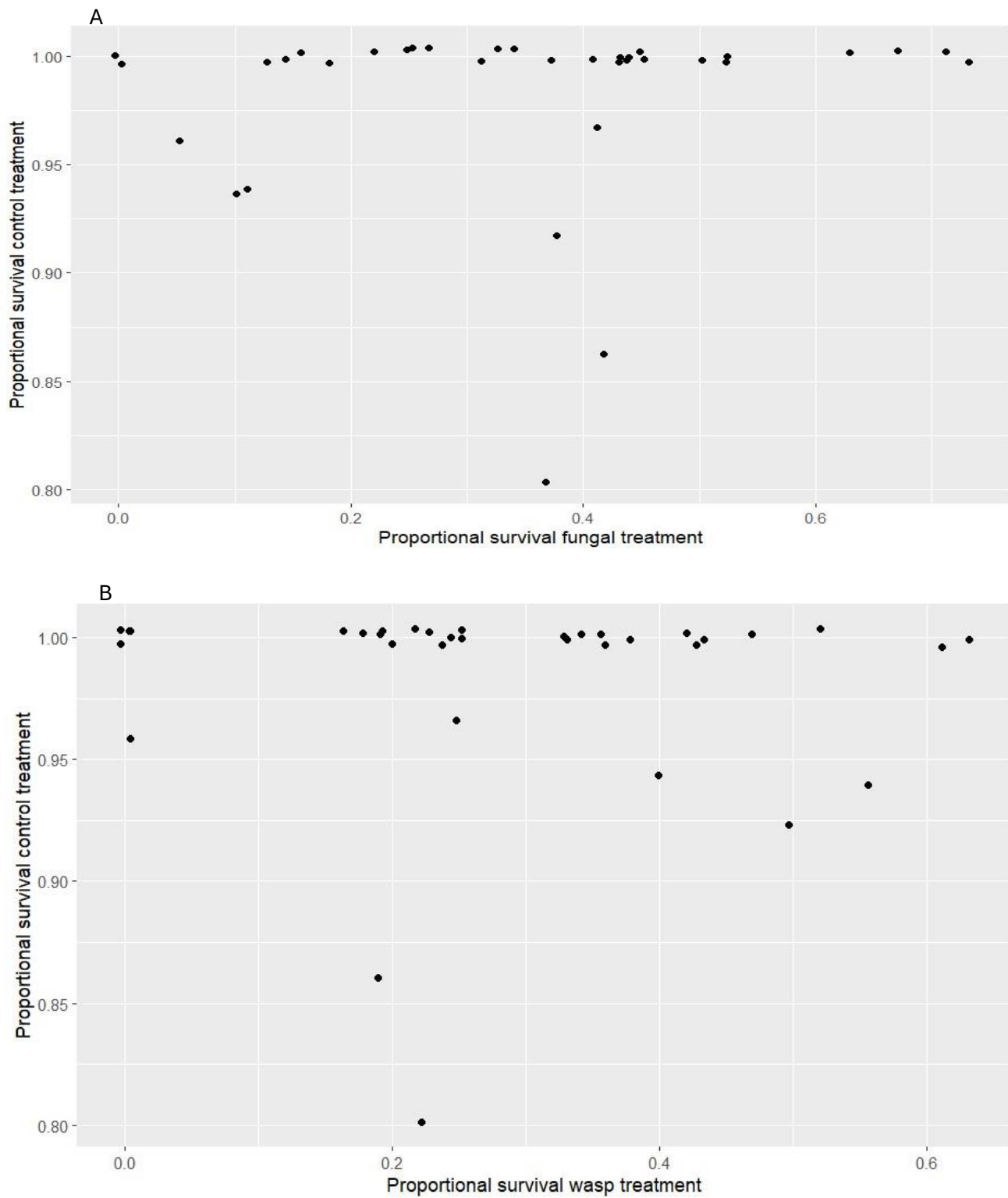


Figure 10: The proportional survival for each replicate line, represented by a point in the A; fungal treatment and B: the *E. formosa* treatment against the control treatment.

Lines did not have consistently high or low survival across control and treatment conditions, nor did lines with low survival in one condition have high survival in another (Pearson's correlation, fungal vs control: $r(34) = 0.074$, $p = 0.673$) and wasp vs control: $r(34) = -0.000078$, $p=1$; figure 10A & B).

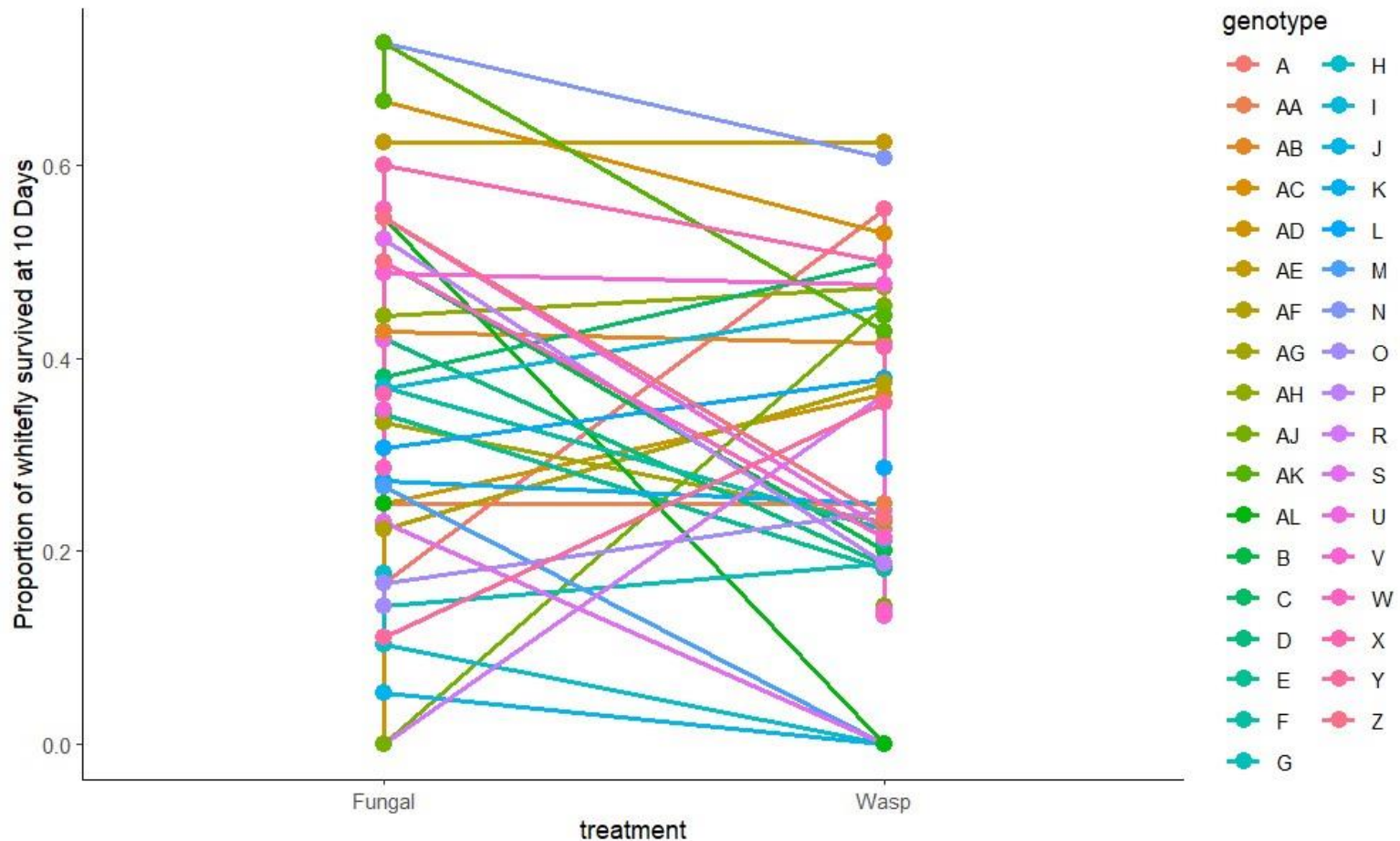


Figure 11: The proportional survival for each line in the fungal and the *E. formosa* (wasp) treatments. For the majority of lines, their survival in one treatment is different to their survival in the second treatment. Each individual point represents a replicate line, with a coloured line connecting the line proportional survival in each treatment.

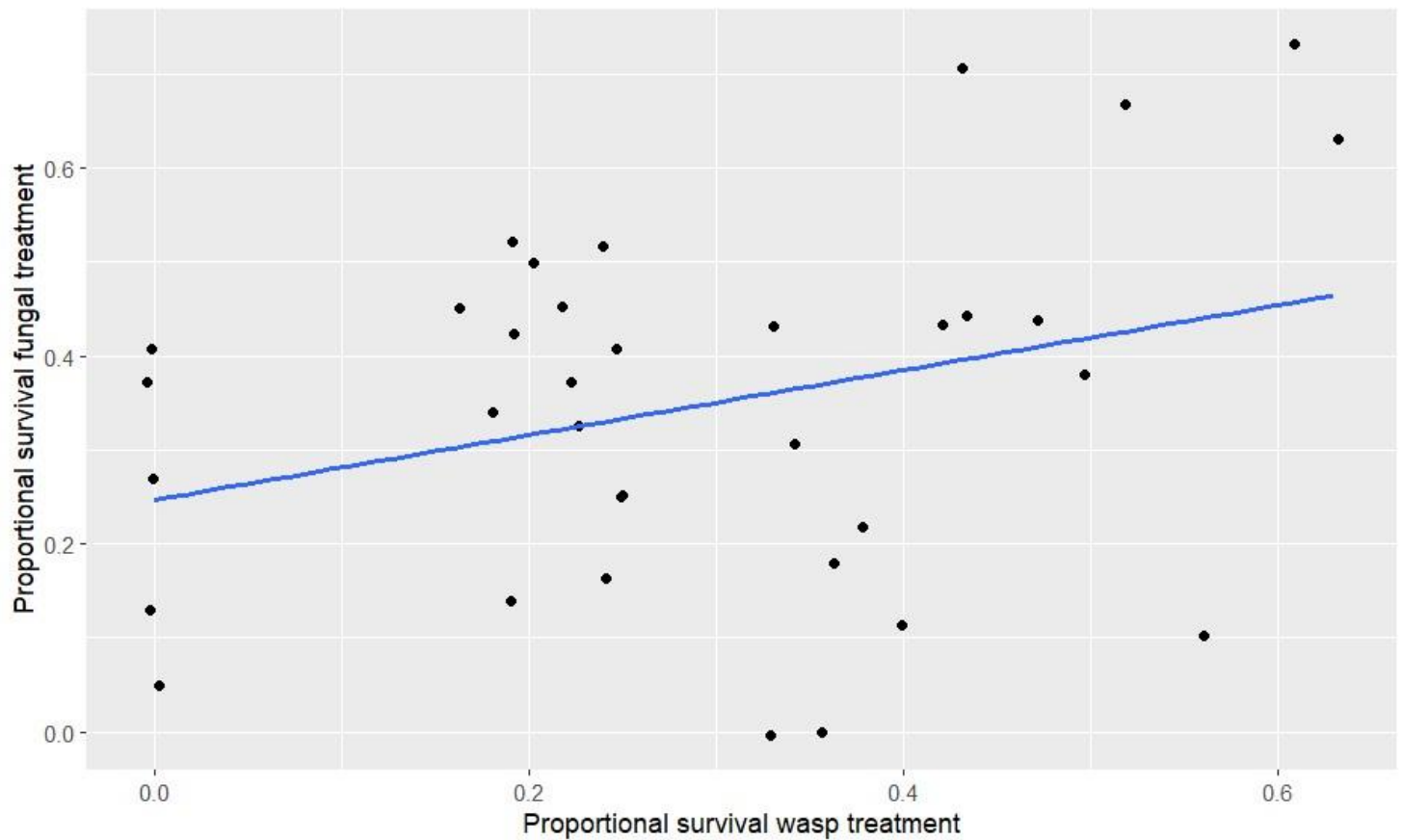


Figure 12 The proportional survival for each replicate line, represented by a point in the fungal treatment against the *E. formosa* treatment. A solid trend line represents the weak positive correlation in survival between the treatments.

I found a weak non-significant positive correlation in proportional survival between the fungal treatment and wasp treatment $r = (34) = 0.32$, $p = 0.069$. This correlation suggests there could be weak cross resistance, with lines that have higher survival in the fungal treatment also having higher survival in the wasp treatment, and low survival lines having low survival in both treatments (figures 11 and 12).

3.3.2 Simulation results

3.3.2.1 Whitefly probability of mortality to three biological control treatments.

The effects of the fungal treatment and wasp treatment on whitefly survival after 140 generations, were simulated alongside a control treatment and an alternating treatment, where each generation was exposed to the fungal treatment and the next generation the wasp treatment. Each simulation was replicated 20 times using mortality values derived from the empirical experiment above. Simulations of whitefly exposure occurred on two different crops: tomato and aubergine, giving 8 different treatment groups in total (see Table 1).

Whitefly probability of survival at generation 140

Across all 20 replicates, at generation 140, average whitefly survival varied depending on the crop (Figure 11). When survival probabilities were compared in the final generation (140) I found significant effects of treatment ($X^2_3 = 932173.5$, $P < 0.001$) and crop type ($X^2_1 = 1711.4$, $P < 0.001$) and the interaction between the two ($X^2_3 = 1662.7$, $P < 0.001$). Survival was significantly higher in the control treatment than in any other treatment. Survival in the control was significantly higher on tomato compared to survival on aubergine ($z = 53.86$, $p < 0.001$) figure 13. Pairwise tests between the treatments where wasps and/or fungal biocontrol were applied indicated that survival was not consistently higher or lower in any treatment but was affected by crop type.

On aubergine crops, survival was significantly higher in the wasp treatment compared to the fungal treatments ($z = 3.22$, $p < 0.05$). Survival significantly reduced in the alternating treatment compared to the fungal treatment ($z = -14.45$, $p < 0.001$) and the wasp treatment ($z = -17.56$, $p < 0.001$) figure 14.

On tomato crops this pattern changed; survival was significantly higher in the fungal treatment compared to the wasp treatment and alternating treatments ($z = -16.64$, $p = 0.227$ and $z = -14.45$, $P < 0.001$ respectively). Survival in the alternating treatment was significantly higher than the wasp treatment ($z = 12.56$, $p < 0.001$), figure 14.

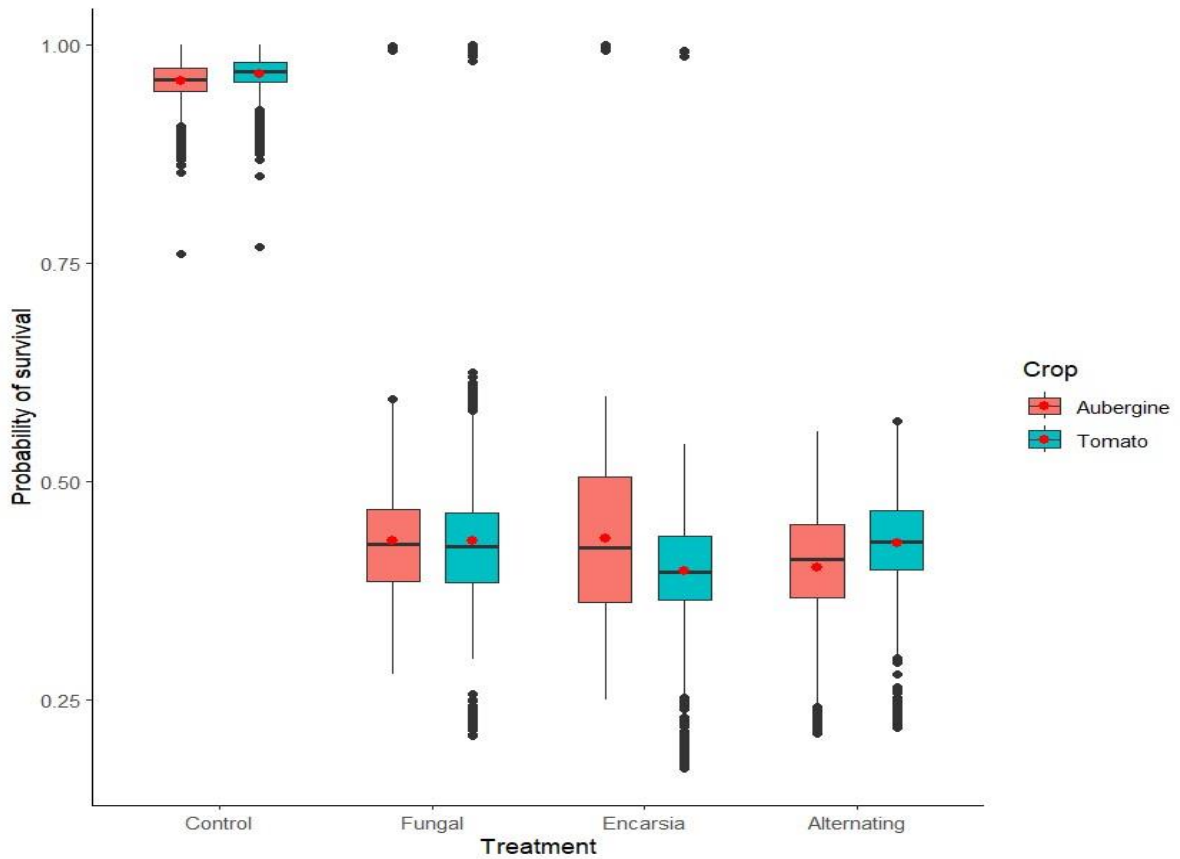


Figure 13: The probability of whitefly survival in each treatment group at generation 140. The red box indicates whitefly reared on aubergine and the blue box indicates whitefly reared on tomato plants. The red points are the mean proportional survival for the 20 lines in each treatment group. The black line indicates the median value, and bars are the 95% confidence intervals.

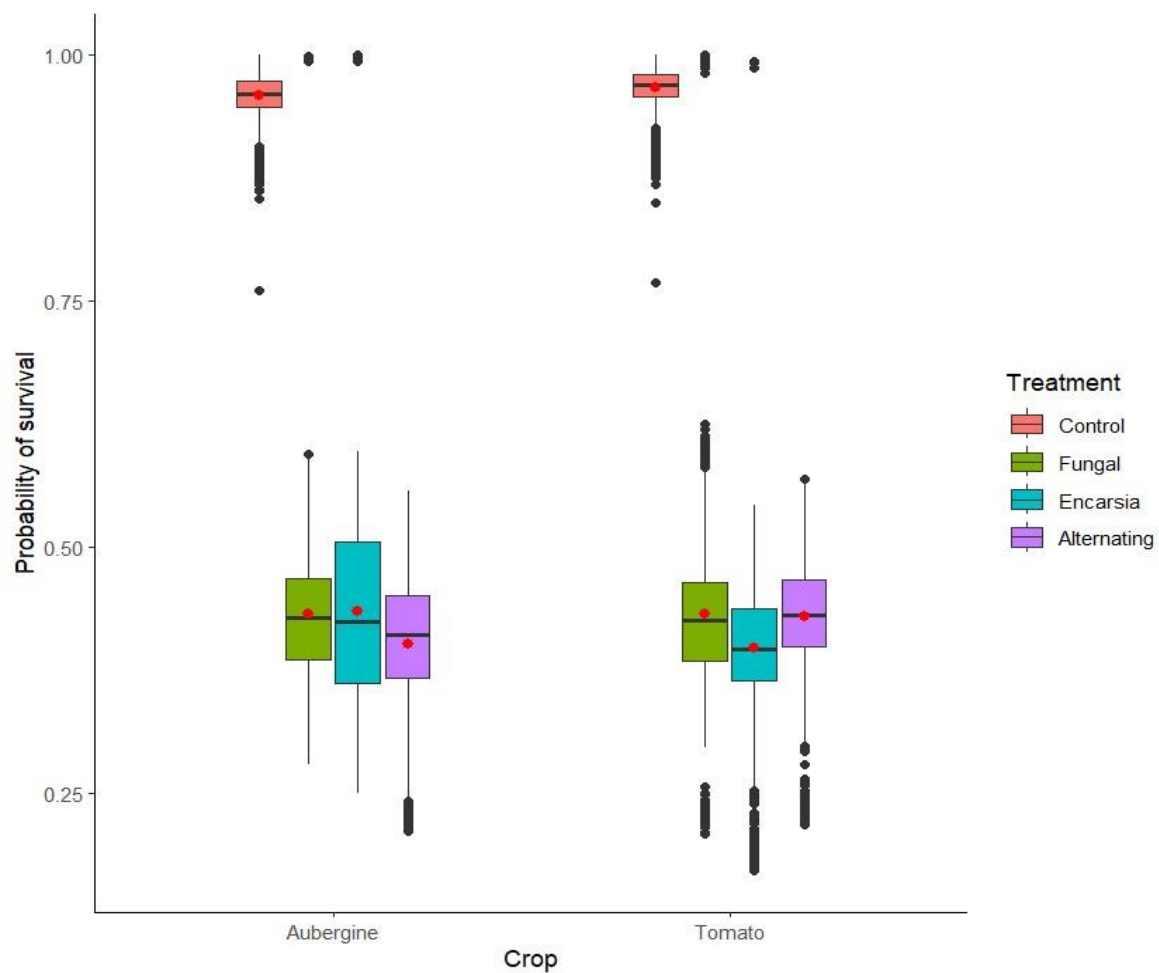


Figure 14: Probability of whitefly survival in each treatment group per crop at generation 140. The red box indicates whitefly survival in the control treatment, the green box indicates whitefly survival in the fungal treatment, the blue box indicates whitefly survival in the wasp treatment and the purple box indicates the whitefly survival in the alternating treatment. The black lines indicate the median value, and the red dots indicate the mean value, the bars represent the 95% confidence intervals.

Whitefly probability of survival between generation 41-140

Average survival across the 20 replicates at generation 41 and 140 were compared across the eight treatments. Treatment exposure occurred from generation 21 onwards. Comparing data at generation 41 removed the 20 burn-in generations where they were not exposed to any treatments and also removed the first 20 generations that were exposed to biocontrol treatments to allow the population numbers to stabilise.

Table 2: The probability of proportional survival in the eight treatment groups where the pest management agent or the crop varies, established in the IBM at generation 41 and 140 and the percentage change between the generations.

Treatment	Crop	% Survival generation 41	% Survival generation 140	Change in % survival between generations 41 and 140
Control	Tomato	97.40	96.77	-0.63
Fungal	Tomato	40.70	42.30	1.6
Wasp	Tomato	33.30	39.80	6.5
Alternating	Tomato	36.40	42.94	6.54
Control	Aubergine	95.90	95.87	-0.03
Fungal	Aubergine	40.50	43.30	2.8
Wasp	Aubergine	40.20	43.60	3.4
Alternating	Aubergine	37.60	40.23	2.63

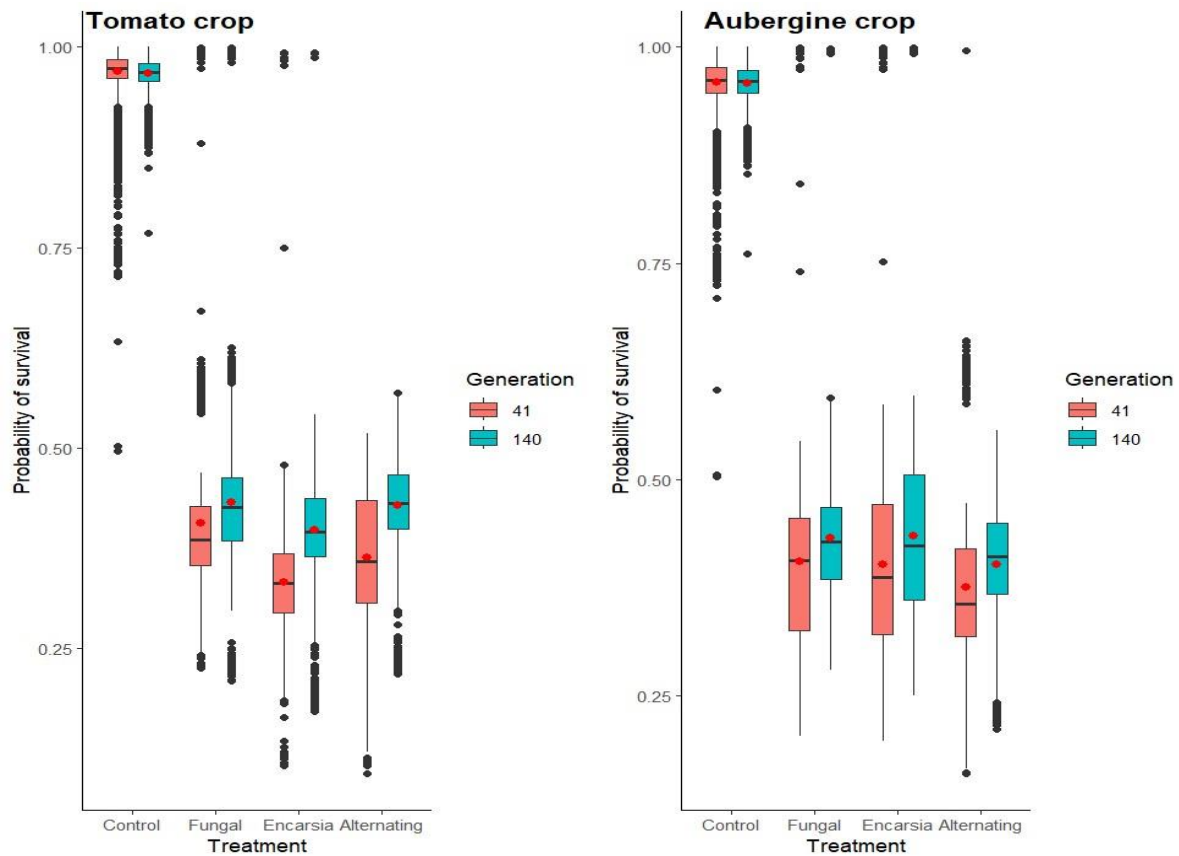


Figure 15: Probability of survival between whitefly at generation 41 compared to generation 140 in all treatment groups reared on 1) tomato crops or 2) Aubergine crops. The red boxes are generation 41 and blue boxes generation 140. The black lines indicate the median value, and the red dots indicate the mean value, the bars represent the 95% confidence intervals.

Average survival was compared and found to increase from generation 41 to 140 for all biocontrol treatment regimes (treatment effect $X^2_3 = 1425365.4$, $P < 0.001$), and the increase was more pronounced on tomato crops compared to aubergine (main effect of crop $X^2_1 = 1689.7$, $P < 0.001$). There was a significant interaction effect between treatment and crop ($X^2_3 = 2715.8$, $P < 0.001$). The smallest increases in survival across generations were seen in the fungal treatments in both crop types (table 2), suggesting a smaller selective response to fungal only treatment compared to treatments including parasitoid wasps (alone or in combination with fungal treatment).

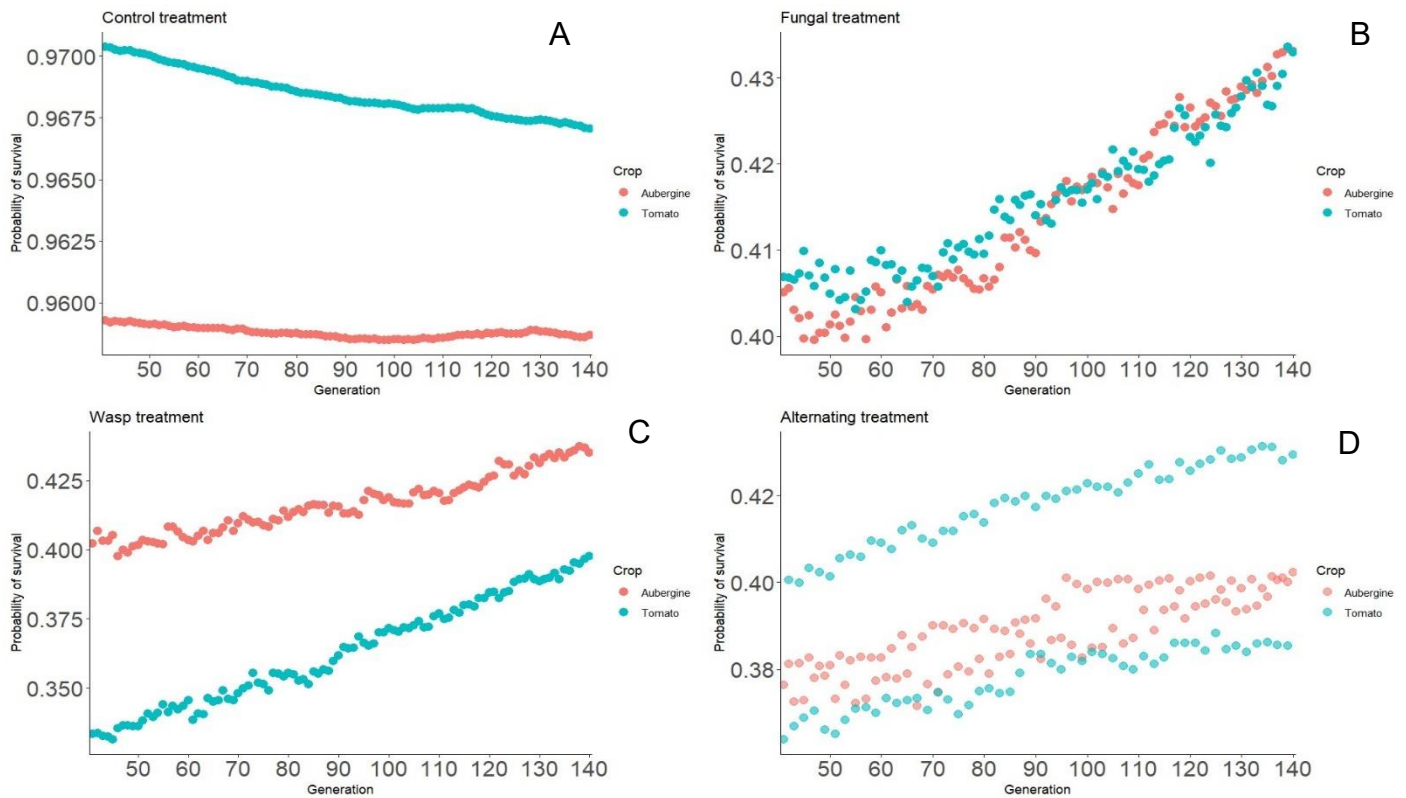


Figure 16: Probability of survival generations 41-140 in all four treatments groups; Control, fungal, *E. formosa* and alternating. Red points indicate survival on Aubergine crops and the blue dots survival on the tomato crop.

Figure 16 shows the trends in survival across all generations for every treatment by crop interaction. Changes in survival were marginal in the control and most pronounced in the wasp treatment figures A and C. The strongest evolutionary response can be seen in the wasp treatment in tomato plants figure 16 C, but survival probability in tomatoes in response to parasitoid wasps never reached the same level as survival in response to parasitoids in aubergine plants (even at the start of treatment). In contrast, changes in survival in response fungal treatment were more similar across the two crop types, particularly after generation 100 figure 16 B. The pattern in the alternating treatments is more complex figure 16 D; survival increased on both crop types, but the variation in survival remained high suggesting more susceptible individuals remained in the population. This was particularly noticeable in tomato plants, where the distribution appeared to be bimodal, indicating that some individuals with lower survival always remained in the population (figure 16d).

3.3.2.1 Whitefly abundance at generation 140 after exposure to three biological control treatments.

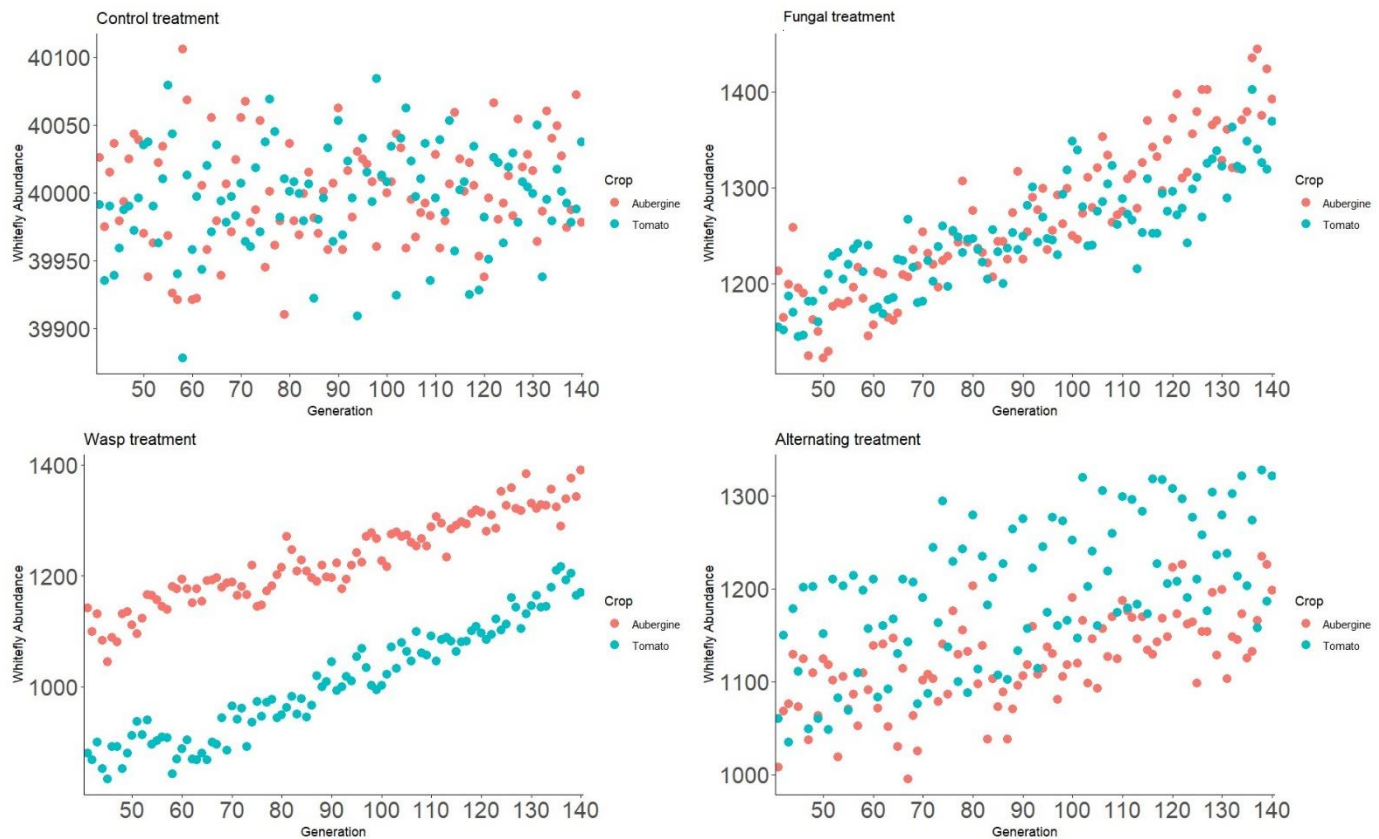


Figure 17: Whitefly abundance in generations 40-140 in all four treatments groups; Control, fungal, *E. formosa* and alternating. Each point is the total abundance for the 20 replicated simulations per generation. Red points indicate survival on Aubergine crops and the blue dots survival on the tomato crop.

Figure 17 shows the survival trends represented by figure 16 impacted whitefly abundance over generations in the simulations. Whitefly abundance remained high and variable across generations in the control treatments and there was complete overlap in abundance on the two crop types. Abundance was highly variable in the alternating treatment but remained lower than in the other single biocontrol treatments at the end of the 140 generations of selection, this was particularly the case for aubergine crops, where whitefly abundance was always below ~1200. In the fungal treatment whitefly abundance reached the greatest levels at the end of the 140 generations and tended to be higher in aubergine plants than tomato but there was overlap between the two crop types. In the wasp treatment the abundance of

whitefly was always higher in aubergine crops compared to tomato, and the variation in abundance within crop types was noticeably lower than in the other treatments.

Table 3: The simulated mean abundance with calculated 95% confidence intervals of whitefly over time from the IBM from generation 41 and 140 in four treatment groups reared on either tomato or aubergine crops. Generation one is set at 99 as this was the founding population abundance.

Treatment	Crop	Mean abundance generation 41±95% CI	Mean abundance generation 140±95% CI	Change in mean abundance
Control	Tomato	1999.60 (1999.38, 2002.72)	2001.85 (1977.18, 2006.52)	2.25
Fungal	Tomato	57.70 (47.61, 67.8)	68.45 (57.47, 79.43)	10.75
<i>E. formosa</i>	Tomato	44.05 (36.89, 51.21)	58.5 (49.2, 67.8)	14.45
Alternating	Tomato	53 (45.89, 60.11)	66.05 (58.90, 73.20)	13.05
Control	Aubergine	2001.30 (1996.47, 2006.13)	1998.90 (1994.23, 2003.58)	-2.4
Fungal	Aubergine	60.65 (50.40, 70.90)	69.60 (61.05, 78.20)	8.95
<i>E. formosa</i>	Aubergine	57.10 (46.61, 67.59)	69.55 (59.16, 79.94)	12.45
Alternating	Aubergine	50.4 (41.42, 59.38)	59.90 (51.65, 68.15)	9.5

3.4 Discussion

Understanding pest resistance is crucial for the effective pest management strategies and food security. Attention has historically focused on the longevity and potential resistance to chemical synthetic pesticides with little focus on the potential resistance to biological control agents. The aim of my thesis was to begin to address this imbalance; using a lab-based experiment and simulation modelling, I demonstrated the potential for glasshouse whitefly to evolve resistance to two commonly used biological control agents a fungal biopesticide, BotaniGard and parasitoid wasp, *Encarsia formosa*.

Significant variation in survival rate between whitefly lines was found in both the fungal biopesticide (0-72.2%) survival and wasp treatments (0%-62.5%). When this variation in survival from the empirical data set was extrapolated over 140 generations in the simulation data set, survival rates increased significantly in all biocontrol treatment groups and crops over 100 generations. These results suggest whitefly do have the potential to evolve resistance over time in favourable conditions. In the lab experiment, the variation in susceptibility was unlikely to be due to uncontrolled environmental factors, as the amount of variation was very low in the control group compared to the fungal and wasp treatment groups.

The whitefly populations showed significant variation in survival rates, suggesting that strong selection pressures could drive the evolution of resistance. Within these populations, some individuals possessed traits that confer a higher likelihood of surviving parasitism or fungal pathogens, most likely to be physiological resistance, enhanced immune responses or behavioural responses, from physiological based mechanisms or the presence of endosymbionts (Georghiou, 1972). When the biocontrol treatments were introduced, fungal sporulation or wasp larvae development occurred in the susceptible individuals, while those with resistance traits survived with the potential to reproduce. As seen in other insects, it is likely when the whitefly are attacked by pathogens, the cellular and humoral responses are activated, lytic activity occurs including the production of toxic molecules such as antimicrobial peptides, lysozyme, and lytic enzymes, which leads to the degradation of the invading pathogen (Contreras-Garduño *et al.*, 2023). This selective survival favours individuals carrying resistance traits, which could lead to a rapid increase in the frequency of these traits

within the population, increasing the survival rate in the population over time, reducing the effectiveness of the biocontrol agents. This potential for resistance has been observed in studies on pea aphid variation in survival to parasitoids and fungal pathogens. Significant clonal variation was found to two parasitoids *Aphidius ervi* and *A. eadyi* and the fungal pathogen *Erynia neoaphidis* (Ferrari *et al.*, 2001).

The increase in survival rate over time was observed in the simulated data set that extrapolated the survival rate of whitefly populations using the average of and range in survival from the empirical data. This suggests that in the successive generations, the frequency of resistance traits increased within the simulated population, leading to the evolution of a more resistant pest population. However, as the empirical data were taken over a single generation, they did not account for the presence of maternal effects that could be responsible for the increased survival, thus this was not taken into account in the simulation model. The IBM simulation model was also limited by preset assumptions affecting whitefly survival rate. Assumptions were set that all adults mated with one other adult and produced offspring that were exposed to a control measure or died before reproduction. For this IBM simulation Haplodiploidy was not taken into account, it was assumed all females mated to produce offspring, and no haploid offspring were produced. In reality whitefly are a haplodiploid species; females can produce male offspring without mating and can also mate with multiple partners. As such, the mortality values may not be realistic, as the 'resistance' passed on to offspring was calculated as the average of each parents 'resistance'. In a haplodiploid system, females could produce male offspring from unfertilized eggs, allowing for a form of asexual reproduction. This would change the sex determination process, where males are haploid and females are diploid, affecting genetic relatedness within the population. The IBM would need to incorporate new parameters, such as the frequency of asexual reproduction and the decision-making process for mating versus asexual reproduction. The inherited mortality value taking an average mortality from both parents would also need to alter, where male offspring take 100% of the mothers mortality value. These changes would increase the complexity of the model and could lead to different evolutionary outcomes and population dynamics, particularly in sex ratios, mortality values and the frequency of resistant traits in the populations. Therefore, the survival rates may differ in reality to the current IBM.

Parasitoid resistance with increasing survival over time has been observed in studies investigating genomic changes during rapid evolutionary adaptation in the model system *Drosophila melanogaster* to strong selection pressures for increased resistance to the parasitoid wasp *Leptopilina boulardi*. The survival rate of *D. melanogaster* after an attack increased from approximately 0.5% to between 40% and 50% over five generations, indicating substantial additive genetic variation in resistance within the population (Fellowes *et al.*, 1998). A similar mechanism of resistance could explain the variation in whitefly survival and the increase in survival rate in my laboratory study and IBM model respectively, suggesting whitefly do have to potential to evolve resistance to both parasitoid wasp *E. formosa* and the fungal pathogen *B. bassiana*. If substantial additive genetic variation in resistance is present within the population, the ability of the whitefly population to withstand attack from parasitoid wasp *E. formosa* and the fungal pathogen *B. bassiana* could be due to the additive effects of individual genes. This means that multiple alleles at different loci contribute independently and cumulatively to the resistance trait. The presence of substantial additive genetic variation is crucial because it allows for effective and predictable responses to selection pressures, thereby enhancing the overall resistance of the population over time when facing strong selection pressures. However, further empirical studies to assess the mechanisms of resistance, due to both biological control agents having different mode of actions and the resistance rate over time would provide a greater insight into the effectiveness and long term effectiveness of these biocontrol agents as pest management methods.

When treatment groups were assessed for their effectiveness, whitefly in the *E. formosa* treatment had the lowest survival rate in both the lab experiment and in the simulation when reared on tomato crops. However, in the simulation, whitefly in the alternating treatment had the lowest survival rate, with whitefly in the fungal and *E. formosa* treatments having similar survival rates. This suggests crop, or crop variance could have an effect on the effectiveness of biological control agents. Threefold interactions between the plant, pest and biocontrol agent plays a large part in the effectiveness of pest management systems (Ortego *et al.*, 2021). Whitefly fitness, the presence of endosymbionts and the effects of the crop on the biocontrol agent could all play a role. However, in the case of this simulation, where whitefly fitness was the

only parameter that could have had an effect, biocontrol effectiveness between crops was affected by whitefly fitness variations between crops. Whitefly have a significant preference for laying and feeding on aubergine crops compared to tomato crops and have higher fecundity and faster more successful development through to adulthood, thus increasing whiteflies fitness (Li *et al.*, 2023).

Glasshouse Whiteflies fecundity rates, thus population densities, are known to alter based on environmental conditions such as temperature and light levels but also can depend on crop type. On tomato and cucumber crops at 26°C, fecundity is around 175-200 eggs; on aubergine crops, whitefly can lay over 500 eggs (Capinera, 2008). Standard advised release rates for *E. formosa* are 10 individuals per m² of crop (Berndt & Meyhöfer, 2008) and for BotaniGard (contains 220 g/kg of the entomopathogenic fungus *Beauveria bassiana* strain GHA) standard application rates are 0.94kg/Ha for protected edible crops such as tomato and aubergine spraying leaves until wet, just before runoff (Parker *et al.*, 2015).

As the application rates for biocontrol agents are standardized per area of crop and don't necessarily account for pest population density, this could influence the effectiveness of the biocontrol agent depending on its mode of. When standard doses of *E. formosa* are released to target whitefly nymphs on aubergine crops, as each individual can only target a set number of nymphs via feeding and oviposition, their effectiveness could be lower compared to their effectiveness on the same size area of tomato crops due to higher nymph densities. The effectiveness could also be reduced in comparison to the fungal biopesticides. As these are sprayed over the entire leaf surface, it would target a similar proportion of the population regardless of crop type.

In my laboratory study I mixed BotaniGard to commercial standards, and applied to the underside of leaves until wet, due to the controlled spray regime, the entire surface of the leaf surface, thus all nymphs were sprayed. For *E. formosa* applications, from consultation with the biocontrol company, *E. formosa* were released on a high ratio of 1:5 wasp to whitefly, thus taking nymphal density into account. As these mortality values were then used for the simulation model, mortality was based on nymphal density, rather than cropping density. Thus, the rate of resistance evolution in the simulation model may be different in the real world due to the potential of higher numbers of whitefly having an interaction with the control agent.

The variation in mortality based on nymphal density could have detrimental effects on pest management strategies for growers. Growers may face inconsistent or less effective pest control results when using biocontrol agents on different crops if applying at the standard rates across all crops. This inconsistency could lead to less effective pest control, resulting in crop damage and yield loss and potential exacerbating resistance evolution.

My empirical data did not show any evidence of a trade-off in survival rates between the two agents; lines with high survival against one biocontrol agent did not tend to have low survival against the other. Conversely a significant weak positive resistance correlation was observed, indicating that whitefly that are less susceptible to the fungal pathogen *B. bassiana* are also less susceptible to *E. formosa* parasitoid wasps. This suggests that the resistance traits in whitefly may be due to general defence mechanisms. This is due to the pathogens *B. bassiana* and *E. formosa* having different modes of action; where the fungal pathogen infects the insect host via cuticle penetration and sporulation in the whitefly nymphs haemocoel, the parasitoid wasp attacks the insect host via endoparasitism, ovipositing a wasp egg inside the whitefly nymphs haemocoel. These general defence mechanisms may include an increase in the density of circulating haemocytes, increased cuticle thickness, and increased levels of detoxification enzymes and antimicrobial peptides that aid encapsulation and detoxifying fungal pathogens (Stoepler *et al.*, 2013). This defence correlation has been observed in the pea aphid when exposed to two parasitoid wasp species *Aphidius ervi* and *A. eadyi* and the fungal pathogen *Erynia neoaphidis*. A significant positive correlation was observed between the two parasitoid species and a non-significant positive correlation between the parasitoid and fungal pathogen was observed (Ferrari *et al.*, 2001). However, correlations are a basic tool to study defence trade-offs as they often based on noisy or non-linear relationships that are hard to detect a pattern (Ferrari *et al.*, 2001). Nevertheless, the significant weak correlation suggesting cross resistance could pose a worrying issue for the effectiveness of combining or alternating the use of these biological control agents. As the resistant trait is likely to be a general defence mechanism, resistant individuals would likely survive in the population when exposed to both the fungal pathogen and parasitoid either

simultaneously or sequentially. Although alternating the treatments could slow the rate of evolution of resistance, resistant traits would still be present in populations.

As the lab-based experiment assessed survival rate variation over one generation, expanding this to a multi-generational experiment would allow for empirical data to be collected to investigate the evolution of resistance and changes to survival rate to be assessed over time. Repeating this experiment on both tomato and aubergine would also allow for empirical data to be collected.

The individual based model was designed to forecast the evolution of resistance in whitefly populations to biocontrol agents over time alongside the effects of alternating biocontrol agents and the rate of resistance dependent on crop type. Assumptions were set based on simplified traits from the empirical data set. Although I can conclude that survival rate does increase over time, the model assumes this is due to resistant traits in the population. However, as the model is simplified and based on few parameters from the empirical data it does not include detailed complex whitefly life history parameters or factors that could affect the success of the two biological control agents studied. Therefore, the results from the IBM simulation may represent over or underestimations of reality. The model was firstly designed to forecast the increase in survival rate over 100 whitefly generations when exposed to the same biological control agents as the empirical study, the parasitoid wasp *E. formosa* and the fungal biopesticide Botanigard. The survival rate assumptions of the model were based on the average mortality values for each biocontrol agent taken the empirical data set for the first generation in the model, with the further generations survival rate extrapolations of this. The model assumed all adults either mated with one other adult and produced offspring, set by a binomial distribution with the average value set dependent on the crop type based on previous literature, or died. Other factors that could affect whitefly fitness and fecundity such as haplodiploidy, where female adults do not mate but produce male offspring, thus skewing the sex ratio and future matings, alongside crop age and health and further environmental conditions that were not taken into account in this model. Developing the model further to incorporate haplodiploidy, where some females do not mate but produce male offspring that have the same mortality value as the mother, that could be exposed to biocontrol agents and go on to mate and produce offspring could enhance the model results and the likelihood of the forecasted results indicating an accurate forecast of resistance

evolution. The model also does not take into account the potential of defensive maternal effects that are passed on to offspring. Adding these assumptions into the model could enhance the results and improve the accuracy of the predictions the model provides on the survival rates over time.

The model assumptions do not take into account the effects of crop, whitefly movement and the environment on biocontrol agent performance. It is known from literature that the parasitoid was *E. formosa* use searching behaviour to find whitefly nymphs to parasitise. The success of parasitisation was based on the average mortality values from the empirical data set. In the empirical data, whitefly nymphs were exposed to *E. formosa* on a single aubergine plant in a single plant pot cage, where the ratio of *E. formosa* to nymphs was high suggesting that there was a greater chance of successful parasitism as searching efforts and time for a whitefly host would be reduced. Fungal biopesticide Botanigard was sprayed on the underside of all leaves in the empirical dataset targeting all whitefly nymphs.

As the IBM was designed to simulate a large-scale glasshouse setting, the model does not take whitefly movement into account, thus the spread of whitefly nymphs across different plants. Whitefly movement and spread of nymphs could potentially reduce the level of successful parasitism as *E. formosa* would have to exhibit searching behaviours spending more time searching for a host, thus parasitism could be reduced, in turn reducing mortality values. The spread of nymphs could also reduce the success of the fungal biopesticide as the likelihood all leaves will receive a lethal dose of fungal pathogens is reduced due to the increase in foliage. The reduced average mortality could therefore increase survival rate as parasitism would be reduced, however this could be due to lack of parasitism or the potential of whitefly behavioural resistance trait over a physiological resistance trait spreading through a population increasing survival. Introducing whitefly movement, nymphal locations and the searching behaviour of *E. formosa* and success of biocontrol exposure into the model as another survival factor would enhance the model development and improve the accuracy of the forecasted predictions of the increase in survival rates due to resistance alongside lack of exposure.

The IBM was also developed to predict the effects on survival rate on different crop types, as whitefly fecundity alters dependent on crop, thus the population and survival

rates would alter. The model takes fecundity based on crop type into account from average fecundity values based on previous literature. However, it does not include assumptions of how the biocontrol agent is affected by crop type. Each crop in the case of this study tomato and aubergine could have differing Tri-trophic interactions with the whitefly and pathogens having an effect on mortality rates. It is known that *E. formosa* searching behaviours can be affected by crop type due to trichomes (Shishehbor & Brennan, 1995). Thus, mortality values could be affected. The simulation used fecundity values based on crop type, but an overall average mortality value as the empirical data set was only conducted on aubergine plants. To enhance the model development, using mortality values from empirical datasets that were used on both crops would provide more accurate values for the model to forecast the increase in survival rates over time.

3.5 Conclusion

Understanding pest resistance to pest management regimes is crucial for global food security and the effectiveness of pest management strategies. In this study, I investigated the potential evolution of resistance to biological control agents in glasshouse whitefly (*Trialeurodes vaporariorum*), a significant pest affecting global crops, particularly protected edible and soft fruits. In a lab-based experiment, assessed variation in whitefly survival when exposed to *Encarsia formosa* parasitoid wasps and a commercial fungal biopesticide containing *Beauveria bassiana*. Additionally, a simulation model was developed to compare whitefly survival rates over time under three different treatments: fungal biopesticide, *E. formosa*, and an alternating treatment between the two biocontrol agents across generations on both tomato and aubergine crops.

Through lab-based experiments and a simulation model, I observed significant variation in survival rates among whitefly lines, with higher survival observed in the fungal treatment. Survival rates increased over time under all treatment conditions, particularly when treatments were alternated across generations. Notably, survival was higher in aubergine crops compared to tomato crops, suggesting that crop type can influence the effectiveness of these biocontrol agents. These findings suggest that

whitefly have the potential to develop resistance to these biocontrol methods, with potential variations in resistance linked to the type of crop. The study highlights the importance of considering resistance management and mitigation strategies in pest management regimes and the need for ongoing research to explore the mechanisms behind the evolution of resistance in detrimental pest study systems and the implications of resistance over time. However, the simplification of the insect pest genetics in the individual based model could affect the heritability, the proportion of variation in resistant traits in the population due to genetic differences. Thus, the simplification of the model could affect the accuracy of the assumptions drawn on predicting the potential for resistance evolution in whitefly populations over 100 generations. Overall, the potential for resistance to biological control agents should be considered as critically as resistance to other pest management options, empirical research and forecasting the rate of evolution is necessary to aid in mitigating and slowing the rate of evolution to biological control agents for crop protection and resistance management.

Chapter four: The potential for resistance evolution to biological control agents over six generations.

Abstract

Pesticide resistance evolution is a continual agricultural concern, causing reduction in crop protection efficacy and economic losses. Overreliance on chemical insecticides has led to pest resistance evolution, amplifying shifts towards use of biocontrol agents. Biocontrol can provide major ecological benefits compared to chemical control due to minimal impacts on non-target organisms. However, we know little about whether insect pests will evolve resistance against biocontrol agents. In UK protected edible agriculture, fungal biopesticides and parasitoid wasps are commonly used to control glasshouse whitefly (*Trialeurodes vaporariorum*). Although commonly used, there are reports of reduced efficiency over a growing season, suggesting resistance evolution could be at play. In a laboratory experiment I conducted a six-generation selection experiment to investigate the evolutionary response of whitefly populations to challenge with the parasitoid wasp *Encarsia formosa*. I found a considerable increase in survival when exposed to *E. formosa* in whitefly that had been subjected to *E. formosa* treatment for six generations, from 35% to 56%, with no significant change in survival in the control (no *E. formosa*) treatment. It was found that some maternal effects played a role in the increase in survival in the *E. formosa* treatment group over six generations, as after a generation of relaxed selection (no *E. formosa* exposure) survival when exposed to the *E. formosa* was significantly higher in the treatment group (47%) compared to the controls (39% survival), but lower than the *E. formosa* treatment group that was exposed for six generations (56% survival). I also found that after 2 generations of selection 10% of whitefly nymphs with a positive oviposition event survived parasitism in generation three. These results indicate that resistance to parasitoid-based biocontrol could evolve rapidly in whitefly if selection is applied strongly and consistently in whitefly, and this is likely primarily due to direct selection, with some maternal effects. This evolution of resistance over a short time frame could threaten effectiveness of these ecologically sustainable biological control agents.

4.1 Introduction

Resistance evolution is an ongoing issue in pest management, severely impacting global agriculture and food security. Over time, as pests are repeatedly exposed to high selection pressures from pesticides, resistance arises through standing genetic variation, mutation and selection; genetic adaptations that provide individuals with a survival advantage under pesticide application are more likely to be passed on to future generations (Hawkins *et al.*, 2019). This ongoing process can lead to resistant pest populations that are increasingly difficult to control, rendering pest management products that were once effective, obsolete. Although the evolution of resistance is often inevitable, the rate of evolution of resistance can be accelerated by factors such as the overuse of chemicals, particularly continual and sequential applications of pesticide products with the same mode of action, insufficient pesticide application rates, and environmental homogeneity (Mangan *et al.*, 2023). Alongside legislative changes, the evolution of resistance has reduced the effectiveness of many current pest management active substances leading to fewer effective active pesticides. This has resulted in higher pest management costs and an increase in crop loss.

Resistance evolution occurs when an organism's population mortality rate decreases over multiple generations due to exposure to a selection pressure, such as pesticide application. In the case of pesticides, initially they are highly effective causing significant mortality in pest populations. However, individuals with mutations that provide survival advantages under pesticide application survive and reproduce. These advantageous mutations are passed on to their offspring, quickly spreading throughout the population. Therefore, resistant genotypes become more prevalent, while non-resistant ones decline leading to a reduction in efficacy of the pesticide (Rex Consortium, 2013). Insecticide resistance mechanisms can be broadly categorised into physiological adaptations, such as enzyme activity changes or target site alterations, and behavioural adaptations, such as altered feeding patterns, avoidance or habitat use (Siddiqui *et al.*, 2022) (see Chapter 1.2-1.4). Indirect maternal effects can also evolve, enhancing the rapid evolution of direct, genetically encoded resistance in populations (Boots and Roberts, 2012). Maternal effects occur when mothers alter the conditions their offspring experience during development, for instance through physiological changes during gestation or by depositing beneficial

components such as antibodies or energy/nutritional reserves in eggs (Boots and Roberts, 2012). These maternal effects can enhance the offspring's ability to survive in environments where the stressor is present (such as a pesticide), even if the offspring haven't been directly exposed to it. Genetically resistant females are more likely to survive and reproduce, thereby passing on their resistance traits to their offspring. If there is also variation in maternal effects, it can further boost the reproductive success of these resistant individuals. Females that possess both resistance traits and beneficial maternal allocation traits will have significantly higher fitness. When these two traits are genetically correlated, it could lead to rapidly accelerated evolution of resistance, as the advantageous combination of traits quickly spreads through the population. This has been shown in pyrethroid resistant Colorado potato beetle *Leptinotarsa decemlineata*. A study investigated the effects of several sublethal pyrethroid insecticide doses on survival, body mass, and reproduction within four generations (F0, F1, F2, and F3) of the Colorado potato beetle. It was found that offspring of insecticide-stressed resistant mothers exhibited higher larval and pupal survival, compared to mothers who were not exposed to insecticides suggesting stressful insecticide exposure can have beneficial transgenerational fitness effects in the offspring independently of genetic resistance (Margus *et al.*, 2024).

Once resistance has arisen in a population stopping its spread is difficult therefore the key is to manage resistance by slowing its evolution and mitigating its effects (Whelan and Cunningham, 2020). The rate that resistance evolves can depend on many ecological factors such as the intensity of selection pressures, the organisms dispersal ability, environmental homogeneity and fitness costs associated with resistance (May and Dobson, 1986) (See Chapter 5). Successful resistance management requires planning and communication between stakeholders to manipulate these ecological factors in ways that slow the spread of alleles for resistance.

The intensified use of pesticides in traditional agricultural pest management has exerted continual exposure to strong selection pressure on pests, key for rapidly driving resistance evolution. The strong selection pressures favour an increase in the frequency of insecticide-resistant alleles in natural pest populations (Bass *et al.*, 2015). This ongoing selective environment drives rapid evolutionary change, leading to a faster emergence and spread of resistance and reduced effectiveness of the control regime over time (Oz *et al.*, 2014). This has been seen in many cases of anti-microbial

resistance evolution in medicine and in agriculture as many pests have rapidly evolved resistance to pest management measures. A classic example of continual selection pressures increasing the rate of resistance is in the codling moth *Cydia pomonella*, where only a few active substances are still effective at controlling populations. The codling moth is a worldwide pest predominantly in orchard and stone fruit production. The first recorded case of resistance in codling moth (CM) occurred in the USA in 1928, when resistance to arsenates was documented (Balaško *et al.*, 2020). Since then, resistance has been reported in nearly all major apple-growing regions worldwide. During the 1980s and 1990s, codling moth control in Europe relied on continual high dose exposure to broad-spectrum insecticides, such as pyrethroids and organophosphates, which are now ineffective due to the intensity of selection on resistance (Balaško *et al.*, 2020). It is now thought that rotating different pesticides or control agents with differing modes of action will mitigate the evolution of resistance, due to relaxing constant selection pressures, or counterselection that can reduce the prevalence of resistant individuals in the population (Tabashnik, 1989) (See chapter 7. 4). Rotating insecticides to mitigate resistance was assessed in a study on a field population of *Bemisia argentifolii*. *B. argentifolii*. was subjected to five insecticide regimens, including continuous applications of bifenthrin, endosulfan, and chlorpyrifos, as well as a rotation of all five and a mixture of bifenthrin and endosulfan. It was found that resistance developed at varying rates, with bifenthrin alone leading to the highest resistance (752-fold by generation F27). In contrast, resistance was significantly delayed by 10 generations in the rotation and mixture regimens. The resistance level was lowest in the rotation and mixture treatments after 23 generations, demonstrating that using rotations and mixtures of insecticides effectively reduces the rate of resistance development compared to continuous single insecticide use (Prabhaker *et al.*, 1998).

To mitigate resistance evolution and to improve environmental sustainability, there has been a global increase in the use of integrated pest management (IPM) regimes that combine chemical controls with biological and mechanical methods, alongside improved planning and communication (Deguine *et al.*, 2021). Biological control provides a sustainable approach to pest suppression, partly because resistance to natural predators and parasitoids is thought to be limited by factors such as spatial or temporal refuges, reciprocal co-evolution by control agents, and varying selection

pressures from other natural enemy species (Tomasetto *et al.*, 2017). However, although the evolution of resistance to many chemical active substances is well recognised, the potential evolution of resistance to biological control agents has often been overlooked (Schulz *et al.*, 2019). There is now increasing evidence to suggest many pests can evolve resistance to biological control agents.

Biocontrol agents can also vary in their success at achieving the desired level of pest management. Various factors contribute to reduced efficacy such as the release of inappropriate control agents that are not formulated to have high efficacy of pest control. Moreover, biocontrol agents can be poorly adapted to the environmental conditions where they need to operate (for instance due to adaptation to the lab environment) resulting in reduced efficacy and potentially enhanced resistance evolution (Tomasetto *et al.*, 2017). For instance, when fungal biopesticides are used in environmental conditions that do not match recommended use, they have reduced virulence reducing the intended dose that the pest receives. Lower doses can increase resistance evolution when resistance is a continuous trait; if a higher proportion of individuals with some level of resistance survive under low doses their offspring will survive and mate assortatively, conferring ever higher levels of resistance in the preceding generations (Bosch *et al.*, 2014).

As agricultural intensification diminishes available refuges and reduces the diversity of natural enemies, the likelihood of pests evolving resistance may increase, especially if control agents face physiological constraints in their own evolution (Tomasetto *et al.*, 2017). At least 27 species of insects have been described as resistant to *Bacillus thuringiensis* toxins, commonly used as insect pest biocontrol agents (Siegwart *et al.*, 2015). After the introduction of granulovirus-based insecticides to control codling moth (*Cydia pomonella*) in European apple orchards in the early 1990s, resistance emerged rapidly by the mid-2000s. This resistance necessitated the development of new products using different viral strains (Jehle *et al.*, 2017). More worryingly resistance has also been found to an endoparasitoid wasp, *Aphidius ervi* in pea aphids (*Acyrtosiphon pisum*). All aphid genotypes tested had some resistance to parasitism, with resistance levels varying significantly (19% to nearly 100%). In the pea aphid, infection with the bacterial endosymbiont *Hamiltonella defensa* is the main factor that determines resistance, not the aphid genotype; when a single *H. defensa* isolate was tested across different aphid clones, resistance levels remained consistent. These

findings suggest that aphids can evolve resistance through genetic mutations, however, the presence of a symbiont increases the presence of parasitoid resistance (Oliver *et al.*, 2005).

As pests evolve resistance to biocontrol agents, these agents may, in turn, evolve new strategies to overcome the resistance, creating an ongoing evolutionary arms race (Arora *et al.*, 2011). However, if biocontrol agents are reared in a laboratory controlled environment it may limit the natural enemies ability to co-evolve in response to the pest due to less genetic variation in biocontrol agents populations than the pest hosts genetic variation. To enhance the effectiveness and long term effectiveness of biocontrol agents, integrating genetic and genomic approaches is increasingly important, especially as the demand for successful biocontrol continues to grow. While these techniques were previously underutilized, recent technological advancements and lower costs have made them more accessible (Leung *et al.*, 2020). Effective biocontrol resistance management is crucial for sustaining the efficacy of biological control agents in pest management programs. Given the challenges in completely preventing resistance evolution, it is essential to focus on mitigating and slowing its progression. Strategies to manage resistance include diversifying the modes of action of control agents, establishing refuges where pests are not exposed to biocontrol agents, and increasing landscape diversity (Bosch *et al.*, 2014).

While there is mounting anecdotal and observational evidence that resistance to biocontrol readily evolves in the field, as yet no studies have demonstrated that resistance evolves in these examples as a direct consequence of the application of biocontrol (Hufbauer and Roderick, 2005). In order to demonstrate resistance evolution *sensu stricto* selection experiments with appropriate control treatments are required. Resistance evolution can be inferred when resistance/survival in response to a selection pressure (in this case a biocontrol agent) increases over generations in populations which have been exposed to it, but not in genetically equivalent control populations. Importantly this result should be replicated across several independent replicate populations to demonstrate how repeatably resistance evolves. The aim of this study was to demonstrate whether the glasshouse whitefly *T. vaporariorum* evolves resistance to its biocontrol agent, the endoparasitoid wasp *Encarsia formosa* (see chapter 2 section 2.1 for an overview of its lifecycle and mode of action), over 6

generations (approx. 6 months). I exposed replicates of 20 families to parasitism by *E. formosa* for 6 generations, and 20 replicates of these families acted as the control (they were not exposed). In the generation prior to final testing (generation 5) each of the 20 *Encarsia* replicates was divided into 2, one of these was exposed to *E. formosa* as normal and the other was not (selection was relaxed), this allowed me to test whether putative resistance was conferred through indirect maternal effects vs direct genetic effects.

4.2 Methods

4.2.1 Host Plants

Aubergine plants used in this experiment followed the growing procedures set out in Chapter two (section 2.2.1) general methods.

4.2.2 Overview

Trialeurodes vaporariorum were collected from the established colony, which was set up and maintained as described in Chapter 2.71-2.73 general methods.

The selection experiment was conducted over six generations, running for approximately 6 months from set up to final resistance assay mortality counts (See figure 17). In generations 1-4, there were two treatments; Control (C) and *Encarsia formosa* (parasitoid wasp) exposed (S). Each treatment was replicated 20 times over using 20 different lines (which were represented in each treatment group), in each treatment each line was reared on an individual aubergine plant, in an individual plant pot cage. To start a new generation a fresh plant was exposed to a minimum of 100 adult whitefly from each line, for generations two to six these were founded from the same lines as the previous generation. In generation five a relaxed selection (RS) treatment group was set up; 2 duplicates of the 20 lines from the *E. formosa* (S) treatment were established, one of which was exposed to *E. formosa* as normal and one which was not (the RS 'relaxed selection' treatment). The purpose of the relaxed selection treatment was to test the relative importance of indirect maternal effects and

direct genetic effects on resistance evolution (if mortality was higher in the relaxed selection than the selection treatment this would indicate maternal effects are operating). In generation six I assayed mortality of all lines by treatment combinations when exposed to *Encarsia formosa* to test whether variation in susceptibility had changed as a result of *E. formosa* exposure over the six generations. See Appendix 8.2.2 For full dates each generation was set up on).

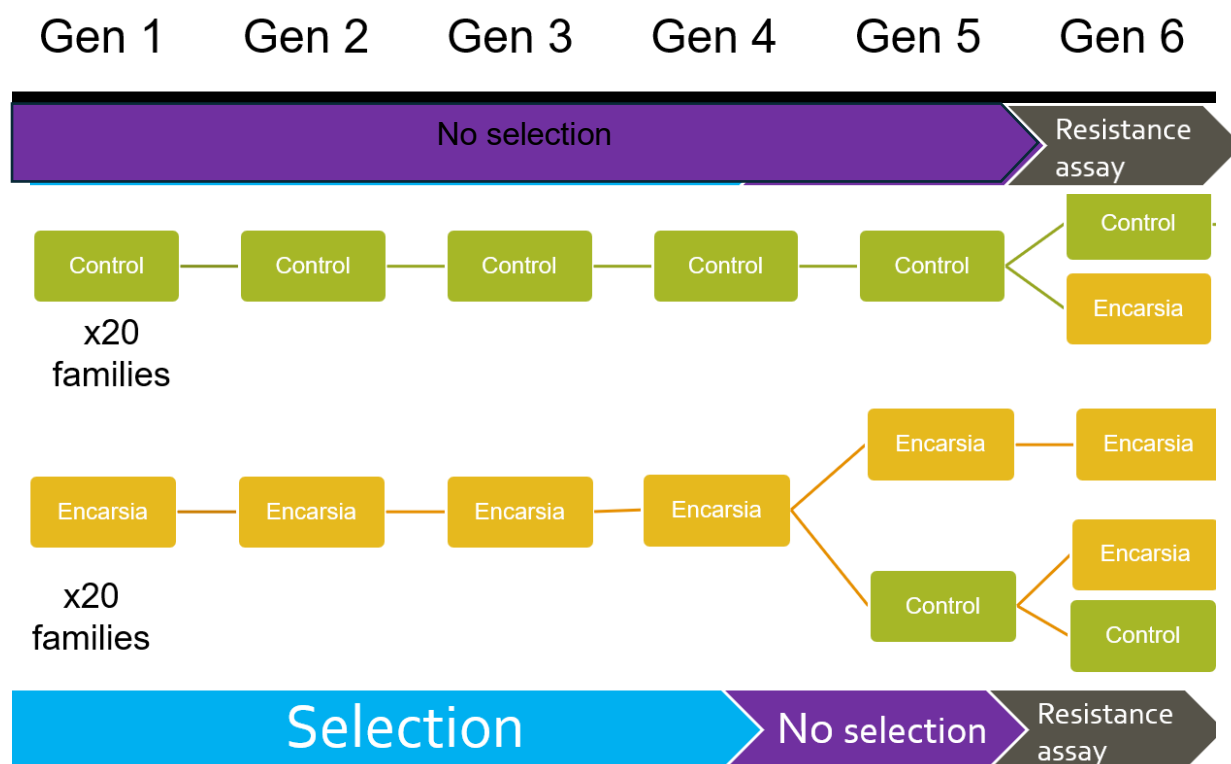


Figure 18: A schematic showing the experimental treatment groups with founding lines and selection pressures for generations 1-6. The top lines had no selection for the first six generations, with the bottom lines having five generations of selection.

4.2.3 Selection experiment set-up and maintenance

Selection experiments on *T. vaporariorum* were conducted using black beauty organic aubergine plants grown from seed following the growing protocols set out in chapter 2. Plants used in this experiment were 10 -12 weeks from sowing when used in the experiment, by which time they had at least four-six large leaves. Plants were watered three times a week by filling the plant tray with water, to avoid opening of cages and disrupting insects or damaging the plants. To found generation one, *T. vaporariorum* adults were collected from a laboratory colony established in 2021 using a mechanical or electric pooter and put into twenty cages containing two plants each. Before being put into cages adult whitefly were anaesthetised by cooling in a fridge, sexed and then 125 females and 125 males were placed in each cage with 2 plants. Cages were placed in a controlled environment facility kept at 16:8 L:D photoperiod 26°C constant temperature and 70% relative humidity. Adult *T. vaporariorum* were left in the cages for four days to mate and lay eggs before being removed by shaking/pootering. Once adults were removed, the plants from each pair were separated and assigned a line number (the same line number was given to plants from the same cage) and treatment group (control, C or *E. formosa*, S), labelled and placed in individual plant pot cages. The C and S treatment groups were kept in separate control cabinets to reduce the risk of cross contamination between treatments.

Plants were then left for a further 10 days for nymphal development before treatments were applied, by which time the majority of the whitefly nymphs had reached second instar stage. I chose to expose second instar nymphs to *E. formosa* in all generations based on the commercial suppliers recommended guidelines. On day 14, nymphs on S treatment plants were counted and adult *E. formosa* wasps were placed in the S treatment cages with a ratio of one wasp to 5 whitefly nymphs (see chapter 2 general methods for details on *E. formosa* rearing). *Encarsia* wasps were removed from the S treatment cages after 48 hours, by shaking/ pootering. Control C cages were also shaken to ensure similar methods between treatment groups. Wasps were removed to promote synchronised parasitisation between lines, as well as to limit host-feeding and superparasitism (laying multiple eggs on one host) which could result in additional mortality so high that subsequent generations would fail to establish. Whitefly nymphs were left for a further 10 days to develop and for whitefly/ *E. formosa* to emerge as

adults. Adult whitefly that emerged were counted, sexed and used to establish the next generation (generation 2). I took the first 64-100 adults (due to variation in the total number of adults emerged) with a 0.5 male to female ratio to initiate the next generation, with the total number added remaining the same for all lines in the same generation (based on the replicate line with the fewest emerging individuals, i.e. all replicates in generation 1 used N=76 whitefly to found generation 2, (see appendices 8.2.3 for all founding values). I added these whitefly to a new cage to start the next generation adding equal numbers of males and females to keep a sex ratio of 0.5. I repeated this for each line*treatment combination sequentially taking care to avoid cross-contamination between lines.

This was repeated from generation two onwards: (i) whitefly were left for 4 days to mate and oviposit on all plants; (ii) adult whitefly were removed from plants; (iii) on day 20 plants in the S treatment group were exposed to wasps (at a ratio of 1:5 wasps to whitefly nymphs); (iv) *Encarsia* wasps removed from S plants after 48 hours; (v) on day 26 emerging whitefly were collected to found the subsequent generation. In generation 3 after removal of *E. formosa* from the S treatment cages, the parasitism status of the whitefly nymphs was recorded; nymphs were classified as: parasitised (which is evident from a melanised black mark on the cuticle where the wasp egg was laid), eaten, or not attacked. In generation 5 the 20 *E. formosa* selected lines (S) offspring from generation 4 were split into two treatment groups, 20 replicate lines were exposed to selection pressures from *E. formosa* for a further generation (S) and the same 20 replicate lines were treated the same as the control treatment and not exposed to *E. formosa* (classified as relaxed selection, RS treatment group). In generation 6 these lines were split into a further 2 treatment conditions; control (no *Encarsia*; C-, RS-) and *Encarsia* exposed (C+, RS+, S+; see figure 18 and table 4). If maternal effects contribute to the reduced mortality in the *Encarsia* selection treatment, S, I predicted that in generation 6 mortality would be higher for the relaxed selection, RS, whitefly compared to the S lines which were exposed to *Encarsia* in generation 5.

4.2.4 Data collection

In every generation, 10 days after exposure to treatment (or not for the control) mortality counts were conducted. Adult whitefly were removed from plants using a pooter and placed into fly vials. These fly vials were labelled with the line ID and treatment on the plant pot they were removed from. For each line all leaves were removed from a plant and the number of whitefly nymphs alive, dead and parasitised on the leaf were recorded using a microscope and counter. The number of nymphs before exposure was estimated from these values as the sum of the alive nymphs, dead nymphs, parasitised nymphs and emerged adult whitefly. The emerged adult whitefly in these vials were used to establish the next generation for each line*treatment combination (see 4.2.3 above).

4.2.4.1 Generation 6 resistance assay

In generation six a final resistance assay was undertaken where every individual line was exposed to *E. formosa* (+) (figure 17 and 18; table 4) to test whether mortality under *E. formosa* parasitism was significantly lower after 6 generations of selection compared to the unexposed controls, indicating resistance evolution. Mortality assays followed the same methodology as the previous five generations, with the same number of adults founding all treatment lines (N = 72, with an even sex ratio). Control adults that emerged from generation five were split into two treatments from the control treatment group (C-; control no *Encarsia* and C+; control lines exposed to *E. formosa*). Adults from the generation 5 S treatment group were again exposed to *E. formosa* in generation six (S+). An S- test where adults from the generation 5 S treatment group were not exposed in generation 6 was not conducted, as it would not be an informative treatment group when assessing the potential survival and presence of the maternal effects. The *E. formosa* lines that were not exposed to *E. formosa* in generation five (Relaxed selection, RS) were split into two treatment groups in generation six (RS+, exposed to *E. formosa* and RS- control, unexposed; see figure 18).

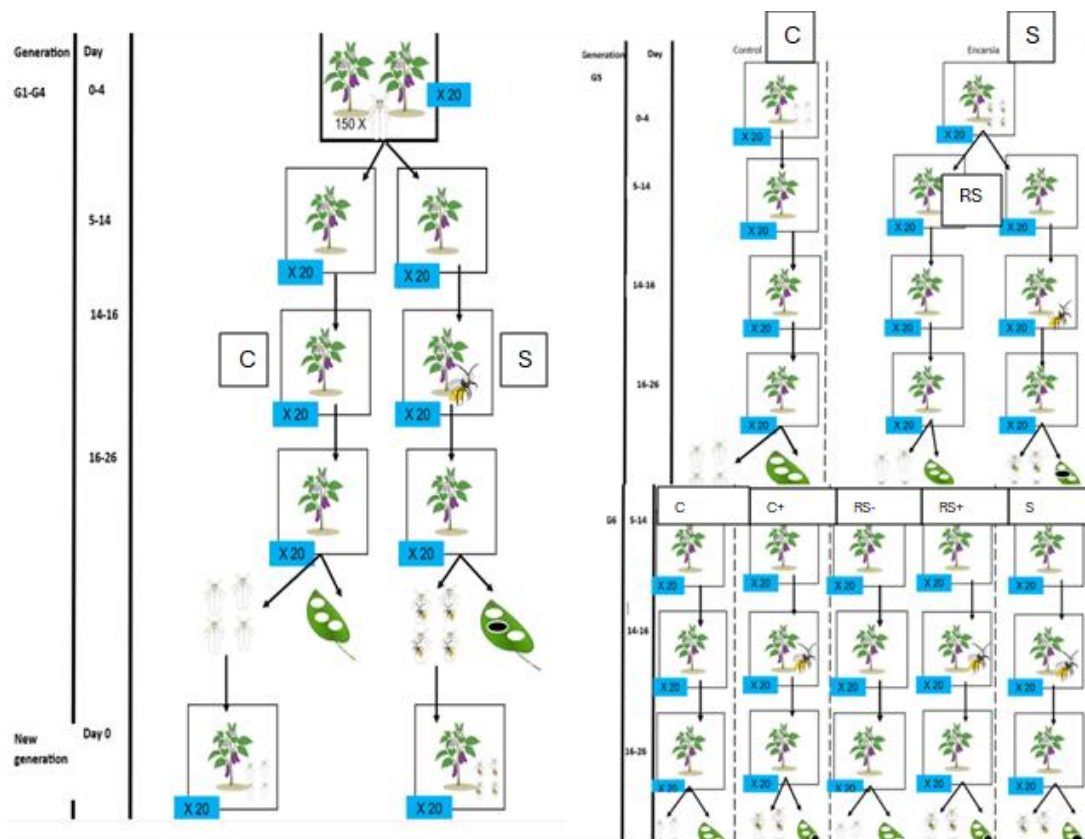


Figure 19: A schematic showing the methodology for generations 1-6, from initial set up to mortality counts and founding the new generation with varying treatment groups in generations five and six. Abbreviations refer to the following: C; control no *E. formosa*, C+; control lines exposed to *E. formosa* at generation 6. S; exposure to *E. formosa*. RS+, exposed to *E. formosa* after no exposure in generation 5 and RS- control, unexposed for generation 5 and 6.

4.2.3 Data analysis

Data were analysed in R version 4.1.3 (R Core Team, 2022). Mortality variation between treatments for all generations was assessed using a generalised linear mixed Model (GLMM) with a negative binomial distribution with a two-vector response (total number dead, total number alive using the cbind function) (Brooks *et al.*, 2017). Models contained two categorical fixed factors, treatment (S or C) and generation and their interaction and a random effect of line. Post hoc tests using the lsmeans package were used to compare mortality across different treatments and generations. Model diagnostics, including residual simulations with the DHARMa package, were employed to validate model assumptions and ensure the robustness of my results (Hartig, 2022).

For generation 6 data analysis, all treatment groups were given a code based on the selection treatment and their exposure (+ exposed to *E. formosa* and – unexposed) in generation 6 (See table 4). Selection is the treatment group, and the selection pressure whitefly experienced before the generation 6 resistance assay. Control (C) is no exposure to *E. formosa*, S, *E. formosa* have been continually exposed to *E. formosa*, Relaxed (RS) are whitefly lines that were exposed to *E. formosa* for generation 1-4 and not exposed in generation five, with re-exposure in generation 6.

Table 4: All treatment groups in the selection experiments codes for data analysis (generation 6 only). No exposure indicates no *E. formosa* exposure for that generation. + indicates exposure, - indicates no exposure.

Selection	Exposure	Code
Control	No exposure	C-
Control	<i>E. formosa</i>	C+
<i>E. formosa</i>	<i>E. formosa</i>	S+
Relaxed	No exposure	RS-
Relaxed	<i>E. formosa</i>	RS+

A separate binomial glmm was used to analyse the results of the mortality assay in generation 6. This included a fixed effect of treatment (S+, RS+, RS-, C+ and C-; See table 4) with mortality (weighted using the cbind function; number dead, number alive) as the response variable and line as a random effect. DHARMA was used to check model fit and post hoc pairwise comparisons were conducted using lsmeans to explore differences across treatment conditions in generation six and test whether maternal effects influence whitefly mortality (Lenth, 2016). If females exposed to *Encarsia* do provision their offspring to enhance their survival under *E. formosa* parasitism I predicted that mortality (when exposed to *E. formosa* RS+) would be significantly higher in the 'relaxed selection' treatment (mothers which had not been exposed to *Encarsia* in generation 5) compared to females in the selection lines (which had been exposed to *E. formosa* in generation 5 S+) (See table 4).

4.Results

4.1 The fate of whitefly nymphs exposed to *E. formosa*

In generation three the rate of parasitism was investigated. All nymphs across the 20 lines in the *E. formosa* S treatment group were assessed to determine if there has been a positive oviposition event. The parasitism status of the whitefly nymphs was recorded; nymphs were classified as: parasitised (which is evident from a melanised black mark on the cuticle where the wasp egg was laid), eaten, or not attacked.

On average in generation three, out of the 7342 nymphs in *E. formosa* treatment 53% had a positive oviposition event. Positive oviposition events varied between the 20 *E. formosa* lines from 41% to 65%. Out of the 7342 nymphs, , 47% were successfully parasitised by *E. formosa* and 5% survived parasitisation (Figure 19).

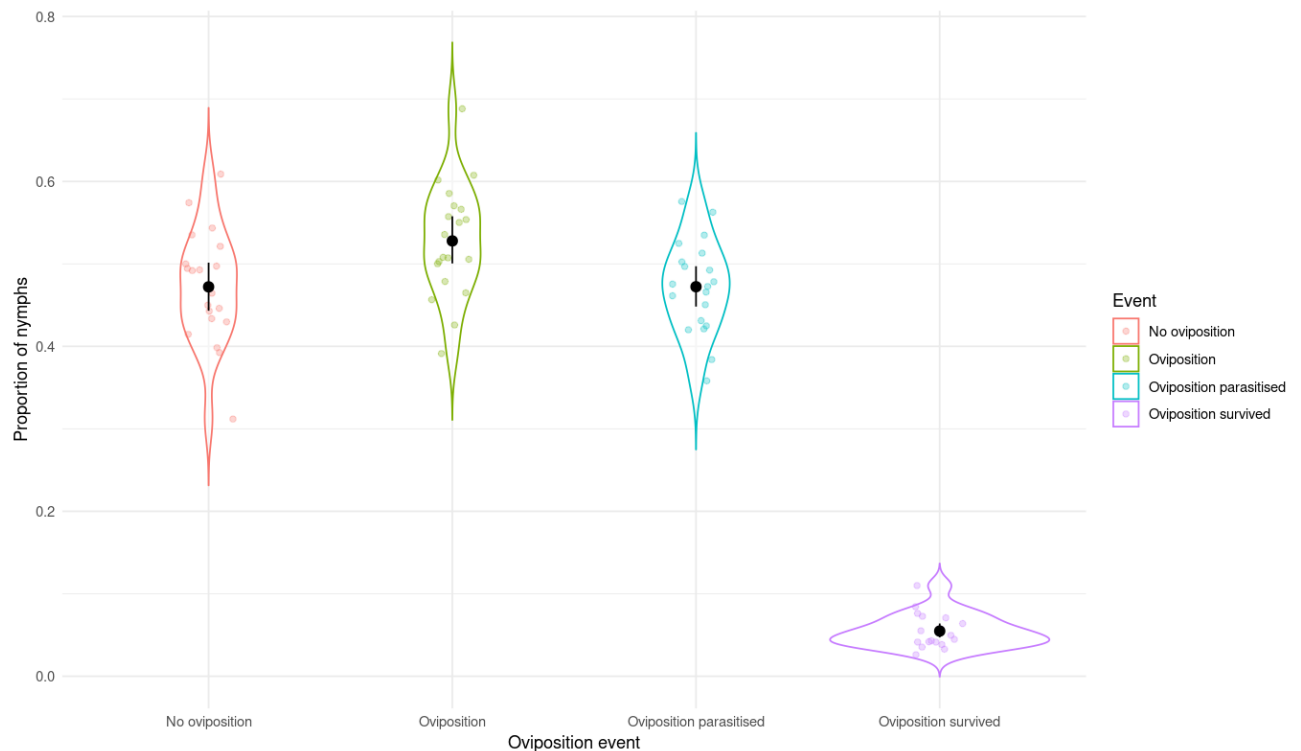


Figure 20 : the proportion of whitefly nymphs in generation three that did not have an oviposition event (red), had a positive oviposition event (green), had an oviposition event and were successfully parasitised by *E. formosa* (blue) and had an oviposition event and unsuccessful parasitisation by *E. formosa* (purple).

4.2 Proportional survival across six generations

Three hundred individual aubergine plants were used for rearing whitefly over the course of the experiment, which used approximately 30,000 adults that laid a total of 88,115 nymphs across the six generations and all treatment groups.

When the proportional survival over six generations was assessed, treatment had a significant effect, ($X^2_1=4064.46$, $P < 0.001$; figure 20) (where whitefly survival was higher in the control treatment compared to the *E. formosa* treatment, table 5).

Survival over time was also significantly affected by generation, with survival increasing in both groups over the six generations. Whitefly mortality was low in the control lines over all 6 generations; 95% of whitefly survived in generation 1 in the

control with an increase to 99% by generation 6 (table 5). Proportional survival changed significantly across generations in the control treatment. Between generation 1 and 2 survival significantly increased ($z = -8.99$, $P < 0.001$) (Figure 20, A and B), decreased between generations 2 and 3 ($z = 6.30$, $P < 0.001$) (Figure 20, B and A), and increased between generations 3 and 4 ($z = -7.88$, $P < 0.001$) (Figure 20, A and B), there were no significant changes between generations 4, 5 or 6.

Whitefly survival increased over the six generations in the *E. formosa* treatment lines, and there was a significant generation*treatment interaction effect ($X^2_5 = 82.66$, $P < 0.001$) (figure 21); survival increased more across generations in the *E. formosa* treatment S lines compared to the control C lines. For the *E. formosa* treated lines in generation 1 only 36% of whitefly survived exposure to *Encarsia*, increasing to 56% in generation 6. Between generation 1 and 2 survival significantly increased ($z = -10.58$, $P < 0.001$) (Figure 21, C and D), decreased between generations 2 and 3 ($z = 11.19$, $P < 0.001$) (Figure 21, D and C), increased between generations 3 and 4 ($z = -12.63$, $P < 0.001$) (Figure 21, C and D), increased between generations 4 and 5 ($z = -6.29$, $P < 0.001$) (Figure 21, D and E) and increased between generations 5 and 6 ($z = -3.50$, $P < 0.05$) (Figure 21, E and F).

Table 5: The average proportional whitefly survival per generation and treatment.

Generation	Treatment	Total nymphs	Nymphs survived	% Survival	SE
1	Control (C-)	6870	6521	95	0.768
2	Control (C-)	7538	7372	98	0.676
3	Control (C-)	7271	6978	96	0.545
4	Control (C-)	7996	7854	98	0.457
5	Control (C-)	5850	5779	99	0.461
6	Control (C-)	2976	2956	99	0.401
6	Control (C+)	3050	1182	39	0.345
1	<i>E. formosa</i> (S+)	7023	2516	36	0.523
2	<i>E. formosa</i> (S+)	7991	3540	44	0.710
3	<i>E. formosa</i> (S+)	7342	2609	35	0.661
4	<i>E. formosa</i> (S+)	7909	3605	46	0.328
5	<i>E. formosa</i> (S+)	3715	1922	52	0.390
6	<i>E. formosa</i> (S+)	3063	1710	56	0.402
5	Relaxed Selection (RS-)	3529	3480	99	0.538
6	Relaxed Selection (RS-)	2904	2882	99	0.373
6	Relaxed Selection (RS+)	3088	1473	47	0.345

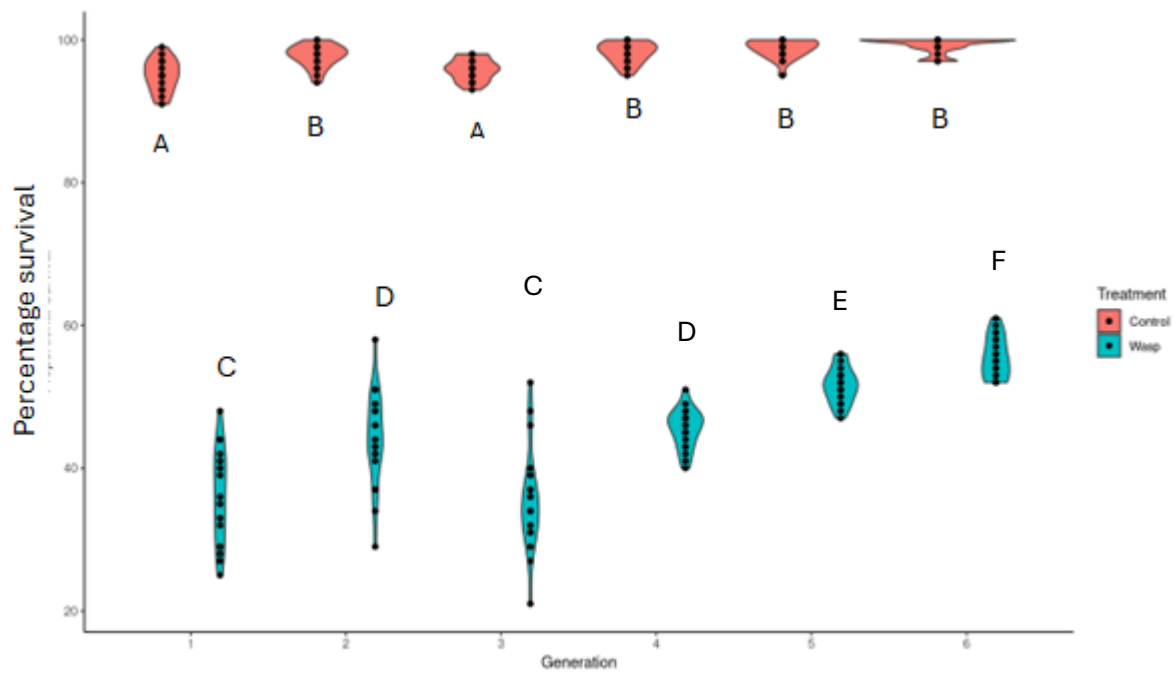


Figure 21: The proportional whitefly survival across the six generations in the control treatment (C) (red) and the *E. formosa* (S) treatment group (blue). All lines are represented by points and different uppercase letters represent groups that are significantly different from each other at $p < 0.05$.

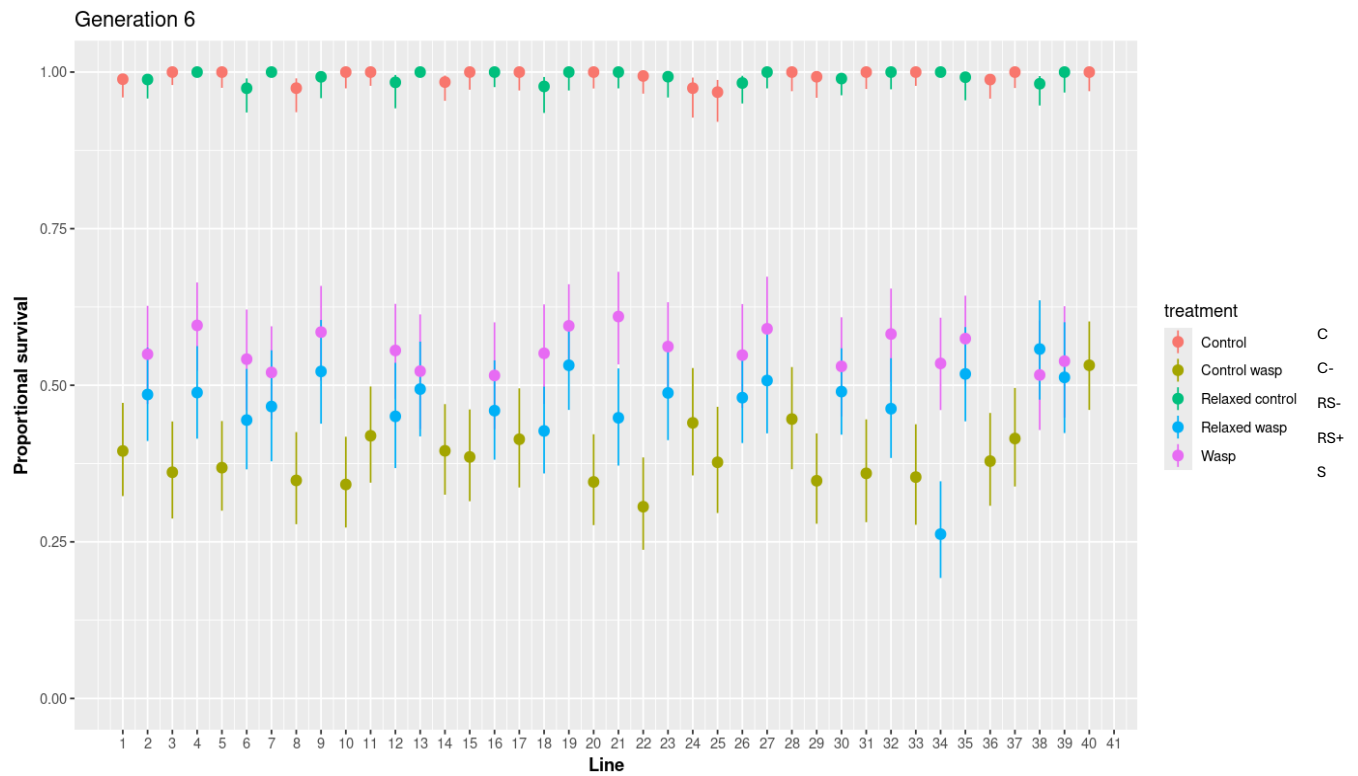


Figure 22: The proportional survival for each whitefly line, with error bars representing binomial confidence intervals in the control treatment (C-) (red) , control exposed to *E. formosa* (C+) , the *E. formosa* treatment (S+) (purple), Relaxed selection (RS-) (green) and the relaxed selection exposed to *E. formosa* (RS+) (Blue) at generation 6 of the selection experiment. Paired lines 1 and 2, 3 and 4 onwards were from the same founding population.

4.3 The potential of maternal effects increasing whitefly survival

In generation 5 lines in the *E. formosa* (S) treatment group were split into 2, one of which was exposed to *E. formosa* (S) as in the previous generations and one of which was subjected to no parasitism (relaxed selection) (RS) (See table 4) . The offspring produced by relaxed selection whitefly in generation 5 were then split further in generation six into 20 lines that were either exposed to selection pressures from *E. formosa* (RS+) in generation 6 and 20 lines were treated as a control treatment (no *E. formosa*) (RS-) (See table 4) .

In generation six treatment had a significant effect on whitefly proportional survival (χ^2_4 1188.1, $P < 0.001$). Whitefly in the *E. formosa* treatment (S+) had significantly lower survival than whitefly in the control treatment (C-) ($z=20.49$ SE= 0.23, $P<0.001$) (figure 22).

Pairwise comparisons between treatment groups in generation 6 were used to determine if maternal effects played a role in whitefly proportional survival. If maternal effects do play a role in increased whitefly survival under *E. formosa* parasitism I predicted that survival in the relaxed selection lines exposed to *E. formosa* (RS+) in generation 6 would have higher mortality than the selection lines exposed to *E. formosa* in generation 5 and 6 (S+). When exposed to *E. formosa* I found significantly lower survival in the relaxed selection whitefly (RS+) compared to the selection lines exposed to *E. formosa* in generation 5 and 6 (S+) ($Z=-6.57$, SE=0.05, $P<0.001$). The relaxed lines exposed *E. formosa* (RS+) had significantly higher survival compared to the control lines exposed to *E. formosa* in generation 6 (C+), ($Z=-6.14$, SE=0.06, $P<0.001$) (Figure 22). This indicates that maternal effects play a role in whitefly survival alongside genetic effects.

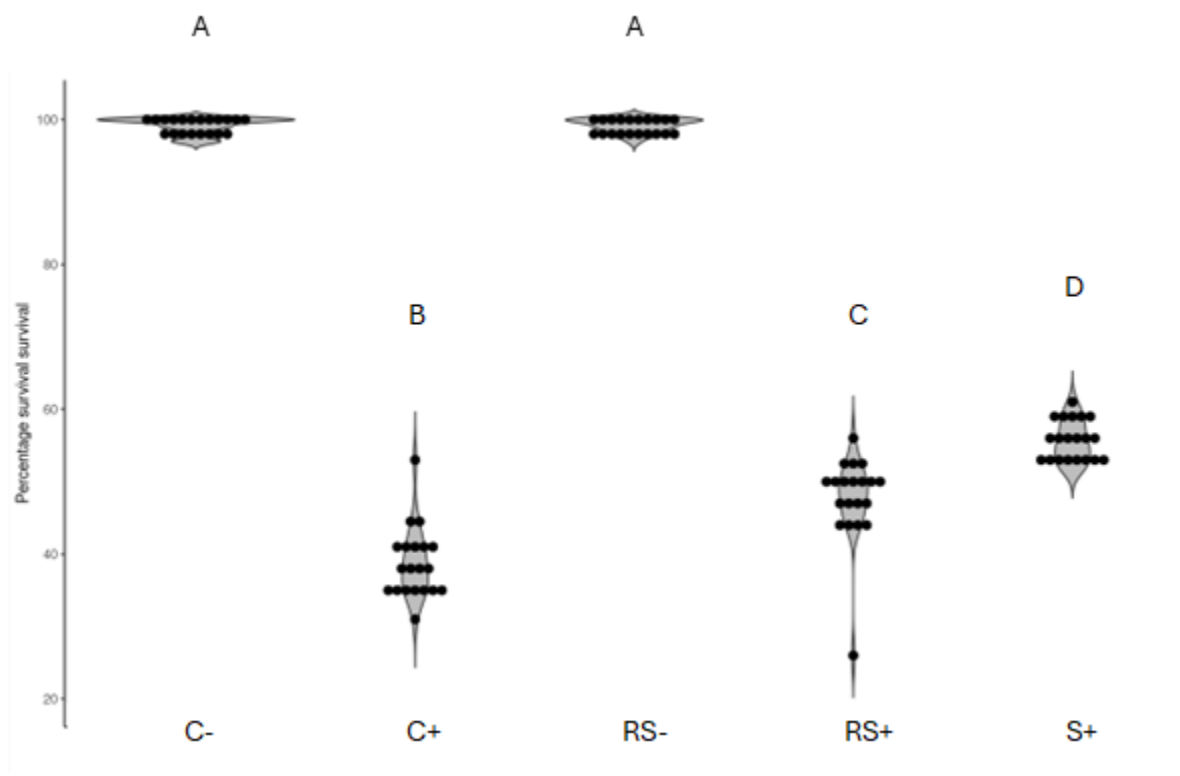


Figure 23: The proportional survival for all 20 lines at generation 6 in the control treatment (C), the control treatment exposed to *E. formosa* (C-), the relaxed treatment exposed to *E. formosa* (RS+) and the *E. formosa* treatment exposed to *E. formosa* (S+). The mean proportional survival for 20 lines is indicated where the violin plot is at its widest. Uppercase letters indicate groups that are significantly different from each other ($p < 0.05$).

4.4.Discussion

Pesticide resistance is a growing agricultural issue, diminishing crop protection, food security and leading to economic losses. The shift towards biocontrol agents, like fungal biopesticides and parasitoid wasps offers ecological benefits, but there's limited understanding of whether pests will evolve resistance against them. Reports suggest declining efficacy of biocontrol agents used to manage glasshouse whitefly (*Trialeurodes vaporariorum*), hinting at potential resistance evolution. In a six-

generation lab experiment, I observed a large and significant increase in whitefly survival when exposed to the parasitoid wasp *Encarsia formosa*, from 35% to 56%, but only small significant changes in survival the control group where average survival ranged from 95% to 99% across generations. In generation 3 found that 10% of whitefly nymphs survived parasitism, when a positive oviposition event was identified by a melanisation mark on the nymphal cuticle, suggesting resistance is conferred through generalised immune defence response. Whitefly surviving a positive oviposition event was also found in Chapter 5, where whitefly with no oviposition event were removed from the experiment, thus, leaving adult whitefly who survived parasitism to determine life history costs. In the generation 6 mortality assay I found evidence that maternal effects may also have some influence on the survival increase in the selected wasps, suggesting that mothers may be able to provision their offspring in a way that enhances their survival under parasitism. Overall, my findings indicate that sustained strong selection pressures can quickly lead to resistance in whitefly populations, potentially undermining the effectiveness of these sustainable biological control methods potentially within a single growing season. This could be damaging to crop health, yield and food security if the potential of resistance is not considered.

This significant increase in survival suggests whitefly can rapidly evolve resistance to *E. formosa* when under high selection pressures. This is supported by the observed reduction in variation in survival in the treated lines; after the 3rd generation the range in survival is lower than in the preceding 3 generations. This is likely due to resistant genotypes/ individuals becoming more prevalent within the lines in the *E. formosa* treatment while the presence of susceptible individuals decline leading to a reduction in variation in resistance (Rex Consortium, 2013). My results are similar to a study which showed that *Drosophila melanogaster* rapidly evolves resistance to its parasitoid *Asobara tabida*. I found a 55% increase in survival over 6 generations, while in *D. melanogaster* larval survival doubled within 5 generations. The *D. melanogaster* study conducted whole-genome sequencing on selection lines at the end of the experiment, which revealed multiple, highly localized genomic changes, with selected regions covering less than 5% of the genome. The authors suggested that high genetic variability and small linkage blocks in the base population likely facilitated the evolution of this complex trait without extensive genomic disruption, even under rapid and intense selection. This is likely because smaller linkage blocks decrease the chances

of linkage disequilibrium, where closely located genes on a chromosome are inherited together. Consequently, these linked regions could be selected more efficiently, allowing for the rapid evolution of resistance without causing significant changes or disruptions in other parts of the genome (Jalvingh *et al.*, 2014).

Although sequencing was not conducted during my study, it was evident that physiological effects were not solely responsible for reduced survival, as in generation 6 I found evidence that the maternal environment did influence whitefly survival. Whitefly survival in the relaxed treatment group (unexposed in generation 5) that was exposed to *E. formosa* in generation six (RS+) had lower survival than whitefly from the *E. formosa* treatment (which were exposed to *Encarsia* in generation 5; S+). Maternal effects were not solely responsible however, because the relaxed selection whitefly (RS+) still had higher survival than control (C+) whitefly in generation 6 when exposed to *E. formosa*. These results, when viewed alongside my observations that a proportion of whitefly do survive positive oviposition events suggests that whitefly resistance to *E. formosa* is likely to primarily due to physiological mechanisms of resistance but enhanced by maternal effects. This selection experiment shows rapid evolution of resistance, supporting the findings of the empirical and simulation results in chapter 3 of this thesis.

The rate of resistance evolution in this experiment was more rapid than the simulated rates shown in chapter 3 however, likely due to the way the experiment was conducted, in particular the numbers used, and the strength of selection applied. As this selection experiment was conducted with lines on individual plants in confined settings, the selection pressures are likely to have been much greater than they would be in the field. This is due to whitefly nymphs being laid on fewer leaves, with higher numbers in a smaller area resulting in higher whitefly density and higher exposure to *E. formosa*. As *E. formosa* find hosts to parasitise through searching behaviour, when nymphs are closer together *E. formosa* will require less time to search, increasing parasitism rates (Romeis and Zebitz, 1997). This pattern was confirmed by a study that examined the foraging behaviour of *E. formosa* using a stochastic simulation model that replicated the parasitoids actions (Roermund *et al.*, 1997). During the simulation, *E. formosa* searched a tomato plant infested with whitefly nymphs (*Trialeurodes vaporariorum*). Simulation outcomes were consistent with real observations of *E. formosa* foraging on tomato plants. The number of encounters and ovipositions increased with host density,

following a type II functional response. At low host densities and clustered distributions, key factors affecting oviposition rates included leaf area, parasitoid walking speed and activity (Roermund *et al.*, 1997). This suggests that in my study the high host density and clustered distributions would increase the oviposition events, thus, increasing the selection pressure. As such, the rate of resistance evolution is likely to be faster in my study compared to real world environments. Nevertheless, anecdotal evidence from personal communications with growers suggests resistance evolves over a six-month growing season. Another factor to consider is whitefly emigration and immigration. In my study whitefly lines were maintained separately and so there was no dispersal through immigration or emigration. The lack of dispersal could accelerate the evolution of resistance compared to field conditions, as the resistance traits were not diluted through immigration of susceptible alleles (Miller and Sappington, 2017). In the field an influx of susceptible individuals from nearby areas could slow down resistance evolution. On the other hand, immigration could also accelerate the evolution of resistance in the field if there are more resistant individuals in nearby areas which disperse and introduce resistant alleles into the local population. Moreover, environmental factors like changing climate conditions, varied plant host availability, and the presence of natural predators or other biocontrol agents could impact resistance development differently compared to the controlled conditions of this controlled laboratory experiment. As such, resistance evolution is likely to be highly context dependent, and realistic rates of evolution would need to be measured by conducting experiments over full growing seasons in a range of conditions.

In generation three the number of positive oviposition events were recorded to determine if whitefly could overcome *E. formosa* parasitisation after a positive oviposition event. The oviposition event was characterised as a melanised mark on the whitefly cuticle. It was found that 10% of whitefly that had a melanised positive oviposition event survived parasitism and emerged as whitefly. The characterisation of melanisation mark indicating positive oviposition event was also used in chapter five, where whitefly without a melanisation mark were removed from the experiment to assess life history costs associated with parasitoid oviposition. This finding suggests that the whitefly could be overcoming parasitism through general mechanisms that improve their immune response. Many organisms overcome parasitism via a cellular response where large pathogens like parasitic eggs

entrapped via encapsulation (Sideri *et al.*, 2008). Parasitoid egg encapsulation and the immune interactions between parasitoids and their host insects, such as in *Drosophila*–wasp models, have been a major focus in insect immunology and parasitology for a long time. In response to parasitoid infestation, *Drosophila* utilises various innate immune defence mechanisms, encompassing both cellular and humoral immunity. Within the hemocoel, cellular immune responses include the proliferation, differentiation, migration, and dissemination of host haemocytes, which are responsible for encapsulating the parasitoid egg, and surviving attack (Yang *et al.*, 2020). However, in my study the characteristic of oviposition was a melanised mark on the nymphal cuticle 48 hours after *E. formosa* exposure, with no further observations until emergence. Future studies which dissect whitefly to detect parasitoid eggs and determine encapsulation are necessary to test whether melanisation results from successful parasitism or the presence of another foreign body also results in the mark.

Another possibility, which is not mutually exclusive, is that behavioural adaptations allow whitefly to escape parasitism. In generation 3, 47% of whitefly in the *Encarsia* treatment were not attacked at all suggesting that attack is not a given even at the high densities of *Encarsia* used in this experiment. While I can only speculate about this as I did not assess attack rates in the control treatment in generation 3 it is possible that whitefly can employ behaviours allowing them to evade parasitism. The aphid *Myzus persicae* shows significant heritable variation in defence behaviour in response to an alarm pheromone produced when conspecifics are attacked by parasitoids. The behaviours in response to the alarm pheromone can include, stopping feeding and dropping off the leaf or walking away to avoid predation, kicking the legs to deter or dislodge predators, clustering together and producing more winged offspring (alates), which can disperse to new locations away from the danger (Vandermoten *et al.*, 2012). In this study, eight parthenogenetic clones of *M. persicae* were tested for their response to alarm pheromone. These clones were then observed in small-scale arenas during exposure to adult female parasitoids, *Diaeretiella rapae*, both with and without the presence of alarm pheromone. The findings showed that clones with a consistently high alarm response exhibited behaviours (moving away from the area and withholding larviposition) which were associated with higher survival rates and reduced parasitism compared to aphids with a low alarm response (Foster *et al.*,

2007). Many pests have developed and evolved various adaptations to evade parasitoids. For instance, some pests pupate underground, within rolled leaves or other protected areas, creating physical barriers that shield them from parasitoid attacks. Numerous pest species are leaf rollers and stem borers, living the majority of their life cycle within plant tissue, that not only protects them from natural enemies but also makes them more invasive and difficult to manage with many pest management regimes (Aoyama and Ohshima, 2019).

Many of these behaviours are not applicable to whitefly however, as the nymphs become immobile soon after hatching and adult whitefly were not present when *E. formosa* were released. In the case of *T. varporariorum*, behavioural avoidance may instead take the form of female preference for oviposition site. For instance, females that oviposit in less accessible areas of the plant (such as trichome/hair dense parts of the plant, or in rolled leaves) would be under strong selection if their offspring were protected from parasitism (Shishehbor & Brennan, 1995). Future studies to test this hypothesis would be a fruitful avenue for research, and would be relatively straightforward and inexpensive to conduct, requiring behavioural observations on the oviposition behaviour of selected and control whitefly and then behavioural tests using *E. formosa* to assess parasitism rates in different areas of the plant.

During generation five of this experiment a relaxed selection treatment group was established to determine if maternal effects were playing a role in the increase in survival over time. I found evidence that increased survival was partly due maternal effects, as the offspring of females from selection lines which were not exposed to *E. formosa* in generation 5 (relaxed selection) had lower survival than the offspring of exposed mothers (normal selection lines) when exposed to the wasps in generation 6. Maternal effects include differential allocation of resources to offspring to enhance their survival under parasitism. If maternal effects act in combination with more direct physiological mechanisms of resistance (such as immune responses in the nymphs) or behavioural avoidance of parasitism, they may have consequences for the rate of resistance evolution. If traits for optimal maternal allocation to offspring under parasitism become genetically linked to traits for resistance, resistance could evolve much more rapidly. If, on the other hand, allocation is a generally plastic response it may allow genetically non-resistant individuals to survive better under parasitism, slowing down resistance evolution. Extending the individual-based model I developed

in chapter 3 to include these maternal effects, and a range of inheritance scenarios would improve the predictive accuracy of the models and test the extent to which maternal effects are likely to alter resistance evolution in *T. vaporariorum* in the field.

4.5. Conclusion

This laboratory study aimed to determine the potential for rapid evolution of resistance in glasshouse whitefly (*Trialeurodes vaporariorum*) when exposed to the parasitoid wasp *Encarsia formosa* over a short time frame, similar to a typical protected edible growing season. The results revealed a significant increase in whitefly survival rates over the six generations, indicating that the whitefly population rapidly evolved in response to the pressure exerted by *Encarsia formosa*. This supports the findings in the simulation model developed in chapter three. The presence of some maternal effects playing a role in survival suggests that this resistance is due to physiological changes passed down through generations, alongside potential phenotypic changes inherited from the mother improving the defence against parasitism, however conducting quantitative genetic studies focusing on the presence of maternal effects in the future would confirm how much of a role they play as well as shedding light on the mechanism and mode of inheritance.

Notably, 10% of whitefly nymphs in the third generation survived after a positive oviposition event by the parasitoid, suggesting the development of a generalised resistance mechanism. However, since the specific resistance mechanisms were not investigated, this assumption could not be confirmed. These findings highlight the potential for biocontrol agents like *Encarsia formosa* to lose efficacy over time due to the rapid evolution of resistance in target pest populations under consistent high selection pressures.

The study emphasises the importance of carefully managing and monitoring biocontrol strategies to maintain their long-term effectiveness. This is crucial for sustainable agriculture and pest management practices, where reliance on biocontrol agents is an environmentally friendly alternative to chemical pesticides. The results suggest that without such management, the effectiveness of biocontrol agents could diminish, leading to challenges in controlling pest populations in the future.

Chapter 5 Whitefly life history costs of parasitism

Abstract

The evolution of resistance is well-documented among insect pests subjected to strong selection pressures from chemical pesticides. Integrated Pest Management (IPM) strategies, aim to reduce the risk of resistance evolution in pests. However, biological control agents, such as parasitoids and pathogens, can also impose significant selective pressures that can drive resistance evolution. While resistance can enhance host survival in the face of parasite pressure, it can often incur associated life history costs. These costs can reflect biological trade-offs and may reduce fitness in the absence of the biocontrol agent. Understanding the link between resistance evolution and associated life history costs can help predict the dynamics of host-parasite interactions and for biocontrol management. This study investigates the potential life history costs associated with resistance to the parasitoid *Encarsia formosa* in the glasshouse whitefly, *Trialeurodes vaporariorum*. Controlled laboratory experiments demonstrated that surviving parasitism attempts comes at a significant cost. Whiteflies that survived parasitism—evidenced by melanisation marks—exhibited significantly reduced fecundity and lower rates of egg-to-nymph development compared to unexposed individuals. These life history trade-offs suggest that while resistance to parasitism may evolve, it carries fitness costs that can constrain pest population growth. Trade-offs such as these are likely to enhance the long-term efficacy of biological control and could delay or mitigate resistance evolution in pest populations. Understanding these trade-offs is crucial for developing integrated pest management (IPM) approaches that anticipate and mitigate resistance evolution.

5.1 Introduction

Life history refers to how an organism allocates its finite resources across different life stages to maximise reproductive success. Key traits such as fecundity, development time, and longevity are shaped by natural selection and vary within and between species depending on environmental pressures. The variation in life history traits is a result of evolutionary pressures that influence how resources are partitioned to ensure the greatest reproductive advantage. The evolution of life history traits by natural selection relies on the presence of genetic variation for these traits, individuals with life history traits that maximise their fitness in their environment have higher evolutionary fitness, resulting in different life history strategies, the most notable examples being *r* and *K* selected life histories (fast growth, high reproductive rate, short lifespan and slow growth, low reproductive rate and long lifespan respectively; Fabian and Flatt, 2012). Because resources are finite, different life history traits must trade-off against one another. If there were no trade-offs, selection would push all traits associated with fitness to their maximum potential, constrained only by the historical and physiological limits of the organism, resulting in individuals which are both extremely long lived and highly fecund. However, life-history traits are maintained well below these limits often due to these trade-offs, the most often cited example being the trade-off between longevity and offspring production seen for *r* vs *K* selected species (Stearns, 1989).

Life history costs describe these trade-offs, examples include allocating limited resources across vital functions such as growth, reproduction, and defence. Trade-offs are often measured as negative phenotypic or genetic correlations between fitness components traits among individuals in a population. These trade-offs occur when an increase or improvement in one life history trait such as survival when infected with a disease is coupled with a decrease or expense in a secondary life history trait such as fecundity (Fabian and Flatt, 2012). Life history traits are often heritable, thus, the genetic variants influencing these traits can be passed from parents to offspring.

In general organisms face three fundamental life-history trade-offs when allocating limited resources. First is the trade-off between immediate and future reproduction—

early reproduction may boost short-term fitness but often reduces longevity and later fecundity, as seen in *Tenebrio molitor* beetles, where early reproducers experienced sharper declines in fecundity with age (Jehan et al., 2021). Second, organisms must balance offspring number and quality (Ponzi, 2017). In fleas, *Xenopsylla ramesis* (host-generalist) and *Parapulex chephrenis* (host-specialist), mothers producing fewer offspring had larger, more resilient young, demonstrating a quality-quantity trade-off, especially in generalist species (Khokhlova et al., 2014). Third, there is a trade-off between offspring production and parental care. R-selected species prioritise quantity, while K-selected species invest more in parental care, producing fewer offspring with increased chances of survival and reproduction can increase the chances they survive (Gilbert *et al.*, 2010). For instance, in *Elasmucha* shield bugs, maternal care enhances egg survival, but smaller females lay smaller clutches to match their defensive capacity (Kaitala and Mappes, 1997). Similarly, *Nicrophorus vespilloides* females with smaller broods retained more resources for future reproduction (Richardson et al., 2020; Gilbert et al., 2010).

Phenotypic plasticity, alongside genetic variation and physiological constraints, plays a crucial role in shaping life history traits such as reproductive timing, growth, and longevity in response to environmental factors (Caswell, 1983). In *Drosophila*, shorter developmental periods improve larval competitiveness in resource-limited environments, while longer development and lifespan enhance dispersal and reproductive opportunities—demonstrating adaptive plasticity (Sevenster et al., 1993). Resource availability also drives plasticity: *D. melanogaster* reared on suboptimal diets were found to have reduced body size, ovariole number, and fecundity, likely prioritising survival over reproduction (Klepsatel et al., 2020; Boggs, 2019; Wong et al., 2013). Predation risk further influences life history strategies. In high-predation environments, early reproduction is favoured to ensure gene transmission before premature death (Magnhagen, 1991). Moreover, the timing of predation stress can differentially affect traits: in the mite *Tyrophagus putrescentiae*, early-life predation stress delayed development and reduced fecundity but increased adult lifespan, while late-stage stress had minimal impact (Wei et al., 2023). This suggests predation pressure can lead to multiple trade-offs between survival and reproduction success.

Physiological and immune defences which protect host organisms from parasitism and pathogen attack are costly to invest in and can have a strong influence on the life-history traits individuals express. For instance, an organism investing resources heavily in reproduction might face a reduced cellular immune function, making themselves more susceptible to diseases (Schwenke *et al.*, 2016). This might occur if an organism allocates resources to immune defence before reproduction, potentially limiting life history traits such as fecundity and offspring development but allowing the organism to survive the pathogen attack (Breiner *et al.*, 2022). An example of this comes from the red palm weevil, *Rhynchophorus ferrugineus*; in this species increases in immune defence in response to pathogens were negatively correlated with fecundity and offspring development. It found that life-history traits often showed temporary declines due to heightened immune activity, indicating that the fitness benefits of immune function may be physiologically traded-off against other fitness aspects, such as growth, development, mating, reproduction, and longevity (Pu *et al.*, 2021).

Conversely, an organism that mates and reproduces, may have increased immunity if the act of reproduction itself triggers an upregulation of the immune system. During reproduction, especially in species with high offspring numbers, an organism may encounter increased exposure to pathogens. As a result, natural selection would favour individuals who have greater immune defences that would ensure the survival of the parent and the survival of their offspring. This has been observed in two ant species, *Lasius niger* and *Formica selysi*, the ants resistance to the fungal pathogen *Beauveria bassiana* varied according to reproductive status in queens (Gálvez and Chapuisat, 2014). Immune defences were upregulated increasing resistance in mated queens compared to virgin queens (Gálvez and Chapuisat, 2014). This showed that immune investment in ant queens is closely tied to reproductive status. However, this increased immune investment may come at a cost to longevity or future reproductive output due to trade-offs in resource allocation

Secondary endosymbiont bacteria can enhance immune defences in some insect species such as whitefly and aphids often helping the species overcome attack (Gross *et al.*, 2021). These secondary endosymbionts are bacteria that reside within the host insect cells that can aid the defence mechanisms of their host insects (Kaltenpoth and Engl, 2014). Secondary endosymbionts can aid the stimulation and

production of antimicrobial peptides or modulating immune pathways, improving the hosts ability to combat pathogens (Wang *et al.*, 2023). They also compete with pathogens for resources within the host, reducing the pathogen abundance and detrimental effects on the host (Pickard *et al.*, 2017). These secondary endosymbionts are often passed to offspring through vertical transmission from mother to offspring and also via horizontal transmission through the environment, infected hosts or vectors such as parasitoids (Eleftherianos *et al.*, 2013). However, in the absence of infection risk, the hosts carrying these endosymbionts can have reduced fitness costs compared to uninfected individuals, suggesting a trade-off between facultative immunity and generalised fitness (Gross *et al.*, 2021). For instance, many aphids carry strains of several endosymbiont species that protect them from parasitoid wasps (Gross *et al.*, 2021).

Endosymbiont-mediated protection against parasitic wasps has been widely documented in aphids. In a study on the pea aphid (*Acyrtosiphon pisum*), Oliver *et al.* (2003) examined the effects of facultative (secondary) symbionts on susceptibility to the parasitoid *Aphidius ervi*, a key natural enemy in the field. The study showed that while symbiont-infected and uninfected aphids were equally likely to be parasitised, infected individuals were significantly less likely to support successful parasitoid development (Oliver *et al.* 2003). This was due to increased mortality of developing wasp larvae in symbiont-harboursing aphids, highlighting a potent form of resistance (Oliver *et al.* 2003). These findings suggest that symbiotic bacteria can influence host-parasitoid interactions and may persist in aphid populations through the selective advantage they confer. This symbiont was classified as *Hamiltonella defensa* in 2005 (Moran *et al.*, 2005).

In the aphid species *Acyrtosiphon malvae* infection with facultative endosymbionts provided protection against one parasitoid wasp, *Aphidius ervi*, but not another *Praon volucre*. (Donner *et al.*, 2024). This suggests that in one host/parasitoid system, the trade off in carrying symbionts offering protection is reduced, but in the second system with a different parasitoid species the fitness trade-off is increased (Donner *et al.*, 2024).

The life history consequences of endosymbiont infection have been well studied in aphid–parasitoid systems because of their significant implications for parasitoid-based

biocontrol. For instance, infection with *Hamiltonella defensa* in the pea aphid (*Acyrtosiphon pisum*) confers strong resistance against the parasitoid *Aphidius ervi*, but this protection often comes with fitness costs such as reduced fecundity and slower development (Oliver et al., 2003; Vorburger & Gouskov, 2011). Similarly, in the black bean aphid (*Aphis fabae*), *Regiella insecticola* provides protection against parasitoids but can reduce host longevity under certain conditions (Tsuchida et al., 2004). What is less clear is how resisting parasitoid attack (with endosymbionts or through other mechanisms) impacts fitness through life-history traits of surviving hosts. In this study I use the glasshouse whitefly (*Trialeurodes vaporariorum*) to test whether resistance to parasitism by the endoparasitoid wasp *Encarsia formosa* is associated with reduced fitness later in life, compared to unexposed controls. *Encarsia formosa* an endoparasitic wasp commonly used in agriculture to control whitefly populations, the wasps oviposit an egg inside a whitefly nymph, that hinders the whitefly nymphs development as the wasp larvae develops. Some whitefly can overcome parasitism to survive and successfully develop and emerge as adults. I predict that whitefly that are exposed to and resist attack by *E. formosa* will invest highly in immune defences, reducing the resources available for reproduction and leading to reduced fitness through life history trade-offs (i.e. reduced fecundity, slower offspring development and offspring emergence) compared to unexposed controls.

5.2 Methods

5. 2.1 Insects and plants for experiments

All insects and plants used in this experiment were reared following methods set out in Chapter 2.7.

5.2.2 Preparation of insect-infested plants

Thirty aubergine plants were placed in individual plant pot cages and infested with *T. vaporariorum* adults that were collected from the laboratory colony using a mechanical pooter; approximately 150 whitefly were put into each individual plant pot cage with one aubergine plant. Two controlled environment facility cabinets were set up to at a

constant 26° C, 70% rH and a 16:8 light dark cycle and 15 plants were placed in each to be used in either the control or *E. formosa* treatment. The 30 aubergine plants infested with adult *T. vaporariorum* were left for four days so that the females could lay eggs before being removed from the cages by being shaken outside. After this the plants were then left for a further 10 days before treatments were applied, by which time most nymphs had reached second instar. The 15 control cages were opened and closed and placed back in the control cabinet. The 15 *E. formosa* cages were removed from the controlled environment cabinet. For each individual plant the number of nymphs was counted and *E. formosa* parasitoid wasps were added to the individual cages in a 1: 5 wasp to whitefly ratio (double the commercial recommend standards to increase the rates of parasitism). After 48 hours *E. formosa* were removed from the treated cages using a mechanical pooter and the whitefly nymphs were checked under a microscope for positive parasitisation signs. A positive parasitisation event was classified as a melanisation mark on the nymphal case. Whitefly nymphs in the *E. formosa* treatment cages that were not parasitised were removed from the plant using a small paintbrush. A proportion of nymphs were also removed from the control cages using small paintbrush for continuity between treatment groups. Plants were placed back in the cages in the controlled environment cabinet and left for a further 10 days to eclose as adults. To collect experimental males and females that were virgins (*T. vaporariorum* do not mate until 1 hour after emergence) I monitored whitefly pupae that were due to emerge on all 30 plants from lights for 8 hours (with hourly checks). *Trialeurodes vaporariorum* adults that had emerged after the eight-hour observation window were discarded. Virgin adults were collected by aspiration as they emerged, anaesthetised by being placed in the fridge and then sexed. To set up experiments two replicates (consisting of one newly emerged adult female and one newly emerged adult male from each plant) were placed on an agar leaf disc to mate and oviposit (N=30 controls and N=30 *E. formosa* treatment; see below). Any remaining adult whitefly not being used in experiments were collected using a mechanical pooter and placed in a fresh colony cage.

5.2.3 Data collection protocol

To measure whitefly fecundity adult whitefly from control (unparasitised) and *E. formosa* (positive parasitism) were placed on an aubergine leaf discs set in agar to lay eggs. Leaf discs were 3cm circles cut from a fresh aubergine leaf and placed in a 5cm petri dish filled with a 1% agar solution. Thirty replicates (1M:1F) from the control and 30 from the *E. formosa* treatment were placed on a fresh aubergine leaf disc. Every 24 hours they were moved to a new disc; this was repeated for 4 days or until adult death. The leaf dish was labelled with the treatment, pair ID and set-up date. The number of eggs on each disc was counted and recorded and labelled on the dish. The leaf discs were then kept in the controlled environment cabinet for a further 7 days from set-up to record development from egg to 1st instar nymphs. Leaf discs were checked every 24 hours from the fourth day after oviposition for four days, classified as development day 1-4 for each leaf disc to record presence/absence of 1st instar nymphs and the number of eggs.

5.2.4 Data analysis

The data were analysed in R version 4.1.3. To assess the effects of treatments on egg laying and hatching rates, a generalised linear mixed model (GLMM) with a negative binomial distribution was used to handle overdispersion and included random effects of ID and Plant (using the glmmTMB package) (Brooks *et al.*, 2017). Post hoc tests using the lsmeans package were used to compare egg counts across different treatments and discs. Model diagnostics, including residual simulations with the DHARMA package (Hartig, 2022) were employed to validate model assumptions and ensure the robustness of my results.

Similarly, for the analysis of egg hatching to 1st instar rates, I fitted a GLMM with a binomial distribution to address the binary outcome of hatching success, modelled as a two-vector response (cbind(hatched, unhatched)). Post hoc pairwise comparisons were conducted using lsmeans to explore differences across treatment conditions and developmental days (Lenth, 2016). Visualisation of predicted values facilitated the

interpretation of interactions and main effects. Throughout, model diagnostics checked using DHARMA to ensure the fit and reliability of the models, confirming that the assumptions were adequately met.

5.3 Results

The effects of *E. formosa* parasitoid wasp (wasp) on whiteflies fecundity and developmental time from egg to 1st instar nymph was assessed.

5.3.1 Fecundity

There were 30 whitefly pairings across the control treatment and *E. formosa* treatment. In total the 30 females laid 881 eggs across 240 leaf discs over a four-day period. In the control treatment 30 females laid 559 eggs in total with a range of 0-48 eggs and a mean 19 eggs per whitefly male and female pair over the four-day laying period, with a standard deviation of 13.84. In the *E. formosa* treatment the 30 females laid 322 eggs in total with a range of 0-39 and an average of 11 eggs per whitefly male and female pair over the four-day laying period with a standard deviation of 11.18. The average number of eggs laid was significantly lower when females had been exposed to parasitism by *E. formosa* (in the *E. formosa* treatment) compared to the control treatment, (main effect of treatment: $X^2_1 = 3.85$, $p < 0.05$) (figure 23).

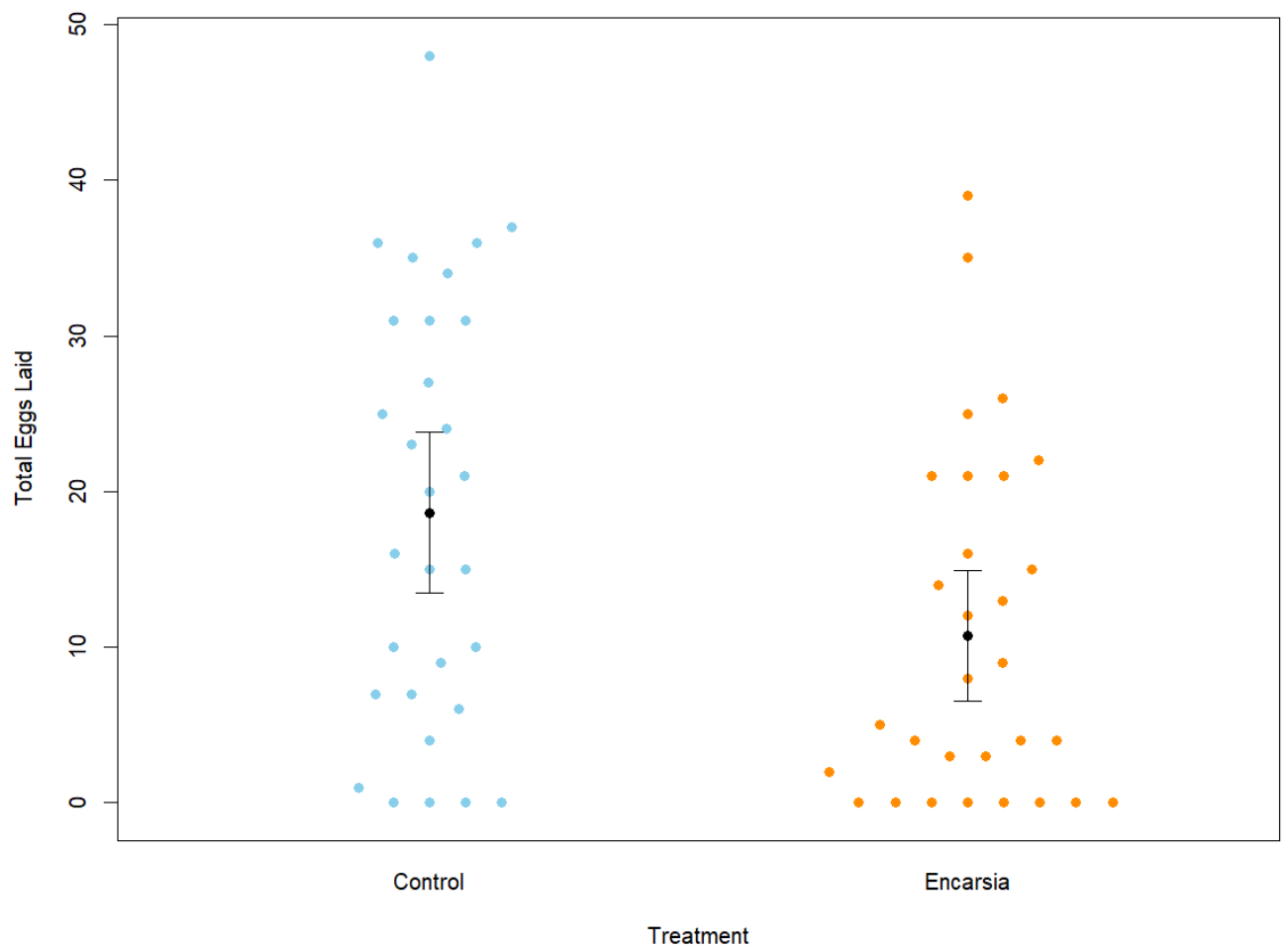


Figure 24: The total number of eggs laid in the control (blue) and the *E. formosa* (orange) treatment group. The total number of eggs laid by individual lines are plotted as points, with the mean number of eggs laid by each treatment group plotted as a black point, with binomial confidence intervals.

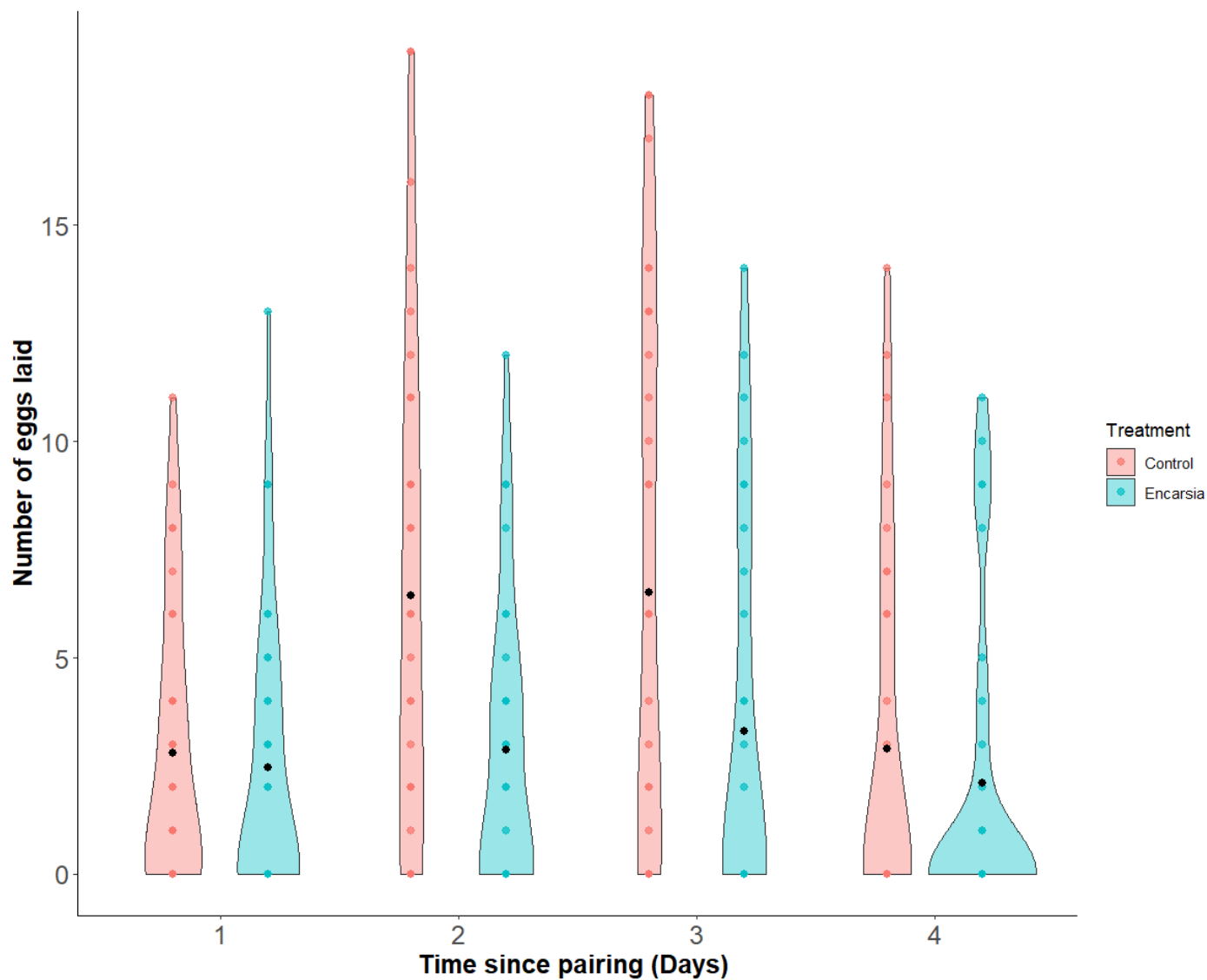


Figure 25: The average number of eggs laid over days 1-4, time since initial set up, for the control treatment in red and the *E. formosa* treatment in blue. The red points indicates the number of eggs laid by each individual, with some overlap, and the black point represents the mean value.

Fecundity varied depending on the day eggs were laid across both treatment groups (main effect of day: $X^2_3 = 15.60$, $p < 0.05$) but there was no significant interaction effect between treatment and laying day ($X^2_3 = 1.84$, $p = 0.61$) (Figure 24).

In both treatment groups fecundity followed a similar pattern where fecundity was the highest on laying day 3. Fecundity was lowest on day 1 in the control treatment and day 4 in the *E. formosa* treatment. In the control treatment pairwise, tests showed that fecundity was significantly higher on days 2 and 3 compared to day 4 ($z = 3.289$, $p < 0.01$, $SE = 0.338$; $z = 2.834$, $p < 0.05$, $SE = 0.335$ respectively; fig 24)

Fecundity in the *E. formosa* treatment varied across days but this variation was not significant between any days (all pairwise $p > 0.05$)

5.3.1 Whitefly development

Whitefly development and hatching rate from egg to 1st instar nymph was assessed, tracking development 4-7 days after the eggs were laid. These were classified as development days 1-4. The proportion of whitefly that successfully hatched from egg to 1st instar over the development days and the time taken were analysed across the control and *E. formosa* treatment groups.

The proportion of 1st instar nymphs that successfully hatched was significantly higher in the control treatment than in the *E. formosa* treatment ($X^2_2 = 61.64$, $p < 0.001$) (Figure 26).

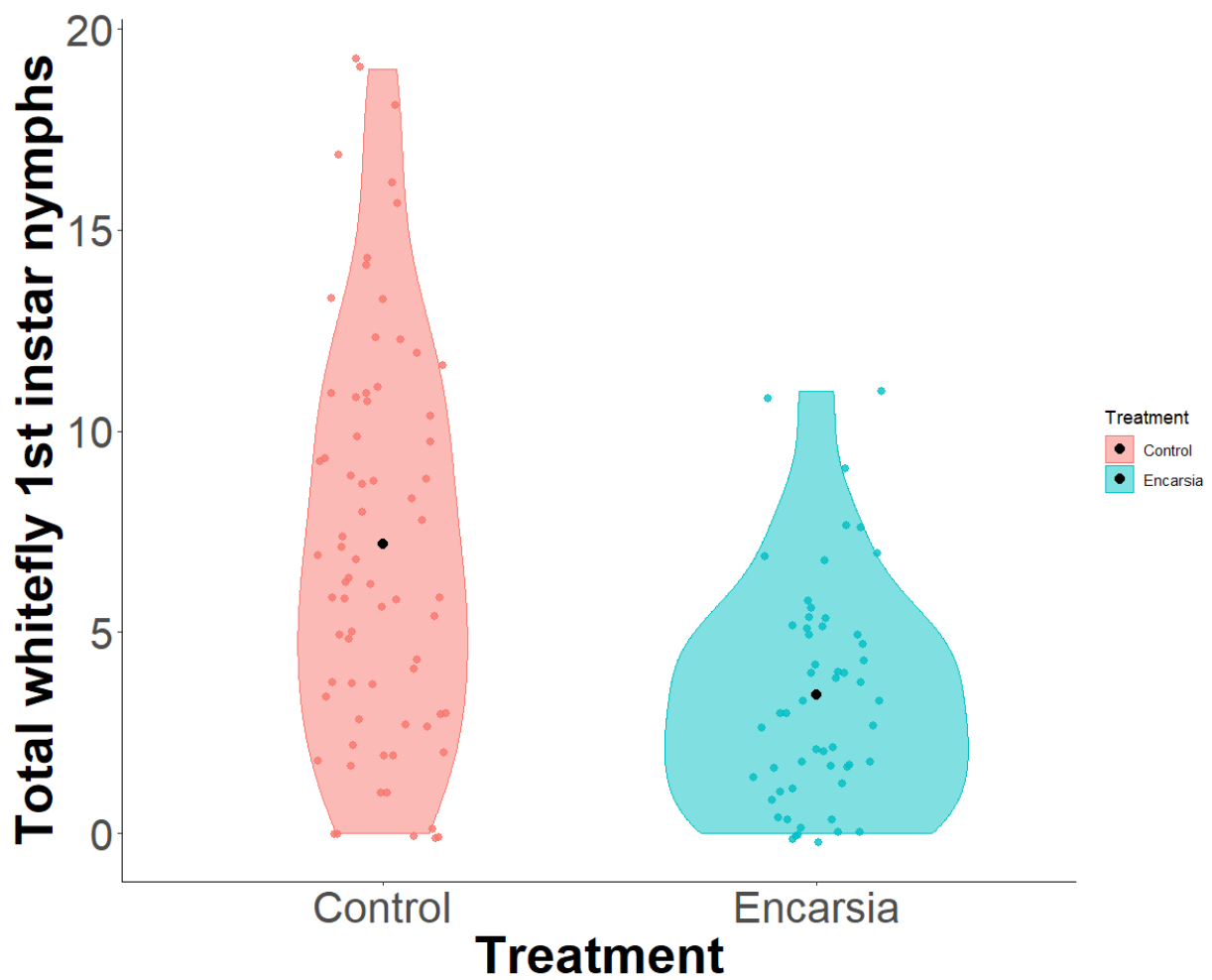


Figure 26: The whitefly 1st instar nymphs that successfully hatched from egg in the control and *E. formosa* treatment by development day 4 (7 days post-laying). The red indicates the control treatment and the blue the *E. formosa* treatment. The black point is the mean number of nymphs successfully hatched.

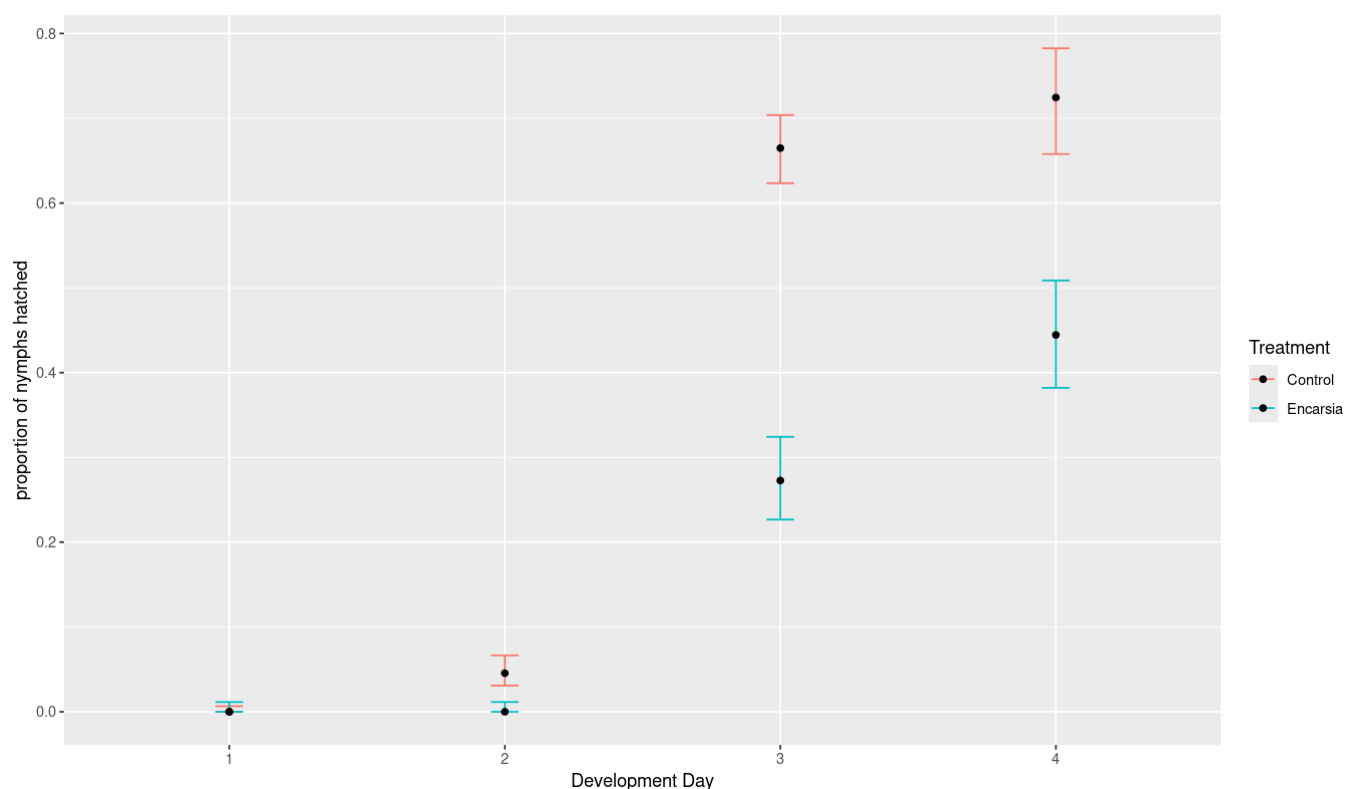


Figure 27: The proportion of 1st instar whitefly nymphs hatched in the control (red) and *E. formosa* (blue) treatment groups dependent on the development day. Error bars represent binomial confidence intervals.

The proportion of nymphs that successfully hatched varied significantly across days post-laying ($X^2_9 = 296.33$, $p < 0.001$) but the pattern was the same for both treatments (day*treatment interaction effect = $X^2_3 = 2.17$, $p = 0.53$). Hatch rate for both treatments followed the same pattern, with the proportion of nymphs hatching increasing over the four development days, peaking on day 4 post-laying (figure 26).

In both treatment groups, the day eggs were laid had a significant effect on the time taken to hatch to 1st instar nymphs (lay day*hatch day interaction effect: $X^2_9 = 35.08$, $p < 0.001$). Eggs that were laid on earlier laying days, one and two, hatched earlier than eggs laid on days 3 and 4 in both treatment groups. The majority of eggs had the highest hatch rate on development day four, except eggs from laying day 2, that had the highest hatch rate on development day 3 (figure 27).

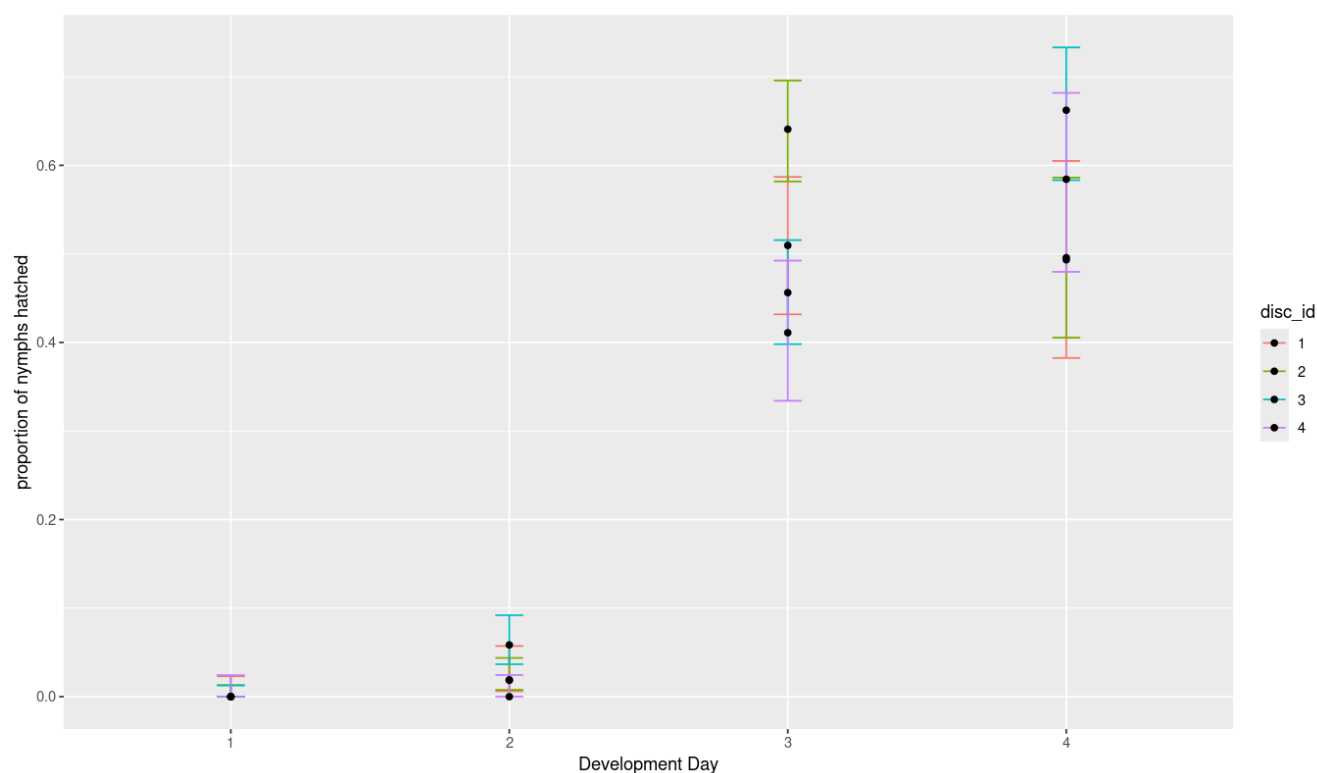


Figure 28: The proportion of 1st instar whitefly nymphs hatched from the four laying days dependent on the development day. Laying days are shown as 1 (red), 2 (blue) 3 (green) and 4 (purple). Error bars represent the binomial confidence intervals.

These developmental results indicate that treatment has an effect, the overall proportion of whitefly that developed to 1st instar nymphs is significantly reduced in the wasp treatment.

5.4 Discussion

The evolution of resistance is an important survival adaptation that has been well-documented in insect pests that face strong selection pressures from chemical pesticides. However, naturally occurring pathogens and parasitoids used as biological control agents also exert strong selection on these species, which must be considered when planning robust IPM strategies. Whilst resistance adaptations are vital for survival of pests, they often entail detrimental life history costs that can influence the future population dynamics and, as such the efficacy of biological control, and the extent to which resistance is a problem for IPM. Although resistance does readily evolve in pests, if the life history costs of resisting control (chemical or biological) have negative effects on the surviving individuals then resistance evolution may not be as detrimental to the long term effectiveness and efficiency of biological control agents as might be expected. This study assessed the potential life history costs of resistance to the parasitoid *Encarsia formosa* in glasshouse whitefly, *Trialeurodes vaporariorum* in terms of fecundity, nymphal development and development time in a lab-based experiment. My results suggested that surviving parasitism is associated with reduced fecundity and reduced eclosion to 1st instar nymphs. Female whitefly that had been exposed to *E. formosa* and showed positive signs of parasitism (melanisation mark) laid fewer eggs which were less likely to hatch into 1st instar nymphs compared to unexposed controls. These results suggest that whilst resistance to parasitism can potentially evolve in whitefly, there are detrimental life history costs, which are likely to strongly impact the whitefly population growth.

In relation to overall fecundity, whitefly in the control treatment laid 73.6% more eggs compared to whitefly in the *E. formosa* treatment. The range in eggs laid per mated female was also greater in the control treatment than the *E. formosa* treatment (0-48, 0-39 in the control and *E. formosa* treatment respectively). The whitefly in the *E. formosa* treatment were adults that had survived and successfully defended themselves against parasitism whilst in the 2nd nymphal stage. All whitefly in this treatment group had a positive oviposition event characterised by a melanisation mark on the nymphs cuticle.

The presence of a melanisation mark on the nymphs' cuticle following a positive oviposition event is not indicative of stinging without parasitism because melanisation is typically a defensive immune response that occurs in the presence of a foreign body or pathogen. However, the melanisation mark could be in response to other foreign bodies introduced on the sting rather than oviposition. However, in a controlled laboratory study, this is unlikely and is thought to be due to positive oviposition (Jacobsen, 2021). In insects, melanisation is a process where melanin is produced and deposited around an invader, such as a parasitoid egg or larva, to encapsulate and neutralise it (Jacobsen, 2021). This suggests that the whitefly in the wasp treatment used resources during their development to trigger an immune response to produce melanin to defend against parasitism and parasitoid development. This suggests there is a trade-off in immunity vs fecundity with a reduction in resources available for offspring production, resulting in lower fecundity and reduced offspring quality. This result aligns with studies assessing resistance fecundity trade-offs in the pea aphid *Acyrtosiphon pisum* when exposed to the endoparasitic wasp *Aphidius ervi*. In *A. pisum* individuals which survived parasitoid attack also produced fewer offspring compared to individuals that were not attacked by parasitoids (Gwynn *et al.*, 2005). It was also hypothesised that aphid immunity was mediated by a secondary endosymbiont, which increased parasitoid egg mortality (Gwynn *et al.*, 2005). It is possible that a similar mechanism may be responsible for my results; whitefly are also known to host secondary endosymbionts, including some that could offer protection against parasitoid attack (Shan and Lieu, 2021), they could also play a vital role in the trade-off in parasitoid defence and fecundity. However, further genetic studies are needed to determine the presence and identity of secondary endosymbionts in this species and test whether their presence is associated with resistance, and trade-offs between resistance and reproduction. For instance, through antibiotic curing and reinfection studies, where insect hosts are treated with antibiotics and reinfected with secondary endosymbionts, these populations are then studied for resistance traits and associated trade-offs. This has been seen in studies investigating endosymbiont-conferred resistance to parasitoids in aphids and associated costs particularly in the absence of parasitoids. In black bean aphids (*Aphis fabae*), introducing 11 isolates of the protective symbiont *Hamiltonella defensa* into previously uninfected aphid clones revealed costs in the form of reduced lifespan and lifetime reproduction. Curing aphids of their *H. defensa* using antibiotics results in increased fitness (when not exposed to

parasitoids), which is comparable to the fitness lost when aphid clones are reinfected with *H. defensa* (Kaech *et al.*, 2022). This suggests that the presence of endosymbionts that offer protection against parasitoids can have associated life history costs when the host is infected not in the presence of a parasitoid. This can be observed through the use of antibiotic curing and reinfection studies.

The whitefly in this study were a maximum 8 hours old before being paired and given four days to lay eggs. The association between treatment and egg laying was assessed by comparing the number of eggs laid per day within and between treatment groups. I found that in both the treatment and control egg laying peaked on day 3 ; previous studies on whitefly life history demonstrated that egg laying peaks between four and seven days after emergence with a skewed right pattern (Wang and Tsai, 1996). In my study egg laying peaked earlier, on day three. As a similar pattern is observed in both treatment groups this suggests that parasitism is not having a strong effect on the rate of egg laying per day. This could potentially be due to different environmental conditions such as lower humidity on agar leaf discs compared to previous fecundity studies that assessed fecundity rate on whole plants.

Nymphal development from egg to 1st instar was also assessed, leaf discs were left for four days from the date laid in the controlled environment cabinet. Nymphal development was assessed for four days, from day five after laying until day eight after laying, classified as day one-four development days. Overall, the control treatment had a greater proportion of nymphs developed with 92.83% of eggs developing into 1st instar nymphs, with an average of 98.74% per replicate by development day four, compared to the *E. formosa* treatment with 59.06% of nymphs successfully developing, an average of 56.03% per replicate over the development window. This suggests that there is a trade-off between survival immune response and the quality of offspring produced having detrimental effects on their development. A similar pattern of trade-offs, reduced emergence and fecundity was observed in insecticide-pyrethroid resistant mosquitos *Anopheles coluzzii*, where resistant mosquitos laid significantly fewer eggs than susceptible individuals, and a lower proportion of these eggs developed successfully. The study suggested that pyrethroid resistance is likely associated with a high fitness cost on *An. coluzzii* populations (Nkahe *et al.*, 2020). Other parasitoid resistance studies have also demonstrated that development time is slower for the offspring of resistant individuals, for instance in the Indian meal

moth, *Plodia interpunctella*, following parasitism by the koinobiont parasitoid, *Venturia canescens* reduced larval growth rate and developmental time as well as overall size of adults (Niogret *et al.*, 2009). In my study I found no effect on developmental time, but it is possible that such a pattern may only have become apparent if the eggs were observed for longer than the normal hatching period. Future studies could continue to observe unhatched eggs for longer periods and observe nymphal growth and final body size which might reveal that patterns seen in other studies are applicable to whitefly as well.

5.5 Conclusion

Overall, these results suggested that surviving parasitism does have detrimental life history costs to whiteflies fecundity and development, however, it could have beneficial impacts on the effectiveness of *E. formosa* as a biological control agent for controlling whitefly. The number of eggs laid and the proportion of whitefly that successfully developed into 1st instar nymphs was significantly reduced in the *E. formosa* treatment compared to the control treatment. Although these life history costs could be detrimental to whitefly populations, they present valuable insights into the interactions between and benefits of using *E. formosa* to control whitefly populations. Understanding these trade-offs and their effects on whitefly population dynamics could be beneficial to the commercial use of *E. formosa* as a biological control agent for controlling whitefly in agriculture. Although the potential for whiteflies to evolve resistance to *E. formosa* is a concern, if the costs associated with such resistance are substantial enough to significantly impact whitefly populations, they may help maintain low population levels. As a result, the evolution of resistance might not compromise the long-term effectiveness and sustainability of *E. formosa* as a biological control agent. This understanding highlights the importance of considering both the costs and benefits of parasitism in developing effective pest management strategies.

Chapter 6: Assessing the impacts of tank-mixing on biopesticide efficacy

Abstract

Biopesticides formulated from live pathogens generally have relatively high target specificity compared to synthetic products. Therefore, in situations requiring management of multiple pest species, it may be advantageous to tank-mix products so that they can be applied at the same time. Tank mixing may also be an important strategy for managing resistance evolution due to the reduced likelihood that individuals are genetically resistant to and able to mount defences against multiple biopesticides with different modes of action, compared to a single agent. However, when organisms are simultaneously exposed to multiple species or strains of pathogen, the resulting coinfection can have unpredictable effects on mortality. In this chapter, I tested how simulated tank mixing influenced biopesticide efficacy in a tomato horticulture system. I measured pest mortality for all pairwise combinations of four commercial biopesticides targeting either whitefly (*Trialeurodes vaporariorum*) or tomato leafminer (*Tuta absoluta*). I found no evidence of synergistic or antagonistic effects of different biopesticides on each other. When products targeting different pest species were mixed pest mortality remained close to the level caused by single application of the most effective agent. When products targeting the same pest species were co-applied, mortality was not enhanced; there was no evidence of either additive or synergistic effects. My results suggest that tank-mixing of these biopesticides is unlikely to compromise pest control efficacy but may not improve pest control effectiveness or help control resistance evolution. Further field trials are necessary to verify this in naturalistic horticultural contexts. Horticultural growers may be able to exploit this result to improve efficiency of biopesticide application in IPM programmes.

6.1 Introduction

The path toward a more environmentally sustainable future in global agricultural crop protection increasingly relies on the uptake of integrated pest management regimes with the adoption of biopesticides and other biorational products, alongside a reduction in reliance on chemical pesticides (Fenibo *et al.*, 2021). Microbial biopesticides, which include parasites and pathogens such as nematodes, viruses, fungi, and bacteria, offer a promising alternative to traditional chemical methods (Lacey *et al.*, 2015). One of the significant advantages of biopesticides is their target specificity; they are designed to affect a relatively narrow range of host pest species with minimal impact on non-target beneficial insects, thus preserving ecosystems and insect biodiversity.

Despite these benefits, the use of biopesticides presents many practical challenges for farmers across the globe. To effectively manage numerous pest species, multiple biopesticides may be required in multiple applications. This requirement often results in increased economic and labour costs. Farmers need to invest in multiple biopesticide formulations and apply them at different times to ensure effective pest management. These additional steps can lead to higher overall costs, labour, time and more complex pest management practices in comparison to the traditional one chemical pesticide fits-all regimes (Oguh *et al.*, 2019). Therefore, while biopesticides represent a significant advancement in sustainable agriculture by reducing reliance on chemical pesticides, the practical implications for farmers, including increased costs and labour, must be carefully considered and addressed to make their widespread adoption more feasible.

In cultivated areas with high agrochemical pesticide use, the repeated application of machinery incurs significant economic costs, demands substantial manual labour, and contributes to greenhouse gas emissions (Lal, 2004). To enhance application efficiency, many users of agrochemicals employ a practice known as tank mixing, where multiple chemical pesticides are combined in the application container with water prior to spraying (Gandini *et al.*, 2020). Tank mixing has been shown to reduce costs and improve convenience for growers, without adversely affecting crop yields (Warnock and Cloyd, 2005) and reducing the carbon footprint of crop production (Fishel, 2020). While guidelines for tank-mixing synthetic pesticides are well-

established, focusing on direct chemical interactions and combined effects on pest physiology (Fishel, 2020), similar protocols for biopesticides are not yet developed.

The compatibility of tank-mixed biopesticides could significantly influence their effectiveness in controlling pests. Although it is unlikely that pathogens from different microbial products will directly interact within a spray tank, and some of these pathogens may target different pest species, co-application means that pests are exposed to multiple pathogens simultaneously and pathogen interaction could occur within the pest. Research suggests that such simultaneous exposure can lead to unpredictable outcomes if it results in coinfection of individual pests with multiple pathogen species or strains (Mideo and Reece, 2012). While some biopesticide or pathogen combinations may enhance efficacy through synergistic effects (Hodgson *et al.*, 2004), others could potentially diminish pest control due to antagonistic effects (Li *et al.*, 2021) (figure 28). This complexity highlights the need for further investigation into the pathogen interactions and effects of tank-mixed biopesticides to optimize pest management strategies effectively.

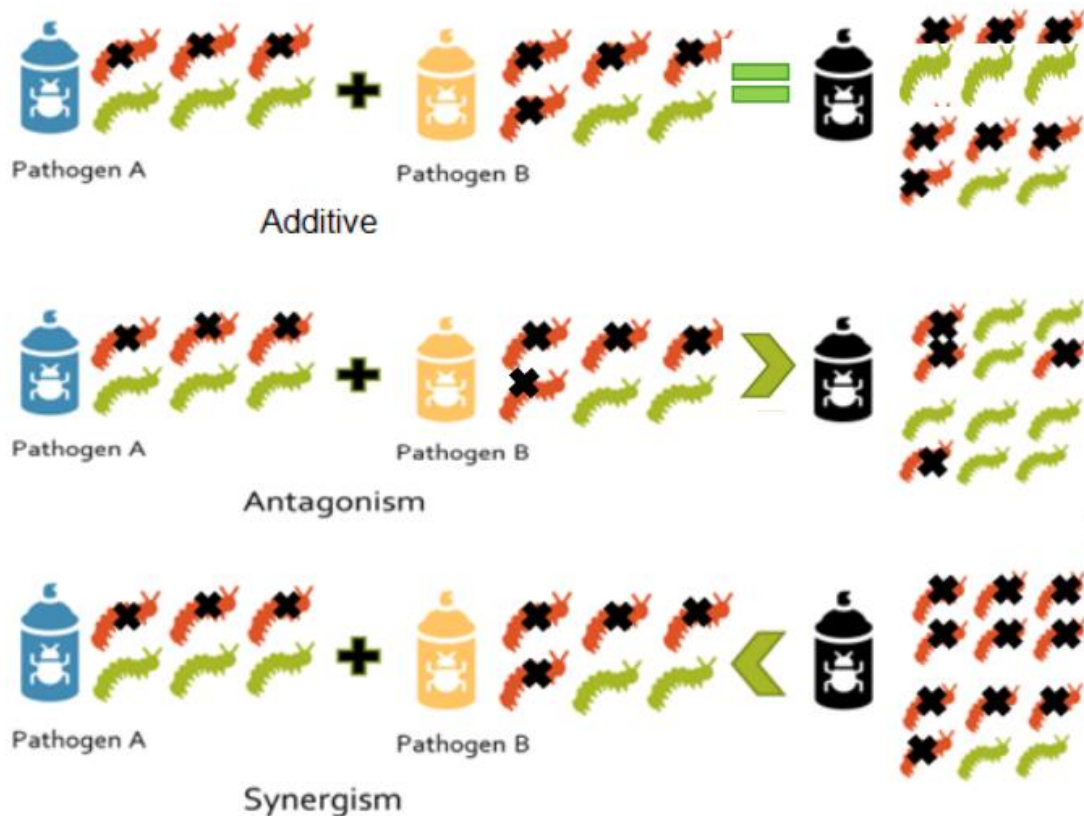


Figure 29: A schematic showing the antagonistic and synergistic effects on pest mortality, when exposed to multiple different pathogens. Dead pests are shown in red and a cross and surviving pests in green with no cross.

An example of antagonistic pathogen interactions comes from the desert locust (*Schistocerca gregaria*) coinfecting with *Metarhizium anisopliae* and *Beauveria bassiana*. Pathogen virulence and reproduction was altered in comparison to single pathogen infections. However, the outcome depended on the order of pathogen infection and environmental conditions (Thomas *et al.*, 2003). When environmental conditions were constant and *M. anisopliae* was applied as a single pathogen, high virulence was observed, whilst when *B. bassiana* was applied as a single pathogen low virulence was observed. When applied simultaneously there was no effect on locust mortality compared to single applications of either pathogen. However, when locusts were pre-inoculated with *B. bassiana* there was an antagonistic interaction

between *M. anisopliae* and *B. bassiana* pathogens resulting in decreased virulence of *M. anisopliae* (Thomas *et al.*, 2003). Antagonistic interactions may have resulted from direct interactions and competition between *M. anisopliae* and *B. bassiana* when sequentially coinfecting the individual desert locust host. Coinfection of pathogens can have implications on the virulence of the pathogens themselves. If a less virulent pathogen, in this case *B. bassiana* is competitively superior to a more virulent pathogen, in this case *M. anisopliae*, *B. bassiana* can outcompete *M. anisopliae* which may reduce the ability and virulence of the *M. anisopliae* fungi to cause harm and sporulate in the desert locust, leading to an antagonistic effect with reduced host mortality (Blaisdell *et al.*, 2015).

Synergistic coinfection effects have been observed in a related species, the migratory grasshopper, *Melanoplus sanguinipes*, when individuals were simultaneously infected by two entomopathogenic fungi, *Metarhizium brunneum* and *Paranosema locustae*. When pathogens were applied simultaneously, *M. sanguinipes* mortality was significantly higher than when they were applied alone (independently of concentration; Dakhel *et al.*, 2019). In this case, the observed synergistic coinfection effects could be due to metabolic resource allocation and depletion. When the host, *M. sanguinipes* is infected by a single fungal pathogen, either; *M. brunneum* or *P. locustae*, metabolic defence resources are allocated to immune defences, such as the production of antimicrobial peptides and the activation of cellular defence mechanisms which engulf and defend against the fungal pathogen. These immune responses are resource-intensive, requiring significant energetic investment for the host. If a second fungal pathogen is introduced simultaneously while the immune system is using cellular defence resources against the initial or more virulent fungal pathogen, the host's immune system can become overwhelmed. The simultaneous presence of the two fungal pathogens that do not compete against each other can exceed the immune capacity of the host, where resources are insufficient to defend against multiple pathogens, resulting in a less effective immune response and subsequently higher overall mortality of *M. sanguinipes* compared to infection from a single fungal pathogen (McLaren & Callahan, 2020).

As the metabolic cost of fighting infection is substantial, infection from a single pathogen depletes the host's resources. If *M. sanguinipes* faces simultaneous coinfection from *M. brunneum* and *P. locustae* the presence of two fungal pathogens enhances resource depletion. The combined demand for defence resources to defend against both fungal pathogens simultaneously can be much greater than the host's metabolic resource capacity. This resource depletion can impair the *M. sanguinipes* ability to sustain necessary immune responses and maintain vital physiological functions, leading to the increased mortality rates observed in coinfecting individuals compared to those infected with a single fungal pathogen. Therefore, the synergistic effects observed in *M. sanguinipes* could be due to the combined impacts of immune system overload and metabolic resource depletion, which together increase the rate of the infection in turn increasing *M. sanguinipes* mortality.

Both antagonistic and additive effects have been observed when lepidopteran hosts *Anticarsia gemmatilis* and *Spodoptera frugiperda* were exposed to a nucleopolyhedrovirus (NPV) and the entomopathogenic fungus *Metarhizium rileyi*. Antagonistic effects were observed in both hosts when pathogens were applied sequentially two days apart regardless of the order. This suggests that the observed antagonistic effects could be due to the viral and fungal pathogens NPV and *M. rileyi* interacting and competing, thus reducing the virulence of the pathogens when sequentially coinfecting the *A. gemmatilis* and *S. frugiperda* hosts. Antagonistic effects were only observed for sequential treatment suggesting the first pathogen applied may be at an advantage and competitively exclude or limit the infectivity of the second pathogen. This could be due to resource depletion where one pathogen does not have access to necessary resources due to competition with the second pathogen (Deschoet and Corey, 2022). However, an additive effect was observed when both pathogens were applied simultaneously. This additive effect was observed in both lepidopteran host species regardless of pathogen concentration applied (Souza *et al.*, 2019). This could be due to differing modes of action from each pathogen independently attacking the host's immune system. The viral pathogen triggers antiviral responses, such as the production of interferons and other cytokines, while the fungal pathogen activates antifungal defences, including the production of antimicrobial peptides and cellular immune responses (McLaren & Callahan, 2020). These immune activations occurring at the same time can strain the host's immune

resources, but due to differing mode of actions, attack from the individual pathogens may not necessarily deplete resources to the point of synergistic failure but result in an additive effect where the overall disease severity and mortality are the sum of the effects caused by each pathogen individually.

The examples outlined above show that the nature of interactions between pathogens can depend on simultaneous or sequential application, suggesting that there can be both beneficial and detrimental effects of combining pathogens for pest control regimes. Antagonistic effects were only observed for sequential, not simultaneous treatment suggesting the first pathogen applied may be at an advantage and competitively exclude or limit the infectivity of the second pathogen. In the case of these pathogens, NPV and *M. rileyi* tank mixing would be beneficial, as it would result in simultaneous applications and additive effects on pest mortality, benefitting growers in terms of both labour and pest control efficacy. However, more studies are needed to provide clear information on what biopesticide products can be mixed for beneficial or at least neutral, interactions.

Tank mixing biopesticides, or combining multiple biopesticides into a single application, presents a promising strategy for managing pest populations and mitigating the evolution of resistance. This approach involves the simultaneous application of different pathogens or biocontrol agents to target insect pests, which can be more effective than using a single pathogen. When pests are exposed to multiple pathogens concurrently, the likelihood of any one pathogen becoming dominant and selecting for resistance is reduced. This diversity in pathogen exposure can help limit the evolutionary pressure that typically drives resistance; using a combination of biopesticides may lead to a more robust and resilient pest control system (Hawkins *et al.*, 2018). Additionally, the simultaneous application of pathogens could lead to synergistic effects and increase the overall population mortality by imposing additional fitness costs on the host through a multi-pronged pathogen attack, particularly when pathogens with differing modes of action are applied in a single application (Abdullah *et al.*, 2017). For instance, if one pathogen targets a specific physiological process of the host pest, it would require high levels of host resources to overcome, reducing the hosts fitness, attack from a secondary pathogen with a different mode of action could then target a secondary process, that the host does not

have enough resources for, making it more challenging for pests to evolve resistance to pathogens that are applied simultaneously (McLaren & Callahan, 2020). Therefore, tank mixing and the co-application of pathogens to host pests could limit the establishment of resistant strains by ensuring that pests are subjected to multiple, potentially synergistic control pressures at once (McLaren & Callahan, 2020). This strategy can limit the chances of pests surviving and reproducing if they encounter several biopesticides with distinct modes of action.

In this laboratory study I investigated how simulated tank-mixing and co-application of pathogens affects the efficacy of four commonly used commercial biopesticides which target these two common global pests of glasshouse horticulture: *T. vaporariorum* and *T. absoluta* ; see 2.1.1- 2.1.4 for a detailed overview of *T. vaporariorum* biology and ecology, 2.1.6 for detailed overview of *T. absoluta* biology and ecology).

I selected two fungal biopesticides that target *T. vaporariorum*, Botanigard and Mycotal (containing *Beauveria bassiana* and *Lecanicillium muscarium* fungal spores respectively). For both pathogens, the route of infection is direct by spraying nymphs with spores, which adhere to the insects cuticle, germinate, penetrate the cuticle, and then grow inside the insect body causing death. In contrast, for *T. absoluta* I chose biopesticides which infect the host through consumption of contaminated foliage as *T. absoluta* damage crops via creating mines in foliage during feeding. I selected the bacterial biopesticide Dipel containing a mixture of live *Bacillus thuringiensis* spores and four different B.t. toxins, and a viral biopesticide, Tutavir, containing *Phthorimaea operculella granulovirus*. These pests were chosen because they both are a significant issue in protected edible horticulture, particularly on tomato crops thus, growers are likely to need to control both pests simultaneously, making tank mixing an attractive option. For this study, I ask whether biopesticide tank-mixing impairs efficacy, when treatments are combined and applied in simultaneous applications than when the more effective treatment of the combination is applied alone.

6.2 Methods

6.2.1 Insects and plants for experiments

All insects and plants used in this experiment were reared following methods set out in Chapter 2.7.

6.2.2 Preparation of insect-infested plants

6.2.2.1 *Trialeurodes vaporariorum*

Experiments on the *T. vaporariorum* model system took place on black beauty organic aubergine plants grown from seed. Individual pest free aubergine plants were double caged in individual plant pot cages, with plant pots placed inside of three larger bugdorm cages, to reduce unwanted pests entering the experiment in the controlled environment plant growth cabinet. *T. vaporariorum* adults were collected from a laboratory colony established in 2021 using a mechanical or electric pooter; approximately 75-100 adults were placed into each individual plant cage with one aubergine plant. Adult *T. vaporariorum* were left in the cages for four days to mate and lay eggs before being removed from the cages (plants were removed from the cages and shaken before being put in a new cage). Plants were then placed back inside cages in the controlled environment facility and left for a further 10 days for development before treatments were applied, by which time most of the whitefly nymphs had reached second instar nymphal stage. This life stage was chosen to fit the commercial suppliers recommended guidelines.

T. vaporariorum experiments were conducted over five treatment blocks, where each experimental block was conducted from *T. vaporariorum* egg laying to mortality, with survival counts conducted before a new block began, approximately 24 days per block. The last three blocks of this *T. vaporariorum* experiment ran synchronously with the three *Tuta absoluta* experimental blocks. During this experiment 90 aubergine plants were infested, with a total of 18,329 whitefly nymphs exposed to the 11 treatment groups (see 6.2.3). Ten-day old second instar *T. vaporariorum* nymphs were exposed to biopesticide treatments whilst on the aubergine plants. See 5.2.3 for biopesticide application procedure. One entire plant was assigned to a treatment group to avoid

cross exposure. Once plants were exposed to a treatment, they were left for 20 minutes to dry off before plant pot cages were replaced. Cages were then placed in the environment facility and left for seven days before mortality counts were conducted. Mortality counts were conducted by removing all leaves from an individual plant, nymphs were examined under a microscope and record as dead, alive, emerged. At this time pathogen death could not be clearly identified as cadavers had not sporulated or shown clear symptoms of pathogen infection.

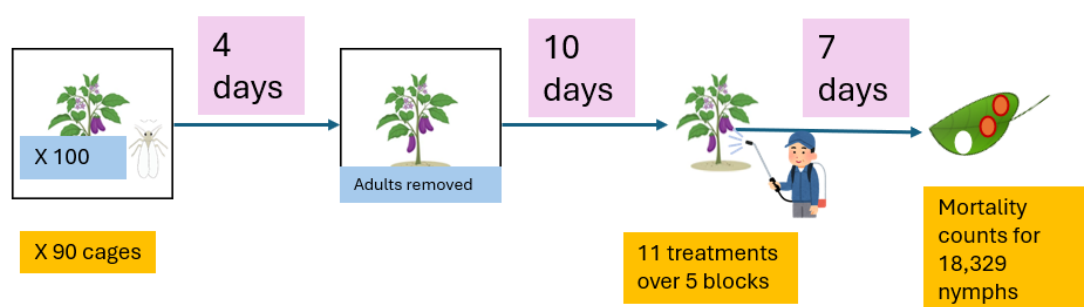


Figure 30: Schematic showing experimental procedure for *Trialeurodes vaporariorum* experiments from initial set up until mortality counts.

6.2.2.2 *Tuta absoluta*

To infest plants with juvenile *T. absoluta* larvae, pupae were supplied by Andermatt Biocontrol AG. Upon arrival pupae were housed in a climate-controlled room at 24°C 70 % rH in a mesh cage until emergence as adults. Once adults emerged, they were kept in the mesh tubs, with a maximum of two adults per tub for up to 24 hours whilst being fed 40% sucrose solution on cotton wool before being transferred onto organic tomato plants used in the experiment. 25-30 adult *T. absoluta* were introduced into mesh cages containing nine tomato plants. Adults were given three days from introduction to mate and lay eggs before the adults were removed, via trapping or a mechanical pooter and placed in ethanol. Tomato plants containing eggs were left for four further days for first instar larvae to develop. Four-day old first instar *T. absoluta*

larvae were exposed to biopesticide treatments whilst on plants, before or when they began to create small mines. This life stage was chosen to fit the commercial suppliers recommended guidelines of biopesticide use. See 6.2.3 for detailed biopesticide treatment procedure.

Once treated, plants were immediately placed into individual plant pot cages to avoid larvae escaping. Plant pot cages were then returned to the large mesh cages in the controlled environment facility. Two cages were used to reduce the risk of escapees. A piece of damp filter paper was then placed over the top of the plant pot, to catch any larvae cadavers or pupae that may fall off the plant once exposed to biopesticide treatments. This was repeated over three experimental blocks, where experiments were conducted from egg laying to mortality count before a new block began. In total 51 plants and 2000 *T. absoluta* larvae were exposed to biopesticide treatment groups. One entire plant was assigned to a treatment group to avoid cross exposure. Seven days after treatment exposure *T. absoluta* mortality counts were conducted. Individual plant pot cages were removed from the controlled environment chamber individually. Before plants were removed from the individual cages, the plant pot cage, filter paper, the topsoil and the entire plant stem was examined for mines, larvae, cadavers and pupae. Once larvae, cadavers and pupae had been collected, the leaves, fruit and flowers if present were removed from the plant and placed on a large piece of damp filter paper. Each tomato plant leaf, flower and fruit if present, were examined for larvae, cadavers and pupae. If mines were present, they were opened using a needle to expose and remove the larvae or cadavers if present. The number of larvae and pupae that were alive and dead was recorded. At this time, the cause of death could not be clearly identified as cadavers had not sporulated or shown clear symptoms of pathogen infection. All plant material and insects from both experiments were disposed of by freezing and autoclaving the waste material.

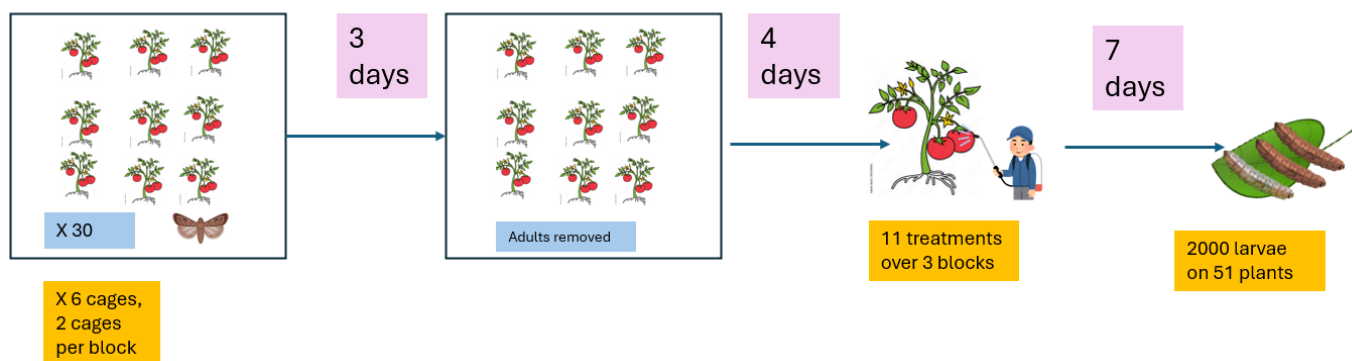


Figure 20: A schematic showing the experimental procedure for *Tuta absoluta* experiments from set up until mortality counts.

6.2.3 Biopesticide application

Four biopesticides were used: Botanigard® WP, Mycotal®, Dipel® DF, Tutavir®. These biopesticides were applied as both single and paired treatments in all combinations, resulting in a total of 11 treatments (Distilled water control, 4 single applications, and 6 mixed applications).

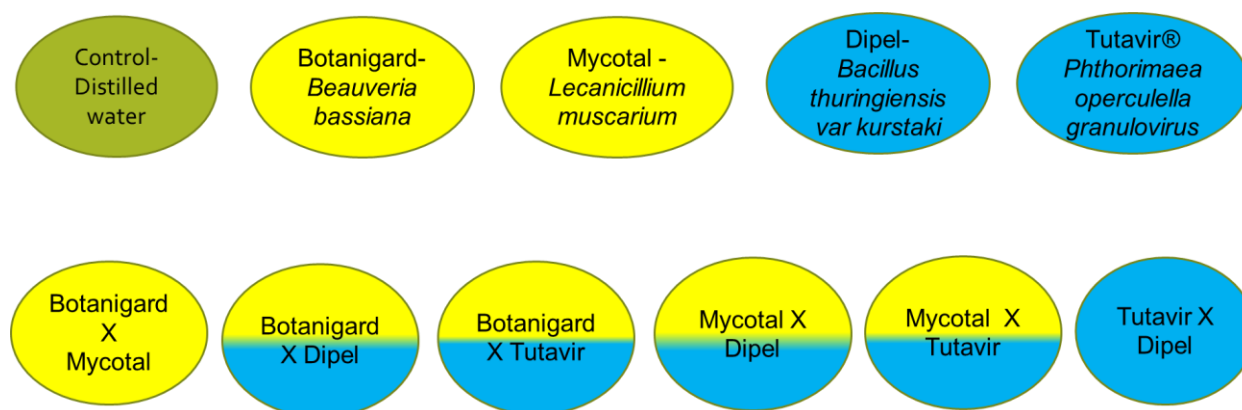


Figure 32: The 11 treatments applied, those coloured green are control treatment. The circles coloured yellow are single treatments commercially produced as whitefly, *T. vaporariorum*, biocontrol agents. The circles coloured blue are single treatments commercially produced as tomato leafminer, *T. absoluta*, biocontrol agents. The circles

coloured half blue and half yellow are the combined treatments that combine pathogens commercially produced to target both pests.

Biopesticides were prepared up to one hour in advance of use, mixed in distilled water at the manufacturers' recommended concentrations; (Botanigard: 0.625g wettable powder/litre; Mycotal wettable powder: 1g/litre; Dipel granules: 2.5g/litre; Tutavir liquid 1ml/litre). For combined treatments, both products were combined at their individual recommended concentrations as above, . For treatment exposure, individual plants were placed in a fume-hood. The treatment formulations were sprayed onto the underside of plants using a Sparmax DH-125 airbrush sprayer attached to a compressor at 50psi. All plants exposed to the same treatment were treated before a new treatment group was applied to avoid cross contamination. The airbrush was cleaned before and after each treatment, by spraying through first with ethanol, then distilled water; and then flushing the new biopesticide formulation through once before spraying commenced. Leaves were sprayed individually to runoff, following manufactures' guidelines. Once sprayed, the plants were placed in individual plant cages to avoid insect contamination, then cages were placed back into controlled environment chambers. Clear control treatments were included for both host species to establish baseline mortality under no-treatment conditions, ensuring that any observed effects could be attributed to the biopesticides rather than environmental variability or handling stress. Control plants were treated with a water-only spray to replicate the physical act of spraying without the presence of any active ingredient. The inclusion of both single and combined biopesticide treatments allowed comparisons between individual agent efficacy and any additive, synergistic, or antagonistic effects when mixed. This design enabled us to determine whether tank-mixing offers any advantage (or risk) relative to using biopesticides alone. Experimental blocks were implemented primarily for logistical reasons, to make experiments a manageable size, as the number of treatments and replicates exceeded what could be managed in a single session and the control facility lacked capacity. The control treatment of water was absent in some blocks due to experiment capacity constraints and the single treatment controls of each biopesticide were of higher value in the experiment blocks than the true water control treatment.

. Treatments were therefore applied and scored in sequential blocks over time, with each block including a Botanigard treatment to assist statistical detection of block effects and in each block, there was a combination of Botanigard with another treatment. The number of treatments per block varied and was dependent on the availability of infested plants. There was a minimum of two infested plants per treatment per block to allow for comparison between plants within treatment and block. As there were limitations on the availability of infested plants, the single treatment was to be present in the same block if it was also present in the combination treatment group. For example, If the Botanigard X Dipel group was present in the block both the single Botanigard and Mycotol treatment groups were also present in the block This approach allowed consistent application and monitoring, while maintaining sufficient replication to allow for statistical comparison between treatments. For *T. vaporariorum*, the trials were set up over five experimental blocks, whereas for *T. absoluta* there were three blocks. The minimum number of treatments per block was four, for statistical comparisons.

Table 6: The treatment blocks for *T. vaporariorum* exposure to either single treatments or combinations. Where the box is coloured green, the treatment was present in that block.

Treatment Block	Control	Botanigard	Dipel	Mycotal	Tutavir	Botanigard x Dipel	Botanigard X Mycotol	Botanigard X Tutavir	Dipel X Mycotol	Dipel X Tutavir	Mycotal X Tutavir
1											
2											
3											
4											
5											

Table 7: The treatment blocks for *T. absoluta* exposure to either single treatments or combinations. Where the box is coloured green, the treatment was present in that block.

Treatment Block	Control	Botanigard	Dipel	Mycotal	Tutavir	Botanigard x Dipel	Botanigard X Mycotal	Botanigard X Tutavir	Dipel X Mycotal	Dipel X Tutavir	Mycotal X Tutavir
1											
2											
3											

6.2.4 Data collection and analysis

Pest mortality and development were recorded by systematically searching plants for live and dead insects when each species was expected to reach the adult stage (*T. vaporariorum*) or pupate (*T. absoluta*), this was seven days after biopesticide application for both species. Data were analysed in R version 4.1.3 (R Core Team, 2022). Mortality variation between treatments was assessed using a generalised linear mixed model (GLMM) with a quasibinomial error distribution (to account for overdispersion) and included random effects of block using the glmer function in the lme4 package (Bates *et al.*, 2015) Plant was the replicate in all analyses (N = 51 tomato ; N = 90 aubergine). Initial models contained two categorical fixed factors: treatment and block, alongside their interaction. Proportional mortality was the response variable and was included as a 2-vector response using the cbind function (number dead, number alive). . Model diagnostics, including residual simulations with the DHARMA package, (Hartig ,2022). were employed to validate model assumptions and ensure the robustness of my results. Significance of model terms was determined on deletion using Likelihood ratio tests. Differences between treatment categories were determined from the significance of individual parameter estimates. Empirical mortality rates in the combined treatments were compared to the expected combined

mortality rates based on an additive model calculation involving random mortality using the calculation $AB = A+B*(1-A)$ where Pathogens A and B both kill the population at random (overlapping subsets of the population die) (McVay *et al.*, 1977). On figures 34 and 35 The red line indicates mortality caused by the most effective biopesticide in the pair, whereas the blue line indicates the mortality rate that would be predicted if the two biopesticides had an additive effect in combination.

6.3. Results

6.3.1 Effects of biopesticide tank-mixing on *T. vaporariorum* control

For *T. vaporariorum* I assessed survival of 18,329 nymphs on 90 independent aubergine plants. Mean number of plants per treatment was 8.2 (range 5 – 9); mean number of nymphs per plant was 203.7 ($\pm$ 12.4 SE). Average nymph mortality in the control treatment was low (5.4%; 95% CI 1.6%—16.5%) and neither Dipel nor Tutavir (which target *T. absoluta*) were associated with significantly elevated mortality risk as single treatments compared to the control treatment (14.9%, $t = 1.60$, $P = 0.12$, and 7.8% $t = 0.15$, $p=0.88$, respectively Fig 34F). Treatment had a significant effect on mortality ($X^2_{10} = 2448.2$, $P < 0.001$) The experiment was conducted over five experimental blocks; mortality differed considerably between blocks ($X^2_4 = 106.30$, $P < 0.001$) but treatments effects were consistent across blocks (block x treatment interaction: $X^2_{23} = 55.27$, $p=0.05$).

Mortality was high (51.1%) across all treatments containing biopesticides that target *T. vaporariorum* (N = 58 plants; Botanigard and Mycotal single treatments, or their combinations with other products). Mortality was very similar when Botanigard (59.3%; 95% CI 46.9—70.6%) or Mycotal (59.1%; 95% CI 45.7—71.3%; Fig 34B) were applied singly. Mortality rates of *T. vaporariorum* were unaffected when Botanigard and Mycotal were combined with Dipel or Tutavir (which target *T. absoluta*; Fig 34A, C, D, E). When Botanigard and Mycotal were applied in combination, mortality elevation was only slight compared to single application and not significant when compared to the control treatment, as the additional mortality did not exceed the confidence limits of the expected control mortality (+4.5%; $t = 0.68$, $p=0.50$; Fig 34B).

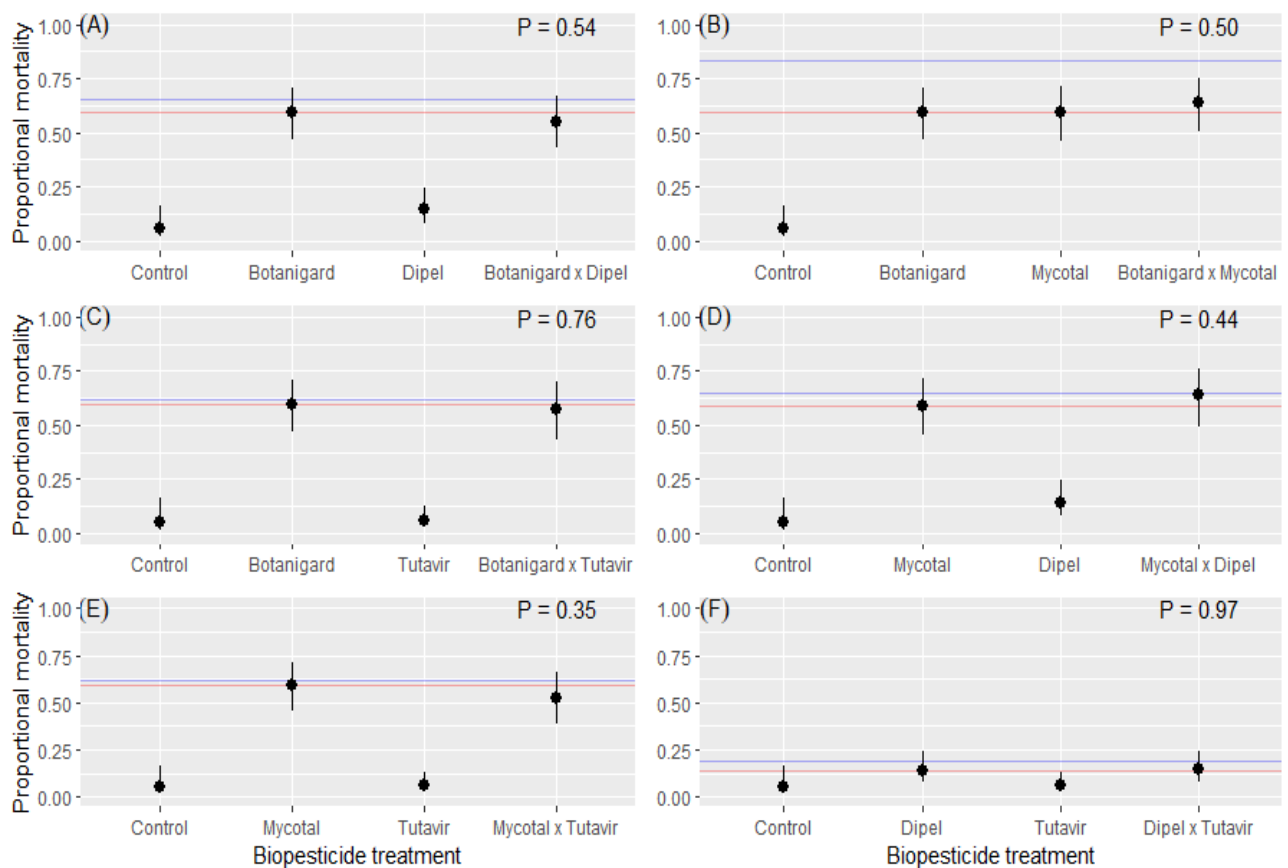


Figure 33. Mortality of *T. vaporariorum* nymphs on aubergine plants 10 days after experimental application of different biopesticide treatments. Each sub-plot (A-F) shows mortality associated with single application of two different biopesticides, alongside the combination treatment. Each treatment is replicated throughout the sub-plots to allow for several pair-wise comparisons. The red line indicates mortality caused by the most effective biopesticide in the pair, whereas the blue line indicates the mortality rate that would be predicted if the two biopesticides had an additive effect in combination (see 6.2.4 for calculation). The P values are for the comparison between mortality in the combined treatment with the more virulent of the two single treatments are shown on each plot: in no cases was mortality significantly affected by biopesticide mixing. Points show model predictions of mean mortality in each treatment group with 95% confidence intervals. Confidence intervals were calculated adding and subtracting the 95% standard error values to the models fitted values on the logit scale, before transforming these bounds back to the original scale.

6.3.2 Effects of biopesticide tank-mixing on *T. absoluta* control

I assessed survival of 2000 *T. absoluta* larvae on 51 separate tomato plants (mean larvae per plant 39; range 14-70; 82% of plants had 30 or more larvae per plant). Across 11 experimental treatments the average number of plants per treatment was five. Mortality in the control treatment was only 2.93 % (95% CI -0.315%—6.18%). The experiment was conducted over three replicate blocks; mean mortality did not differ between the blocks ($X^2_2 = 3.91$, $P = 0.14$).

Mean mortality across all treatments containing products that principally target *T. absoluta* (Dipel and Tutavir singly or in combination mixes) was 86.44% (95% CI 82.63-90.24%, $n = 32$ plants). Treatment had a significant effect on mortality ($X^2_{10} = 492.07$, $P < 0.001$). There was a significant interaction between treatment*Block $X^2_{11} = 54.40$, $P < 0.001$).

In single application, Tutavir caused the highest *T. absoluta* mortality (92%; 95% CI 87.9—96.1%), followed by Dipel (76.2%; 95% CI 60.59—91.81%). Of the two products not intended for *T. absoluta* control only Botanigard was associated with significantly elevated mortality (24.12%; 95% CI 15.92—32.32%) (Figure 35)

Treatment with Mycotal on the other hand was not associated with significantly elevated mortality compared to the control; 12.15%; 95% CI 7.85—16.45; $t = 1.14$, $p = 0.16$). Application of mixed combinations of these biopesticides had little impact on *T. absoluta* mortality. For Tutavir and Dipel, mixing with Botanigard or Mycotal were not associated with significant changes in virulence (all P values > 0.35 ; Figs 35 A, B, D & E). Despite Tutavir and Dipel both causing high mortality when applied singly, mixing these products had little effect on the mortality rate relative to Tutavir single application (-0.1%; $t = 0.02$, $p = 0.99$; Figure 35 C). Whilst Botanigard caused 24.12% mortality singly, this reduced to 22% when combined with Mycotal, although this change was not significant ($t = 1.82$, $p = 0.08$; Figure 35 F).

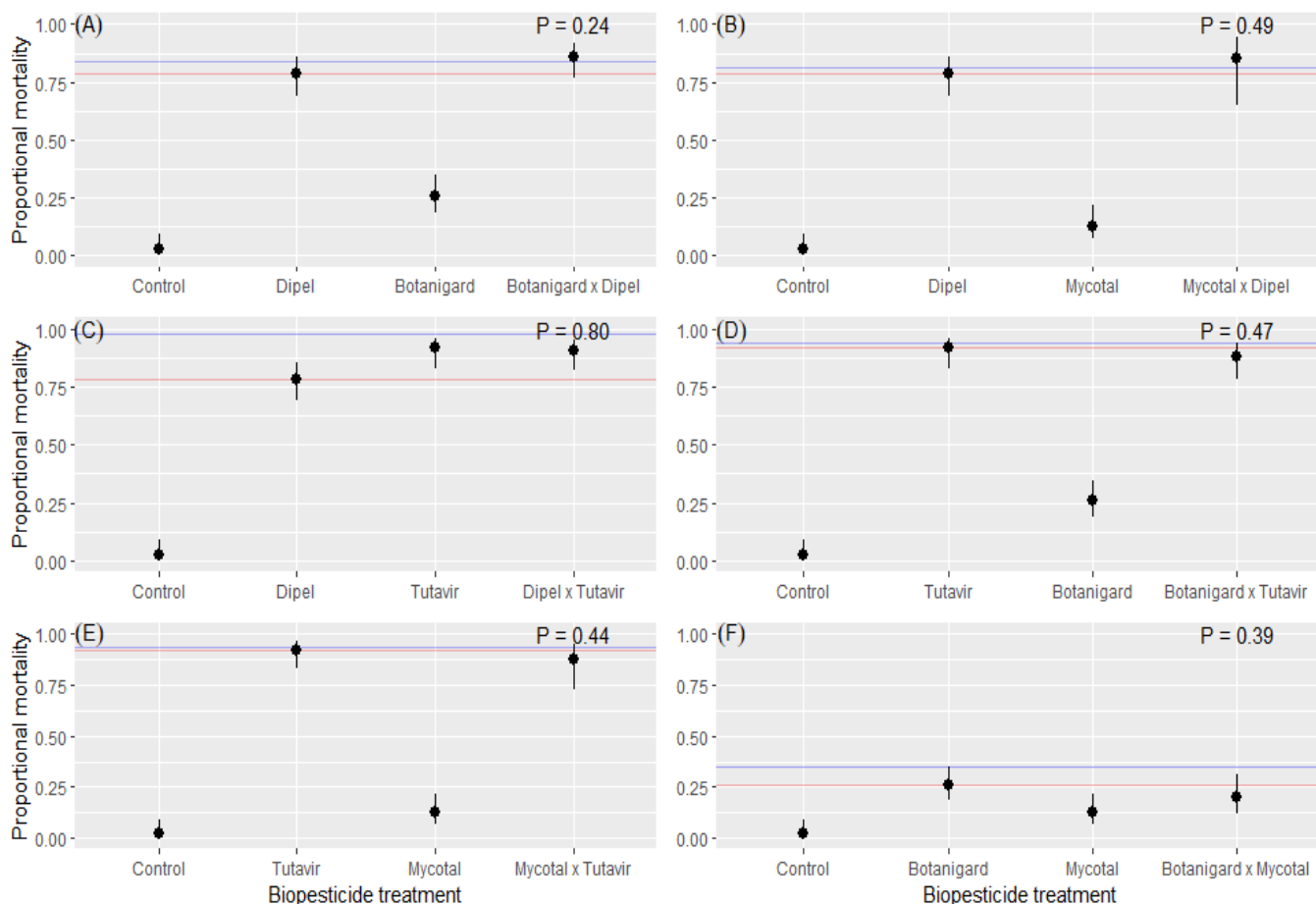


Figure 34: Mortality of *T. absoluta* larvae on tomato plants seven days after experimental application of different biopesticide treatments. Each sub-plot (A-F) shows mortality associated with single application of two different biopesticides, alongside the combination treatment. The red line indicates mortality caused by the most effective biopesticide in the pair, whereas the blue line indicates the mortality rate that would be predicted if the two biopesticides had an additive effect in combination. The P values associated with the comparison between mortality in the combined treatment with the more virulent of the two single treatments are shown on each plot: in no cases was mortality significantly affected by biopesticide mixing. Points show model predictions of mean mortality in each treatment group with 95% confidence intervals. Confidence intervals were calculated adding and subtracting the 95% standard error values to the models fitted values on the logit scale, before transforming these bounds back to the original scale.

6.4 Discussion

Tank mixing is a highly efficient and cost-effective method to apply multiple, usually chemical, pesticides to crops, reducing costs and labour associated with controlling multiple pest species and strains at a time. In this chapter, I show that tank mixing could be applied to biopesticides used to control two pests in tomato horticulture without compromising the effectiveness of pest management. In this study, I tested the effects of tank mixing four commercial (bacterial, fungal or viral) biopesticides in pairwise combinations to target two of the most detrimental pests in tomato horticulture, glasshouse whitefly (*Trialeurodes vaporariorum*) and tomato leafminer (*Tuta absoluta*). Overall, I found no evidence that combining any of the four biopesticide products resulted in antagonistic or synergistic effects compared to the single biopesticide applications. This suggests that tank mixing is unlikely to compromise pest control in this system, but it is also unlikely to enhance control. My results are beneficial to both the agricultural and horticultural industries. Tank mixing biopesticides does not impair pest control and reduces machinery and labour costs, increasing the economic value of the crop. Biopesticide tank mixing also has important environmental benefits, such as reducing water use and greenhouse gas emissions, ensuring pest management regimes remain environmentally sustainable and fit within the integrated pest management strategies (Gandini *et al.*, 2020).

The first pairwise combination in this study evaluated the effectiveness of two commercial fungal biopesticides, Botanigard and Mycotal, used for controlling the whitefly *Trialeurodes vaporariorum*. Both fungal biopesticides demonstrated significant efficacy, achieving approximately 59% mortality when applied in single applications. When these biopesticides were combined in a tank-mix and applied simultaneously their effectiveness remained comparable to when they were applied in single applications. Whilst there were no observed antagonistic effects, there were also no synergistic or additive effects found; tank mixing these two biopesticides did not increase their effectiveness at killing *T. vaporariorum* but did not compromise pest control either. A lack of synergism or additive effects is not uncommon and has been observed in many studies on both viral and fungal pathogen coinfection (Imrie *et al.*, 2023). Explanations for the lack of synergism and additive effects have mainly

focussed on competitive exclusion; in another study using the fungi *B. bassiana* and *Metarhizium robertsii* the latter outcompeted the former, dominating the infection resulting in similar mortality when these pathogens were applied alone or in combination (Li *et al.* 2021). This suggests that in cases of competitive exclusion, the combined application of biopesticides may not offer additional benefits compared to individual treatments.

A similar mechanism could explain the findings of my study; both Botanigard and Mycotal are fungal biopesticides with the same route of infection. An additional possibility, however, is that individuals which are susceptible to the effects of one of these products are equally susceptible to the other. Since Botanigard and Mycotal have the same mode of infection, by penetrating the insects cuticle before spore germination in the haemocoel, similar mechanisms, for instance involving the prophenoloxidase cascade, an innate immune response which involves producing melanin to limit fungal penetration of the cuticle (see 1.4; Read *et al.*, 2008; Susi *et al.*, 2015) may determine which individuals are susceptible and which are resistant. Essentially, the pathogens target and kill identical subsets of the population, those individuals with poor immune defences; the same individuals that are killed by Botanigard are killed by Mycotal, and resistant individuals can resist attack from both pathogens. Thus, resulting in no synergistic or additive effects when both biopesticides are applied in combination. Consequently, growers may not gain added benefits in terms of efficacy from tank-mixing Botanigard and Mycotal for controlling *T. vaporariorum*, but the lack of antagonism means that they will reduce the costs associated with treatment.

Further research to disentangle the mechanism underlying the non-additive, non-synergistic relationship will be important for understanding the significance of these results for the potential of resistance evolution. For instance, in the case of Botanigard and Mycotal the implications tank mixing has for resistance evolution in the *T. vaporariorum* system might vary depending on the mechanism underlying the lack of synergistic effect that I observed. For instance, if the lack of synergism occurs because the same immune mechanisms provide resistance against both pathogens, then the same individual hosts will survive attack and reproduce regardless of whether biopesticides are applied singly or tank mixed. In this case the same resistance genes

will be under positive selection in response to Botanigard and Mycotal regardless of application method. If, on the other hand, competitive exclusion occurs between the biopesticide pathogens, only one of the biopesticide products is actually effective. This results in selection against the biopesticide pathogen that is competitively superior diminishing the efficacy of the second biopesticide when applied simultaneously under tank mixing. If competitive exclusion is occurring in this system, sequential biopesticide applications may be a more effective pest management regime. This approach would target and kill any individuals that resisted the first biopesticide application, and limit resistance evolution.

In the *T. absoluta* system, different classes of biopesticides, one viral (Tutavir) and one bacterial (Dipel), that were commercially approved for lepidopteran pests, were most effective at controlling *T. absoluta*. Both Tutavir and Dipel were formulated to attack the pest's gut when they are ingested via plant material, resulting in infection and death of the host (Gill, *et al.*, 1992). When these products were applied in the *T. vaporariorum* system they were not effective, presumably because the *T. vaporariorum* larval stages targeted are phloem feeders which don't ingest contaminated leaf matter, thus do not ingest the product, limiting infectious exposure of Tutavir and Dipel (Capinera, 2008). The high specificity for lepidopteran hosts, particularly in the case of Tutavir (Tiba *et al.*, 2019) may also play a role here. The effects of Tutavir and Dipel, when applied in combination to the intended pest, *T. absoluta*, were qualitatively similar to those seen in the *T. vaporariorum* system when Botanigard and Mycotal were applied in combination.

Tank mixing and combined application of the key biopesticide products (Tutavir and Dipel) designed to control *T. absoluta* had no effect on efficacy compared to single applications. These products are both ingested from contaminated plant material but are different organisms (a virus and a bacteria). The lack of synergistic effects may arise due to competitive exclusion, whereby the pathogens compete for the same resources within the hosts gut. This competition for resources can lead to one pathogen limiting the proliferation of another, potentially reducing the overall pathogenic load, leading to no antagonistic or synergistic effects on host mortality (Pauli *et al.*, 2018). This type of competitive exclusion has been observed in studies on the sugarcane borer *Diatraea saccharalis* mortality when coinfecting with

entomopathogenic fungi and a viral pathogen. Co-inoculating the fungal pathogen *Beauveria bassiana* and the granulovirus (*DisaGV*) led to antagonistic effects on pathogen virulence, reducing their combined effectiveness compared to each pathogen when applied in a single application. In contrast, when the fungal pathogen *Metarhizium anisopliae* was combined with either *B. bassiana* or *DisaGV*, the effects were additive, increasing the overall pest mortality. When cadavers from dual, *M. anisopliae* and a secondary fungal pathogen *B. bassiana* or *DisaGV* or triple infections, the two fungal pathogens and the viral pathogen, were examined they mostly showed evidence for infection with only one pathogen. Specifically, the presence of viral infection was more noticeable when larvae were infected with *M. anisopliae* and *DisaGV*, particularly at higher inoculum rates (Pauli *et al.*, 2018). This suggests that competitive exclusion is taking place, with the viral pathogen tending to dominate and suppress the expression of symptoms caused by other pathogens. Competitive exclusion could be occurring in my study, on *T. absoluta*, as mortality did not significantly change when simultaneous applications of the key biopesticide products Tutavir and Dipel, were applied compared to single applications of both biopesticides. The viral and bacterial pathogens could be negatively interacting with each other. The pathogens could be competing for resources, limiting the proliferation of another, potentially reducing the overall pathogenic load, leading to no synergistic effects on host mortality (Pauli *et al.*, 2018). The lack of antagonism I observed, may occur because under competitive exclusion if the competitively superior pathogen is not suppressed by the interaction, no antagonistic effects would be observed. A follow up study which dissects cadavers and identifies the pathogen present would help to elucidate whether competitive exclusion (and by which agent) explains my results.

Synergistic effects can arise when viruses and bacteria interact mutualistically, where viruses enhance bacterial fitness, which can make their bacterial hosts more virulent potentially leading to synergistic effects on host mortality (Pauli *et al.*, 2018). Coinfection by multiple pathogen can stretch a hosts defence resources and, in theory, place more complex and multifaceted selection pressures on the target organism, making it considerably more difficult for the pest or pathogen to evolve highly specific resistance mechanisms (Raymond *et al.*, 2006). However, cross-resistance or general increased immunity may occur in the host when the host's immune system encounters multiple pathogens with differing mode of actions, leading to a broader and more

robust immune response and increased host survival (potentially at a cost to fecundity, however, see chapter 5). If a bacterial pathogen in a host can stimulate the host's immune system to produce generalised antibodies, these antibodies could offer protection and defensive resources against secondary pathogens, leading to cross resistance and potentially antagonistic effects.

Antagonistic effects from coinfection with a viral and bacterial pathogen with differing modes of action was observed in coinfection mortality and resistance mitigation studies on the diamondback moth *Plutella xylostella*. Two diamondback moth populations a Cry1Ac-resistant population and a Cry1Ac-susceptible population were coinfecting with a nucleopolyhedrovirus (AcMNPV) and the Bt bacterial toxin Cry1Ac. At low doses of Cry1Ac, antagonistic interactions were observed between the viral and bacterial pathogens AcMNPV and Cry1AC in Cry1Ac-resistant *P. xylostella* and in Cry1Ac -susceptible *P. xylostella* populations. This antagonistic effect was prevalent in susceptible *P. xylostella* larvae, while interactions in resistant larvae populations were generally additive. The stronger antagonistic interactions between Bt Cry1AC bacterial pathogen and AcMNPV viral pathogen in susceptible larvae, compared to resistant ones, may suggest that defence mechanisms are pathogen specific and to be resistant to one pathogen will reduce the relative fitness of Bt-resistant larvae when exposed to a different pathogen with a differing mode of action. Additionally, selecting for Cry1Ac resistance in two *P. xylostella* populations with different resistance mechanisms did not result in any correlated changes in resistance to AcMNPV. This study found that there is no evidence of cross-resistance between the pathogens, the viral and bacterial pathogens AcMNPV and Cry1AC. In applied settings this suggests that in Bt resistant populations using AcMNPV would be an effective pest management option due to the lack of cross resistance (Raymond *et al.*, 2006).

In my study, one notable qualitative difference between *T. vaporariorum* and *T. absoluta* systems was that in the *T. absoluta* system, the fungal biopesticides Botanigard and Mycotal, which are primarily formulated for targeting *T. vaporariorum*, unexpectedly enhanced mortality rates in *T. absoluta* when either fungal biopesticide was used in combination with Dipel. This outcome was surprising because, fungal pathogens, while effective against *T. absoluta*, typically require direct application methods, such as spraying directly onto the eggs and larvae (Chouikhi *et al.*, 2022).

In contrast, the current study employed indirect spraying on the underside of leaves, adhering to commercial application protocols, which was not expected to yield high efficacy.

Presumably additional mortality observed for *T. absoluta* when treated with either fungal pathogen in combination with Dipel occurred as a result of larvae coming into contact with or consuming the pre-treated foliage despite the indirect application method. Regardless of the route of infection, and even though the effects were not synergistic or additive, this result does suggest that combining fungal with bacterial or viral biopesticides that have different modes of action may be a promising method for efficiently controlling pests even when different treatment protocols are recommended.

The enhanced mortality from the coinfection of the bacterial pathogen *B. thuringiensis* and fungal pathogen *B. bassiana* has been previously observed in many studies in the field and the laboratory on many insect hosts. In a laboratory study, increased mortality of the agricultural lepidopteran pest *Helicoverpa armigera* was found when the larvae were exposed to simultaneous applications of *B. bassiana* (1×10^8 conidia ml⁻¹) and *B. thuringiensis* (0.75 µg ml⁻¹). *H. armigera* ingested *B. bassiana* via inoculated chickpea foliage that were dipped in *B. thuringiensis*. Combined treatments of the bacteria and endophytic fungi were compared with single-pathogen treatments. The combined treatments resulted in increased larval mortality, reduced pupation, adult emergence, and egg eclosion compared to the single treatments. Synergistic effects on mortality were observed when larvae were exposed to simultaneous applications of *B. bassiana* and *B. thuringiensis*. These findings suggest that the simultaneous application of endophytically colonised *B. bassiana* and *B. thuringiensis* can be effectively used against *H. armigera* (Wakil et al., 2020). Synergistic effects could be observed because of attack from multiple modes of action. When pathogens with different modes of action are used simultaneously, the combined attack on the target organism is more effective. This could be the case with simultaneous application of bacterial pathogens *Bacillus thuringiensis* (Bt), which produces gut-disrupting toxins, and a fungal pathogen *Beauveria bassiana*, which invades through the cuticle and proliferates within the host. This combined approach not only increases the overall mortality rates but also places a more complex and multifaceted selective pressure on the target organism, making it considerably more difficult for the pest or pathogen to

develop resistance. Simultaneous applications of pathogens with differing modes of action leading to synergistic effects on pest mortality could be the cause of the increased mortality in my study on *T. absoluta*. Increased mortality was observed when the lepidopteran pest *T. absoluta* was exposed to simultaneous applications of the Botanigard fungal biopesticide and Dipel bacterial biopesticide. In the single Botanigard (*B. bassiana*) treatment mortality was 25%, in the single Dipel (*B. thuringiensis*) treatment mortality was 76% and in the combined treatment mixed with the Dipel mortality increased to 87% mortality. Although this was not significant, it suggests the synergism could occur and be host specific as *T. absoluta* individuals are susceptible to both fungal and bacterial pathogens when applied in single applications, and mortality is enhanced when applied in combination (Sayed and Behle, 2017). Conducting this study again with a larger number of individual, increasing the power would determine if synergism did occur and was host specific.

In contrast, there were no effects of combining Tutavir, a viral pathogen formulated from *Phthorimaea operculella granulovirus* with either fungal pathogens (Botanigard and Mycotol formulated from *Beauveria bassiana* and *Lecanicillium muscarium* spores respectively) or the bacterial pathogen (Dipel formulated from *Bacillus thuringiensis*). Both Botanigard and Mycotol are formulated to penetrate insect cuticle and sporulate while Dipel and Tutavir spread through the hosts midgut via ingestion of the pathogen (bacteria or virus respectively) present on foliage. Previous studies suggest that, depending on the host species, simultaneous bacterial and viral coinfection rarely result in synergism or additive effects due to the competitive dominance in the pathogens transmission stages, where one pathogen will outcompete the other and cause host mortality (Deschodt and Cory, 2022). Competitive dominance may also occur when Tutavir is combined with fungal pathogens (Mycotal and Botanigard), explaining the lack of additive effects or synergism which were present when these fungi were combined with the bacterial pathogen Dipel. In this study Tutavir had the highest mortality when applied singly, which suggests Tutavir may be competitively superior, limiting the ability of further infections (fungal or bacterial) to take hold. However, without dissecting cadavers I could not be certain. To control *T. absoluta*, I have no evidence to suggest mixing the biopesticides in any combination would change the effectiveness of the biopesticides efficiency, thus would not be a beneficial

option to growers but could significantly reduce costs associated with application of multiple biopesticides.

6.5 Conclusion

This laboratory studies investigated the effects of tank-mixing pairwise combinations of four different biopesticide products on pest management efficacy and efficiency and considered the potential for resistance evolution in two non-model study systems involving *T. vaporariorum* and *T. absoluta*. The results indicated that tank-mixing these biopesticides would not have detrimental consequences for managing either agricultural pest. For growers dealing with both *T. vaporariorum* and *T. absoluta* on the same crop tank-mixing biopesticides could be advantageous, offering economic, ecological, and efficiency benefits while maintaining the same level of pest control. This approach could simplify pest management practices and reduce the need for multiple separate applications.

However, the study found no major synergistic effects from tank-mixing biopesticides for controlling a single pest species. Therefore, if pest management is required to control a single pest species, applying simultaneous applications of two biopesticides would not be beneficial, if the single biopesticide is effective. Tank mixing in this case would not be economically viable due to the increased cost of using two products without a corresponding improvement in pest control efficacy. However, if more than one pest species is present, tank mixing two products to control both pests would be beneficial and economical due to the reduction in multiple application. The findings suggest that tank-mixing biopesticides would have minimal impact on the potential for resistance evolution in the *T. vaporariorum* system. However, tank mixing could in theory reduce the selection pressure for resistance, potentially reducing the speed of evolution of resistance to any single biopesticide. When used alone, a biopesticide imposes strong selection pressures on the insect pest, favouring the survival of resistant individuals. In contrast, if pathogen competitive exclusion is a factor when tank mixing biopesticides in these systems, sequential applications of biopesticides might prove more effective than simultaneous tank-mixed applications. Sequential applications could help manage pest populations more efficiently by targeting individuals that survive the initial biopesticide treatment, potentially reducing resistance evolution.

This study emphasises the need to consider both economic and ecological factors when devising pest management strategies and application methods. Although tank mixing does not harm pest management, and can be economically beneficial, its benefits depend on the presence of insect host and pathogen interactions. Therefore, additional research is needed to explore more pairwise combinations and interactions in field conditions.

Chapter 7 Discussion

7.1 Overview

Understanding how pests evolve resistance to control methods is crucial for developing effective and sustainable pest management strategies. Historically, the emphasis has been on studying resistance to chemical pesticides.

Resistance evolution in pest populations is a multifaceted process. It not only involves physiological adaptations, such as changes in enzyme activity or target site alterations but also can include behavioural adaptations, which can be highly plastic and influenced by maternal effects. Importantly, behavioural plasticity can influence feeding patterns, habitat use, and overall pest behaviour all of which can feedback into resistance evolution to change the selection pressures acting on pests (Siegwart *et al.*, 2015). Resistance evolution is complicated when in the case of resistance to pesticides, but the use of biocontrol agents introduces the potential of co-evolution, more diverse interactions between co-evolving organisms, making resistance evolution more complex. These adaptive responses enable pests to withstand pesticide exposure, complicating efforts to manage and control their populations effectively (Siddiqui *et al.*, 2022).

The evolution of resistance is not without its costs to the pests. Maintaining resistance traits can be energetically expensive and may lead to trade-offs in other aspects of the pest's life history, such as reduced reproductive success or impaired dispersal abilities. Studies have shown that in over 60 % of insecticide- resistance cases insecticide-resistant pests experience some form of fitness cost, particularly affecting fecundity and reproductive success (Freeman *et al.*, 2021). These fitness costs, while significant, are often not enough to counterbalance the survival advantages provided by resistance traits, allowing resistant populations to persist and even thrive under continued pesticide application. However, it suggests that increasing research into resistance trade-offs and incorporating these trade-offs into resistance management techniques could be beneficial, although this would need to be tested on a case by case basis (Freeman *et al.*, 2021).

Mounting concerns over insecticide resistance evolution, alongside the environmental and health impacts of these chemicals has led to a significant shift towards safer, more sustainable approaches, such as biological control used within integrated pest management regimes (Angon *et al.*, 2023). Biological control, which involves using natural enemies to manage pest populations, has gained popularity as a more environmentally friendly alternative (Ehler, 2006). Despite the growing importance of the biocontrol industry for a sustainable future, the potential for pests to evolve resistance to these natural enemies is not widely recognised or understood (Onstad and Knolhoff, 2023).

This gap in awareness and research is concerning because, just as pests can develop resistance to chemical pesticides, they can also adapt to resist biocontrol agents. The evolution of resistance to biocontrol agents is often diminished within the field, with the theory that evolution will not occur in naturally occurring control agents (Hawkins *et al.*, 2009). However, there is mounting evidence from research and anecdotal evidence from growers and biocontrol companies in the field, with biocontrol efficacy decreasing over time.

Resistance evolution is complicated and hard to mitigate when it's resistance to chemical pesticides, but the potential for biocontrol resistance is much more complex when co-evolution is factored in. Biocontrol agents can potentially evolve themselves, and the ways they act can be much more diverse, making the picture of biocontrol resistance more complex, but it also giving more scope for resistance management (Shields *et al.*, 2022). However, because of the way many biocontrol products are formulated and maintained, they can lack the evolutionary potential to co-evolve with pests. Therefore, increasing the understanding and addressing this potential for resistance is essential for the long-term efficacy and sustainability of biological control agents.

The emergence of resistance to biocontrol agents is particularly concerning because it threatens the long-term sustainability and effectiveness of these alternatives to synthetic pesticides. Resistance to *Bacillus thuringiensis* (Bt) toxins, a widely used biopesticide, has been documented in at least 27 species under laboratory conditions and in the field, including in economically important pests such as *Plutella xylostella*

and *Diabrotica virgifera virgifera* (Tabashnik *et al.*, 1994; Gassmann, 2021). Resistance to biopesticides can involve various mechanisms, such as degradation of biopesticide compounds, altered target site binding, or even immune responses that neutralize the biocontrol agent before it can exert its lethal effects (Siegwart *et al.*, 2015). Resistance to viral biopesticides, such as *Cydia pomonella* granulovirus in codling moths, and fungal biopesticides like *Beauveria bassiana* in the greater wax moth, has already been observed, indicating that resistance can evolve against a wide range of biocontrol agents (Asser-Kaiser *et al.*, 2011; Dubovskiy *et al.*, 2013). Additionally, many aphid species have developed resistance to parasitoids through symbiont-mediated protection, further complicating pest management efforts (Schmid *et al.*, 2011).

The evolution of resistance to biocontrol agents can significantly reduce their efficiency and long term effectiveness, potentially leading to the resurgence of pest populations. This poses a serious threat to crop production and food security, calling for the development of new and more effective pest control strategies. In fundamental research, studies on the evolutionary ecology of insects have demonstrated that co-evolution with natural enemies is a common phenomenon, often ignored in relation to the use of biological control agents. This knowledge is often not fully integrated into applied pest management practices. Many biocontrol companies do not disclose information on the genetic diversity of their biocontrol formulations or stock, with anecdotal evidence suggesting many lines of parasitoids and predators have not had new lines introduced for over 50 years. This lack of diversity demotes the benefits of using living systems which can evolve too. Thus, from a resistance perspective, when pests are exposed to high biocontrol selection pressures, that makes the use of biocontrol agents not much better than chemical pesticides. Acknowledging the potential of co-evolution and the advantage of biocontrol agents being genetically diverse would aid resistance management techniques.

7.2 Results overview

In Chapter 3, I conducted a laboratory-based experiment to quantify the variation in resistance to parasitism in the glasshouse whitefly (*Trialeurodes vaporariorum*) against its biocontrol agents: the parasitoid wasp *Encarsia formosa* and a commercial fungal biopesticide containing the fungal pathogen *Beauveria bassiana*. Through a series of assays, I was able to quantify the level of resistance displayed by *T. vaporariorum* when exposed to these biocontrol agents under different conditions. The data obtained from these experiments were then used to parameterise a simulation model designed to predict the potential evolution of resistance within this system under various application scenarios.

The results from the empirical and individual based model revealed that resistance to *E. formosa* in *T. vaporariorum* was highly variable and significantly influenced by the type of crop. This variability suggests that the whitefly can indeed evolve resistance to biocontrol agents, but the rate at which this resistance develops is likely to be affected by environmental factors, such as the specific crop type in which the whitefly are reared. The simulation model also indicated that while resistance evolution is possible, its progression will depend on the environmental conditions, including the agricultural practices employed. I found that alternating between biocontrol agents with differing mode of actions each generation had improved efficacy, however this depended on the crop type whitefly were reared on. When compared to whitefly continually exposed to *E. formosa*, whitefly that were exposed to alternating treatments each generation had lower proportional survival after 140 generations when reared on aubergine plants, whereas survival increased when reared on tomato compared to whitefly exposed to *E. formosa* continually for the 140 generations. This suggests that crop type is more influential on biocontrol efficacy than predicted (Gatarayihya *et al.*, 2010). This research highlights the importance of considering crop type and other environmental variables when deploying biocontrol agents in order to delay the evolution of resistance and maintain the effectiveness of these pest management strategies over time.

In Chapter 4, I conducted a six-generation laboratory selection experiment to further investigate the evolution of resistance in *Trialeurodes vaporariorum* when exposed to intense selection pressures from the parasitoid *Encarsia formosa*. This experiment was designed to assess the rate at which resistance can evolve and to complement the simulation results presented in Chapter 3. By subjecting *T. vaporariorum* to consistent, strong selection pressures over multiple generations, I observed a notable increase in proportional survival, rising from 35 % in the initial generation to 56 % by the sixth generation. This significant increase in survival suggests a rapid evolution of resistance in response to the sustained pressure exerted by *E. formosa*. The findings from this experiment align with the predictions made by the individual-based simulation model developed in Chapter 3, providing empirical validation for the model's projections. Together, these results highlight the potential for rapid resistance evolution in *T. vaporariorum* under high selection pressures, highlighting the need for careful management of biocontrol agents to mitigate the development of resistance in pest populations.

In Chapter 5, I determined whether *Trialeurodes vaporariorum* individuals that successfully resist parasitism by *Encarsia formosa* experience any life history trade-offs which might affect the course of resistance evolution. To investigate this, I conducted fitness assays on whitefly that survived *E. formosa* attacks under controlled environmental condition in the laboratory. I found that resistant whitefly experienced several fitness costs; they had reduced fecundity and lower hatching success compared to non-resistant individuals. These findings suggest that although *T. vaporariorum* can evolve resistance to parasitism, this resistance comes at a significant biological cost. The observed life history trade-offs imply that resistant whitefly populations may experience reduced overall population growth. This could, in turn, constrain the long-term evolution of resistance within the population, potentially limiting the spread and persistence of resistance over time. Thus, beneficial to the continued effectiveness of biocontrol strategies.

In chapter 6 I investigated whether tank-mixing multiple biopesticides may be a viable option for pre-emptive management of resistance evolution and targeting multiple pest species at the same time. In this experiment I used the whitefly (*T. vaporariorum*) and the tomato leaf miner (*Tuta absoluta*) and applied different biopesticides alone or in combination and measured pest mortality. I found no evidence that mixing

biopesticides targeting different or the same pest species had any antagonistic or synergistic effects. These findings suggest tank-mixing biopesticides does not compromise control efficacy and would improve application efficiency if targeting multiple pest species, but in the scenario, I tested is unlikely to influence resistance evolution.

Overall, my research findings indicated that *T. vaporariorum* are able to rapidly evolve resistance to both *E. formosa* and *B. bassiana* over a short timeframe. The individual based model (IBM) found this resistance to be influenced by the type of crop on which the whiteflies were reared, suggesting that the agricultural environment plays a significant role in the evolutionary trajectory of pest populations. The study determined that the observed resistance is likely due to some maternal effects alongside physiological mechanisms of resistance.

Moreover, the research uncovered a positive correlation in survival rates among replicate lines of *T. vaporariorum* that had been exposed to the fungal biopesticide and then to *E. formosa*. This correlation suggests that the resistance mechanism in whiteflies may be a broad-spectrum defence, capable of providing protection against different types of biological control agents despite their distinct modes of action. Notably, the study found that approximately 10% of *T. vaporariorum* exposed to *E. formosa* survived the critical oviposition event, indicating that the whiteflies' defence mechanism likely involves an immune response.

In addition to resistance mechanisms, my thesis explored life history trade-offs associated with resistance. It was observed that resistant whitefly exhibited decreased fecundity and egg hatching success after successfully overcoming *E. formosa* parasitisation. These findings suggest that the evolution of resistance in *T. vaporariorum* may be constrained by trade-offs that could limit the long-term success of resistant individuals, potentially slowing down the spread of resistance within populations. This would be a beneficial for pest management regimes and resistance management as population numbers would decline even where resistance is present, still controlling pest numbers.

Furthermore, the thesis investigated the effects of tank-mixing biopesticides, a strategy commonly employed to enhance pest control efficiency be economically beneficial. The results showed that there were no antagonistic or synergistic interactions between the biopesticides, indicating that this approach could improve the efficiency of pest management applications without compromising the effectiveness of control if control of multiple pests was required.

Although this thesis scratches the surface on the effectiveness and long term effectiveness of a few biocontrol agents under laboratory conditions, it provides valuable insights into the need to consider the potential for resistance evolution and the importance of combining biological control agents with an understanding of their ecological and evolutionary impacts, vital for ensuring the long term effectiveness and effectiveness of biocontrol agents in pest management strategies.

7.3 The evolution of parasitism resistance and life history costs

The evolution of resistance could threaten the long term effectiveness and use of current pest management regimes without appropriate planning. Understanding and acknowledging when and how resistance evolves over time will enable the adoption of more informed and adaptive pest management strategies, protect the long term effectiveness of pest management regimes whilst improving the sustainability and effectiveness of agricultural practices. In general, resistance evolution and the associated life history costs and trade-offs to traditional pesticides in pest species have been well studied, but resistance to biocontrol agents has often been overlooked and ignored due to them being naturally occurring organisms that could co-evolve. However, biocontrol formulations and stock may often having reduced genetic diversity, the potential for co-evolution is hampered, thus evolution of pest resistance is increasing similar to that of chemical pesticides. Over 500 insect and mite species are now resistant to over 400 pesticide compounds across the globe, and the evidence of pests evolving resistance to biocontrol agents is rising (Siddiqui *et al.*,2023). This resistance evolution primarily occurs due to genetic mutations fuelled by high genetic variation and the intense selection pressures exerted by repeated pesticide

applications. Pests with resistant traits survive, reproduce, and rapidly dominate the population, rendering the pesticide ineffective (Hawkins *et al.*, 2018). The potential of resistance evolution can often initially be studied through variation in survival.

A 2013 study examined the susceptibility of 31 populations of lady beetles (Coccinellidae) to lambda-cyhalothrin, a commonly used pyrethroid insecticide in cotton fields. After treating adult lady beetles with lambda-cyhalothrin, the study found significant variations in susceptibility between species and populations. These results suggest that field selection for resistance to lambda-cyhalothrin is occurring in common lady beetles (Rodrigues *et al.*, 2013). This study suggested that variation in survival between populations can indicate the evolution of resistance. In chapter 3 of my thesis, I tested whether a similar pattern existed in the whitefly *T. vaporariorum*, only in response to two biocontrol agents, the parasitoid was *E. formosa* and a commercial fungal biopesticide containing *Beauveria bassiana*, rather than a chemical pesticide. I found that there was significant variation in survival between whitefly lines in both the fungal and wasp treatment groups, with higher survival rates in the fungal treatment. The presence of high variation in survival within this pest population suggests the existence of resistant traits which could facilitate rapid evolution of resistance to these biocontrol agents over time.

Resistance mechanisms in pests can evolve through various strategies, including physiological, behavioural, and more general defences. When there is substantial genetic diversity, some individuals may possess traits that confer a survival advantage, such as mutations that could enhance detoxification or alter the pesticide's target site (Georghiou, 1972). These resistant individuals survive and reproduce, leading to a rapid increase in resistance alleles within the population under strong selection pressure. Behavioural and physiological plasticity also contribute to survival differences, further driving resistance evolution (Rex Consortium, 2013). Behavioural adaptations, such as laying eggs in protected environments like rolled leaves or inside plant stems, can shield offspring from predators or chemicals. Some insects may also pupate underground, avoiding exposure altogether (Aoyama and Ohshima, 2019). Additionally, general immune defences, such as encapsulation, where the pest's immune system surrounds and neutralises a foreign object, can offer protection

against a range of biocontrol agents (See chapters 1.3-1.4, 4.4) (Sideri *et al.*, 2008). These diverse mechanisms make managing resistance a complex challenge.

I used the data I collected in chapter 3 to parameterise an IBM simulation to test whether the variation in survival under the different biocontrol agents would lead to the evolution of resistance over time, and to forecast how different management scenarios might alter the rate of resistance evolution. The IBM confirmed resistance would evolve in the population over time with survival rates significantly increasing in all scenarios over multiple generations. This suggests that in the successive generations, the frequency of resistance traits is likely to increase within the population, leading to the evolution of a more resistant pest population. I tested this explicitly in chapter 4, showing that resistance to the parasitoid wasp *E. formosa* can evolve rapidly, in as little as 6 generations, a short time period (6 months) which is comparable to a growing period. This result matches anecdotal real-world evidence from growers who comment on the reduced efficacy of *E. Formosa* during a growing season and increasing whitefly abundance.

Whilst my results from chapter 3 and 4 showed a stark increase in resistance over generations, I also found significant environmental effects which could be helpful in developing strategies which mitigate resistance evolution. For instance, in chapter 3 the crop type whitefly were reared on had an effect on the survival rate under biocontrol, with higher survival for whitefly reared on aubergine compared to tomato crops. Another study tested the impact of crop type on biocontrol efficacy using five different crops (beans, cucumber, aubergine, maize, and tomato) on the persistence and control effectiveness of *Beauveria bassiana* (Balsamo) Vuillemin against *Tetranychus urticae* Koch mites. Plants were deliberately infested with *T. urticae*, and *B. bassiana* conidia were suspended in a 0.01% Break-thru® before being sprayed onto the plants. Control plants were treated with Break-thru® solution alone. The number of conidia and mite mortality rates were assessed. Mite mortality was concentration-dependent and influenced by the host crop, with lower control observed on maize and tomato compared to aubergine, beans, and cucumber. The results were consistent across trials and confirmed that crop type can affect the efficacy of *B. bassiana* against *T. urticae* (Gatarayihya *et al.*, 2010). Interestingly, this shows that pest survival is affected by host crop type, with higher survival on tomato than aubergine, the opposite of what was found in my IBM in chapter 3. This suggests that the influence

of crop type is species specific. However, the overarching result of host crop influencing the biocontrol efficacy suggests that the use of intercropping or crop-rotation could reduce the presence of some resistant genotypes, reducing and adding a new selection pressure that makes it harder for resistant genotypes to completely take hold. This could be classed as genotype-by-environment interactions (GEIs) in which the fitness of resistance alleles in the pest species depends on the specific environment a pest inhabits. By intercropping, developing heterogeneous environments, selection for resistance at a landscape scale will not be directional; as the agricultural environment shifts, the alleles favoured by selection will also change. Consequently, local selection in any given generation will drive evolution away from the optimal genotype for surviving pest control measures in other distinct patches within the heterogeneous landscape, mitigating resistance evolution (Mangan *et al.*, 2023). Conducting empirical studies comparing whitefly parasitoid survival dependent on crop type would confirm the results of the IBM from my thesis chapter 3.

7.4 The potential for cross resistance

The emergence of insecticide resistance not only reduces the lifespan of the existing pest control agents but could also undermine the efficacy of novel developed pest control products through cross-resistance or multiple resistance (Machani *et al.*, 2020). Cross-resistance occurs when an organisms resistance to one, in this case insecticide confers resistance to another insecticide, even where the insect has not been exposed to the latter product (IRAC, 2024). There have been several cases of resistance to the insecticides spinosyns, a group of macrolide insecticides with increasing reports of cross resistance particularly to two spinosyns spinosad and spinetoram. Many of these reports of resistance to spinosyns in the field occur in either the diamondback moth or western flower thrips. There have been over 85 studies investigating cross-resistance to the spinosyns, where just under 50 % of studies found cross resistance (Sparks *et al.*, 2012). In my thesis, in chapter 3, there was some evidence of whitefly exhibiting cross resistance to the fungal biopesticide Botanigard and the parasitoid wasp *E. formosa*. There was a weak positive correlation suggesting there could be weak cross resistance in the whitefly lines, with lines that have higher survival in the fungal treatment also having higher survival in the wasp treatment. This suggests that the

resistance traits under selection could involve generalised defence mechanisms, for instance general up-regulation of immunity or behavioural evasion (Chapter 1.3-1.4, Chapter 7.2), as the two biocontrol agents have differing modes of action, fungal pathogen sporulation and endoparasitism. Future studies would be beneficial to test whether alternating crops and biocontrol agents may be a viable strategy for mitigating resistance evolution in whitefly and perhaps other pests. As cross resistance was not the main focus of my thesis, studies were not conducted with the aim to assess cross resistance potential, specific quantitative genetic cross resistance studies should be conducted in the future.

It is well known in literature that resistance evolution can be costly, resulting in trade-offs with other fitness traits that may limit its evolution. Life history costs describe these trade-offs where organisms must allocate limited resources among essential functions like growth, reproduction, and defence. These trade-offs are typically identified through negative phenotypic or genetic correlations between fitness-related traits within a population. They arise when an enhancement in one life history trait, such as increased survival during disease infection, leads to a reduction or compromise in another trait, such as fecundity (Fabian and Flatt, 2012). This has been seen in many cases of insecticide resistance, for instance, a study on the oblique banded leafroller (*Choristoneura rosaceana*) found significant variation in pesticide resistance within a local geographic area. Both resistant and susceptible populations were not exposed to pesticides, however it found that resistant populations, exhibited lower 16-day live mass, smaller pupal mass, and longer development times compared to populations and which were highly susceptible to pesticides. This indicates that in *C. rosaceana*, pesticide resistance is associated with life-history costs of carrying a resistant trait that is potentially not expressed. These findings suggest that alternating insecticides, to which the oblique banded leafroller has not developed resistance, could be an effective strategy for managing this species (Carriere *et al.*, 1994). I found such a life history cost in *T. vaporariorum* in chapter 5; similar to the study on *C. rosaceana*, however in my study resistant whitefly were those whitefly that had survived parasitism. I found that resistant whitefly laid less offspring, and the offspring had significantly lower hatch rate than the control treatment that were not exposed to the parasitoid wasp. It would be interesting to repeat the study with resistant lines that had

not been exposed to parasitism to determine if the associated cost is through carrying or expressing the resistant trait. Nevertheless, the presence of life history costs could be beneficial to pest management as although resistance is present the pest populations would be decreasing due to reduced fecundity and successful development.

The potential for cross resistance to biocontrol agents could be more complex if the potential for co-evolution was considered. The diversity of biocontrol agents, could affect cross resistance potential if biocontrol agents co-evolve, thus understanding biocontrol genetic diversity is crucial for cross resistance management techniques.

7.5 Biocontrol efficacy and efficiency

Resistance in biological control systems can significantly impact the efficacy of biocontrol agents, posing a major challenge to sustainable pest management. When pest populations evolve resistance to a biocontrol agent, the agent's ability to suppress the pest population diminishes, potentially leading to increased pest numbers and reduced effectiveness of the control strategy. This resistance can arise through various mechanisms, such as physiological changes in the pest that enable it to evade or tolerate the effects of the biocontrol agent, or through behavioural adaptations that reduce the agent's efficacy. The development of resistance not only undermines the immediate control efforts but can also lead to a reliance on more intensive or less environmentally friendly pest management practices, such as chemical pesticides. To mitigate resistance and maintain biocontrol efficacy, strategies such as rotating different biocontrol agents, or tank mixing biocontrol agents, and simultaneously applying the biocontrol agents can reduce and diversify selection pressures and target the pest with multiple modes of action. Tank mixing chemical pesticides is a well know approach to improve pest control efficiency without compromising efficacy (Gazziero, 2015).

Tank mixing involves combining multiple pesticides directly in the applicator's container shortly before application, optimising operational efficiency and reducing overall costs. This method minimizes the number of times machinery enters the

production area, thereby decreasing fuel consumption, water usage, and the time required for solution preparation. By consolidating applications, tank mixing also reduces soil compaction, which can enhance plant root development. Additionally, it lowers the risk of worker exposure to potentially harmful chemicals by reducing the frequency of product applications. The streamlined approach not only contributes to environmental sustainability but also improves safety and operational efficiency (Gazziero, 2015). Applying simultaneous applications of biopesticides was often overlooked, with limited guidance and research into the effects of simultaneous applications on efficacy. This is partly due to many biocontrol agents containing pathogens and concerns about the potential for competition depending on the pathogens that are combined in the tank mix. However, as early as 2005 there has been evidence that tank mixing has the potential to increase pest control efficacy. Commercial biopesticides based on the fungal pathogen *Beauveria bassiana* strain GHA and the bacterial pathogen *Bacillus thuringiensis tenebrionis* were tested alone and in combination against larval populations of the Colorado potato beetle (*Leptinotarsa decemlineata*) over three field seasons. When applied alone, *B. thuringiensis* achieved 50–85% control of beetle larvae within 14 days of application, while *B. bassiana* provided no more than 25% control. The highest level of control was consistently achieved with the combination of both products. Combined treatments resulted in a statistically significant 6–35% greater reduction in larval populations compared to what would have been expected if the biopesticides acted independently. This observed low-level synergistic interaction was consistent across all field seasons and rates tested, including the lower rates of each product (Wraight and Ramos, 2005).

In chapter 6 of this thesis, the effects of tank mixing biopesticides with differing modes of action on two pest species was assessed. I found that there was no detrimental effects on the biopesticide efficacy and tank mixing does not adversely affect the management of either glasshouse whitefly (*T. vaporariorum*) or tomato leaf miner *Tuta absoluta*. For growers dealing with both pests on the same crop, combining biopesticides in a tank mix offers economic, ecological, and efficiency benefits while maintaining pest control efficacy.

However, in contrast to the study by Wraight and Ramos (2005) on *L. decemlineata* I did not find any synergistic effects from tank-mixing biopesticides for controlling either pest species. If management is required for only one pest, using two biopesticides

simultaneously does not improve biocontrol efficacy beyond that of a single effective biopesticide. In such cases, tank mixing may not be economically viable due to the increased cost of using two products without a corresponding improvement in pest control efficacy. Conversely, when multiple pest species are present, tank mixing can be both beneficial and economical due to the reduced need for separate applications.

The findings suggest that tank-mixing biopesticides would have minimal impact on resistance evolution in the *T. vaporariorum* system. While tank mixing might reduce the selection pressure for resistance, slowing the development of resistance to any single biopesticide, sequential applications of differing biocontrol agents could potentially be more effective, due to competitive exclusion where one agent has a competitive advantage and nullifies the effect of the second agent (Chapter 6). Sequential applications of differing biocontrol agents might better manage pest populations by targeting individuals that survive initial treatments, thereby reducing the rate of resistance evolution, thus maintaining the efficacy of the product. However, this could reduce the efficiency of biocontrol applications compared to applying simultaneous applications. In Chapter 3 of my thesis the effects of sequential applications, alternating between a fungal biopesticide and the parasitoid wasp *E. formosa* on whitefly survival was assessed in a 140 generation IBM simulation. My simulations showed that alternating the two biocontrol types could reduce resistance evolution, presumably by reducing the strong and continuous selection pressure to resist any one agent. My results suggested that efficacy of both biocontrol agents remained high on both tomato and aubergine crops under alternating sequential applications compared to single applications of either agent. However, the simulations suggested there may be exceptions; on tomato crops the efficacy of fungal biopesticide remained high when they were used alongside the parasitoid wasp, but the parasitoid *E. formosa* became less efficient in the alternating treatment compared to single application. This suggests that efficacy and resistance mitigation may depend on the biological control agent mode of actions and how they are affected by environmental conditions and crop. Thus, recommendations for tank mixing or rotating biocontrol agents should be developed on a case-by-case basis, with thorough investigation as to how the efficacy and efficiency of different biocontrol agents can be affected by a multitude of factors.

7.6 Resistance management

Preventing or mitigating the evolution of resistance to biological control agents in pest populations is crucial to ensure the long term effectiveness and efficacy of current biological control agents used. Key approaches for mitigating resistance to chemical pesticides often include implementing refuges where a portion of the pest population remains untreated to preserve susceptible genes. Other approaches include applying multiple pesticides with differing modes of action simultaneously and rotating different types of pest control agents or biocontrol agents to rotate and alternate the control agents mode of action (Hobbs *et al.*, 2023). For instance, rotating three commonly used pest control agents, *Bacillus thuringiensis* (Bt) proteins, spinosad, and indoxacarb, within a single generation has been shown to slow resistance evolution and limit population growth in the diamondback moth (*Plutella xylostella*) (Zhao *et al.*, 2006). The results showed that rotating insecticides more frequently, every generation, was more effective at reducing resistance and population management compared to rotating every third generation or using a mosaic approach. These findings suggest that frequent insecticide rotation is a more effective strategy for limiting resistance evolution.

The results of the simulation models I present in chapter 3 point to a similar general pattern; alternating between a fungal biopesticide and *E. formosa* parasitoid wasps each generation reduced the proportional survival of whitefly over 140 generations compared to a single treatment applied every generation, however, this was affected by crop type. Three explanations have been proposed for how rotating control agents can delay the evolution of resistance. The first, and most common explanation is counterselection, due to the costs associated with resistance. Counterselection can help prevent or delay the evolution of resistance by reducing the prevalence of resistant individuals in the population during periods when the control agent is not applied. By releasing the selection pressure, susceptible genotypes, which do not pay the costs of resistance, can increase in frequency and outcompete resistant individuals due to their superior fitness in the absence of the control agent (Madgwick and Kanitz, 2024).

The second explanation is that rotating control agents relaxes the selection pressure between rotations. By applying a different toxin with a differing mode of action for some period of time, the selection pressure imposed on the pest is reduced (Sudo *et al.*, 2018). The reduced selection pressure associated with rotating pesticides with different modes of action can potentially prolong the effectiveness of these pest control agents. This was demonstrated in a study on alternating fungicides to slow the rate of resistance evolution in the economically important crop fungal pathogen *Zymoseptoria tritici*. It found that alternating fungicides can reduce resistance selection by limiting exposure time to a specific fungicide and by diversifying selection pressure, which can modify the overall resistance evolution rate. Resistance to a fungicide, with a high resistance evolution potential was reduced by 9% when alternated with a fungicide with a lower risk of resistance evolution. Similarly, alternation with a low-risk fungicide decreased the selection pressure of a medium-risk fungicide by 22%. This suggested that pairing fungicides with lower resistance risks in alternation strategies can effectively reduce the rate of resistance evolution (Ballu *et al.*, 2023). However, ongoing selection pressure, even with different modes of action, may enhance the fitness of resistant individuals through cross-resistance to multiple pathogens and selection pressures ultimately leading to more stable resistance, having a detrimental effect on multiple control agents (Cloyd, 2010).

Thirdly, alternating pest control agents has been proposed to slow the rate of resistance evolution by creating multiple intergenerational killing. This theory is similar to the redundant kill effect seen with pesticide mixtures applied within a single pest generation that works by the killing of an individual that is resistant to one pesticide by a second pesticide. This concept, however, operates by killing the offspring of an individual resistant to one insecticide through exposure to a different insecticide with a differing mode of action, thereby preventing the spread of resistance across generations (Madgwick and Kanitz, 2024).

Another key tool to mitigating pesticide resistance is through tank mixing control agents, applying them in combination treatments, exposing the pest to multiple chemicals/ pathogens simultaneously, potentially complicating the evolutionary pathway for resistance (Bosch *et al.*, 2014). Tank mixing could potentially mitigate resistance in some cases due to the reduced likelihood that individuals are physiologically resistant to and able to mount defences against multiple biopesticides

with different modes of action, compared to a single agent. When pests are exposed to multiple pathogens simultaneously, the chances of any single pathogen becoming dominant and driving resistance are reduced. This diversity in pathogen exposure helps limit the evolutionary pressure that typically leads to resistance, making the pest control system more robust and resilient (Hawkins *et al.*, 2018). Moreover, applying multiple pathogens at once can result in synergistic effects, increasing overall pest mortality by imposing a multi-faceted attack that strains the pest's fitness. This is especially effective when pathogens with different modes of action are used together (Abdullah *et al.*, 2017). For example, if one pathogen targets a specific physiological process, the host may need significant resources to resist it, lowering its fitness. A secondary pathogen attacking a different process can further overwhelm the host, making it more difficult for pests to evolve resistance to both pathogens applied concurrently (McLaren & Callahan, 2020). I did not find any evidence for such synergy when applying multiple biopesticides to two detrimental pest species, glasshouse whitefly (*T. vaporariorum*) and the tomato leaf miner (*T. absoluta*) in chapter 6, but the biopesticides did not have any antagonistic effect either. A potentially fruitful follow-up could be to repeat the study using insect lines known to be resistant to some of the biopesticides being mixed; this would not only show whether mixing can mitigate some of the issues with resistance but would also shed light on the mechanisms underlying the lack of synergy.

Resistance management is crucial for sustaining the long-term effectiveness of pest control strategies. As resistance can develop rapidly with continuous use of a single control agent, it is essential to employ diverse approaches. The findings of this thesis suggest that applying multiple agents simultaneously could reduce the likelihood that individuals are able to defend against attack from multiple control agents with different modes of action. From chapter 3 showing variation in survival based on varying genotypes, understanding the benefits of the natural genetic diversity of biocontrol agents in the wild, and adapting that for the production of biocontrol agents would aid resistance management and the production of novel biocontrol agents (Leung *et al.*, 2020). Currently coevolution between biopesticides or inundatively released biocontrol is impossible because the control agent is grown from stock in the laboratory or factory (Mangan *et al.*, 2023). These strategies delay the onset of resistance, reduce its magnitude, and prolong the efficacy of available insecticides.

7.7 Practical recommendations for pest management

To preserve the efficacy and long term effectiveness of both chemical and biological pest control strategies, resistance management must be proactively integrated into pest management programs. The following suggestions are supported by empirical evidence and simulation modelling from this thesis.

7.7.1 Rotate Control Agents Frequently

Rotate pest control agents every generation rather than every few generations. Alternating between different biocontrol agents (e.g., *Encarsia formosa* and fungal biopesticides) in successive generations to slow resistance evolution and long-term pest survival, as suggested by simulation models in Chapter 3.

7.7.2 Exploit the Costs of Resistance

Design rotations to exploit the fitness costs associated with resistance traits. Resistant individuals often have reduced fitness in the absence of the control agent. Alternating agents can reduce the frequency of resistant individuals through natural selection favouring susceptible genotypes (Madgwick & Kanitz, 2024). Including untreated refuge areas or pesticide free intervals within cropping systems can help maintain susceptible pest genotypes, thereby, slowing the evolution of resistance to control agents (Gould, 1998; Tabashnik et al., 2008; Carrière et al., 2016)

7.7.3. Use Agents with Different Modes of Action

Rotate or mix pest control agents with differing modes of action. Alternating or combining differing biocontrol agents, can help reduce continuous selection pressure on any single resistance mechanism (Biondi et al., 2018). Each biocontrol strategy exerts distinct selective pressures, leading to different evolutionary responses: predation typically selects for behavioural resistance, such as hiding, reduced movement, or altered feeding times (Crowder & Jabbour, 2014); endoparasitism often drives physiological or immunological resistance, including encapsulation of parasitoid eggs or enhanced immune responses (Schmid-Hempel, 2005); and ectoparasitism

may lead to cuticular defences or active defensive behaviours like increased grooming to dislodge attackers (Vincent et al., 2001). Therefore, strategic rotation and combination of biological control agents, planned according to their differing mode of actions and evolutionary risks, along with the inclusion of selective biopesticides and untreated refuge areas, is critical to sustaining long-term pest suppression and minimising resistance development (Gould, 1998; Biondi et al., 2018).

7.7.4. Implement multi-generational management strategies

Use sequential applications of different control agents that act across generations. This “intergenerational kill” strategy ensures that offspring of resistant individuals are targeted by a different control mechanism (Madgwick & Kanitz, 2024). This method targets the offspring of individuals that survive one control mechanism with a distinctly different mode of action in the following generation. This strategy is designed to prevent resistant alleles from accumulating in the population by ensuring that survivors of one generation do not successfully reproduce without their progeny facing a new, unrelated selective pressure (Madgwick & Kanitz, 2024). From cross resistance studies, applying a biopesticide in one generation and a parasitoid in the next had a lower survival rate of offspring, thus disrupting resistance inheritance. The first agent may select for physiological resistance mechanisms, but these do not confer protection against parasitism, which acts through a different biological mechanism. This non-overlapping selective pressure aids in disrupting the inheritance of resistance traits by targeting genetically variable survivors.

7.7.5. Tailor Resistance Management to Crop Type and Ecosystem

Adapt rotation and mixing strategies based on crop type, pest life cycle, and local environmental conditions, as these factors critically influence the dynamics of pest pressure and control efficacy. Introducing different crop types into a system imposes distinct ecological and evolutionary challenges on pests, such as variations in plant architecture, chemistry, phenology, and microclimate, which disrupt the stability of selective pressures. This environmental heterogeneity can slow or limit the evolution of resistance, as pest genotypes that are favoured in one crop environment may be neutral or even disadvantageous in another. Coupled with strategic rotation of control

agents (e.g., biopesticides, parasitoids, predators) aligned with pest developmental stages and local climatic constraints, this approach enhances the resilience of integrated pest management (IPM) strategies and supports the long-term efficacy of both chemical and biological controls. Simulation models showed crop type influences the success of rotation strategies (Chapter 3). Develop crop-specific IPM protocols that consider ecological interactions and the biology of both pest and control agents.

7.8 Limitations and future research

This thesis begins to highlight the resistance evolution potential in glasshouse whitefly and the effects on the effectiveness of commonly used biocontrol agents. Future laboratory and field experiments could enhance and expand the current findings and address the existing limitations of this thesis. Conducting quantitative genetics experiment in the laboratory and in the field to investigate the long-term effects on whitefly resistance evolution and life history costs would corroborate and enhance the findings of my laboratory experiments. The use of quantitative genetic experiments would also provide heritability estimates, that could be used to parametrise the IBM more accurately. Expanding IBM simulations to incorporate a broader range of environmental variables and biological interactions, would more accurately model real-world scenarios. As it was found in chapter 5 that resistance evolution has associated life history costs on population abundance, incorporating this as an additional parameter into the IBM would improve the accuracy of the IBM forecast on resistance evolution and population dynamics. Additionally, integrating molecular techniques to explore the genetic and maternal underpinnings of resistance mechanisms could enhance the understanding of how these traits manifest and spread within whitefly populations. This thesis acknowledges the absence of field trials to validate the findings, particularly in biopesticide efficacy and efficiency when applied in combination.

Although the mechanisms of resistance were not studied in this thesis, the results of multiple experiments suggest that general immune defence mechanisms could be at play, due to the potential encapsulation of the parasitoids egg, and the potential cross

resistance potential found in chapters 3. Using molecular techniques to sequence the whitefly would provide clear information on the potential mechanisms, genetic mutations, presence of endosymbionts are the cause for resistant traits.

As all of the experiments in my thesis were conducted under controlled conditions in a laboratory, the results may be an inflated evolutionary response compared to the real world. In the field there will be dispersal of potentially resistant or susceptible genotypes through immigration into the resistant population or emigrating out of the population, altering the rate and potential of resistance evolution (Pélissié *et al.*, 2018). (Chapter 3.1). The potential of resistant genotypes overwintering from previous generations, and increasing resistance potential, and the associated overwintering resistance costs (Carrière, *et al.*, 2001) could also play a role in the field. In the field any resistant individuals from the previous season that overwinter will emerge the following season, life history trade-offs might mean resistant individuals are lower in numbers early in the season, but their populations could rapidly increase when resistance becomes more important than surviving overwinter. Thus, conducting experiments in the field would allow for a better understanding and comparison to the anecdotal evidence.

Future research conducting comprehensive field trials would assess the real-world effectiveness and potential ecological impacts of tank mixing these pathogens. They would corroborate lab-based findings and consider the challenges in translating controlled experimental conditions to diverse agricultural settings. Addressing these limitations in future research efforts will be crucial to advancing sustainable pest management strategies and ensuring their practical application in agriculture.

7.9 Conclusion

While this thesis scratches the surface and provides an initial insight into understanding the effectiveness and durability of the biocontrol agents studied, it offers valuable insights into the necessity of considering resistance evolution and resistance management strategies when designing pest management regimes. The findings underscore the complexity of resistance evolution and the importance of

integrating ecological and evolutionary perspectives into biological control practices to maintain the long-term effectiveness of these methods.

This overarching theme of this thesis is that pests can evolve resistance to multiple biocontrol agents, however resistance evolution is complex and affected by a multitude of factors that do not necessarily occur in synthetic pesticide resistance evolution.

While both chemical and biological control strategies are vulnerable to resistance evolution, the inherent complexity and co-evolutionary dynamics of biological control agents often delay resistance development compared to the rapid, target-specific adaptations seen with chemical pesticides.

However, when the biocontrol agents are formulated or stocked in laboratories and factories the genetic variation is often unknown and lacking, thus the potential for co-evolution is likely to be low. Thus, in terms of the evolutionary potential for overcoming resistance, the biocontrol agents may be no better than chemical pesticides. This highlights the importance of proactive resistance management and integrated pest management strategies to sustain the long-term effectiveness of both approaches in agricultural systems

To get ahead and mitigate resistance requires biocontrol researchers and companies who formulate and produce biocontrol stock to be open and engage with researchers to develop resistance management techniques. By improving and maintaining genetic variation within the biocontrol stock, it could improve the long term effectiveness and slow the rate of resistance evolution. This would also benefit the companies economically in the longer term by improving the long term effectiveness of their products.

To achieve the broader goal of sustainable pest management, which is essential for ensuring crop protection and food security in the face of evolving pest populations, the long term effectiveness and efficiency of biocontrol agents needs to be ensured for an increase in grower uptake. Alongside resistance management strategies, the efficiency of the products must be considered to be less labour intensive and economically viable, with information available on potential tank mixes to aid growers and improve uptake.

This would ensure the long term effectiveness and efficiency of biocontrol agents, allowing a greater move to achieving sustainable pest management, which is essential for ensuring crop protection and food security.

7.10 Summary

I have demonstrated that glasshouse whitefly *Trialeurodes vaporariorum* can rapidly evolve resistance to a fungal biopesticide and the most commonly used biocontrol agents *Encarsia formosa* parasitoid wasps in a controlled environment setting. I have forecasted this rate of evolution over 140 generations in an IBM simulation factoring in crop type and alternating treatments as a potential resistance management technique. I ran a six generation selection experiment under controlled conditions exposing whitefly to strong *E. formosa* selection pressures, demonstrating that over 6 months replicable to a protected edible growing season whitefly can evolve resistance. In studying the efficacy and efficiency of biocontrol agents I demonstrated that under laboratory conditions 11 pairwise combinations of biopesticides had no detrimental effect on controlling two pests, glasshouse whitefly and the tomato leaf miner *Tuta absoluta*.

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8. Appendices

8.1 Clip cage design for chapter three

Before chapter three experiments commenced, clip cage trials were undertaken to ensure the damage to plants was minimal and whitefly adults could not escape. Three clip cages were created all using the same plastic tubs, with fine mesh fitted, with differing lid/clips (image 1). From the trials it was found that using a solid Perspex lid with a sponge ring ensured the lead was not ripped or damaged and the lid did not lift under the humid environmental conditions ensuring whitefly did not escape. This cage allowed the highest successful fecundity with eggs laid within the cage (image 3)

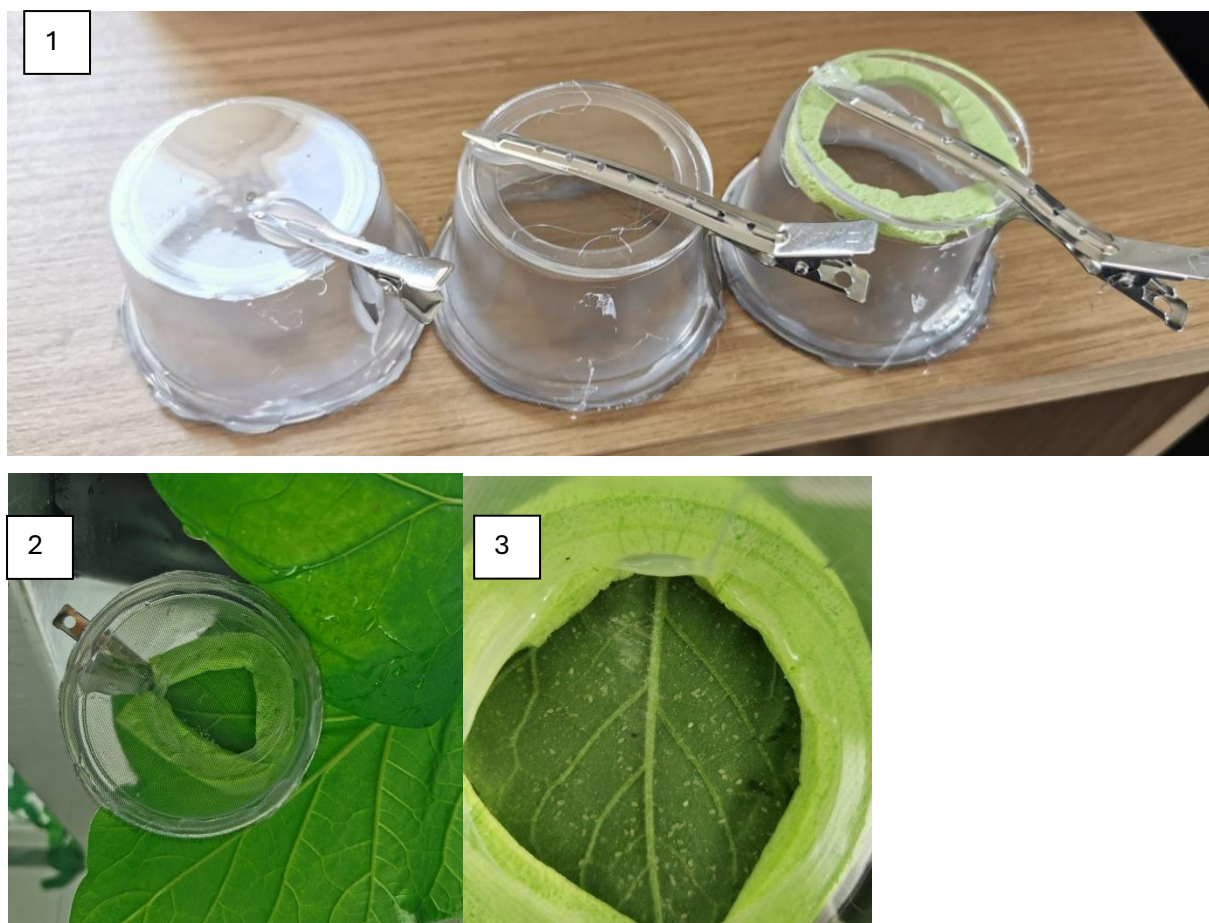


Figure 35: Image one shows the three clip cage designs trialled, image two shows the cage attached to an aubergine leaf, image three shows second/ third instar nymphs on a leaf inside the clip cage.

8.1.2 Reciprocal crossing scheme for line generation in chapter 3

The data showing the reciprocal crossing scheme used to generate individual lines. Initial founding populations were given an ID, where males from one population were crossed with females from a second population in both directions to give a Line ID, e.g females from population 30 were crossed with males from population 33, and females from population 30 were crossed with males from population 33, these were all given a Line ID of A. Clutch ID represents the Line, Female ID, Male ID and replicate ID. Clutch ID represents the successful clutch ID laid by the replicate of the line. Replicates of each line were split into one of the three treatments.

code	genotype	F	M	clutch	plant	treatment
A3033.1	A	30	33	1	A	Control
A3033.2	A	30	33	1	D	Fungal
A3033.2	A	30	33	2	D	Fungal
A3330.1	A	33	30	1	L	Wasp
AA2934.1	AA	29	34	1	F	Fungal
AA2934.1	AA	29	34	2	O	Wasp
AA2934.3	AA	34	29	1	J	Control
AA2934.3	AA	34	29	2	P	Control
AB1448.2	AB	14	48	1	P	Control
AB1448.1	AB	14	48	1	F	Fungal
AB4814.1	AB	48	14	1	R	Wasp
AC26.2	AC	2	6	1	J	Control
AC26.3	AC	2	6	1	H	Fungal
AC26.2	AC	2	6	2	O	Wasp
AC26.3	AC	2	6	2	R	Wasp
AD1147.1	AD	11	47	1	F	Fungal
AD1147.1	AD	11	47	2	L	Wasp
AD4711.3	AD	47	11	1	J	Control
AD4711.1	AD	47	11	1	S	Fungal
AE623.2	AE	62	3	1	J	Control
AE623.1	AE	62	3	1	G	Fungal
AE623.2	AE	62	3	2	O	Wasp
AF3582.1	AF	35	82	1	K	Control
AF3582.1	AF	35	82	2	AG	Fungal
AF3582.2	AF	35	82	1	L	Wasp
AG2075.1	AG	20	75	1	K	Control
AG2075.1	AG	20	75	2	AG	Fungal
AG2075.2	AG	20	75	1	L	Wasp
AH6145.1	AH	61	45	1	K	Control
AH6145.2	AH	61	45	2	AC	Fungal
AH6145.2	AH	61	45	1	L	Wasp
AJ6668.1	AJ	66	68	2	AH	Control
AJ6668.2	AJ	66	68	2	AI	Fungal
AJ6668.1	AJ	66	68	1	X	Wasp
AJ6668.2	AJ	66	68	1	Y	Wasp

AK1170.1	AK	11	70	1	Z	Fungal
AK7011.1	AK	70	11	2	AH	Control
AK7011.2	AK	70	11	2	AI	Fungal
AK7011.1	AK	70	11	1	X	Wasp
AK7011.2	AK	70	11	1	Y	Wasp
AL6487.1	AL	64	87	1	AA	Control
AL8764.1	AL	87	64	1	AH	Control
AL8764.3	AL	87	64	2	AJ	Fungal
AL8764.3	AL	87	64	1	Z	Fungal
AL8764.2	AL	87	64	1	Y	Wasp
B82.1	B	8	2	1	A	Control
B82.1	B	8	2	2	E	Fungal
B82.2	B	8	2	1	D	Fungal
B82.3	B	8	2	1	L	Wasp
C510.1	C	5	10	1	A	Control
C510.2	C	5	10	1	E	Fungal
C105.1	C	10	5	1	I	Wasp
D2513.3	D	25	13	1	H	Control
D2513.2	D	25	13	2	E	Fungal
D2513.2	D	25	13	1	B	Wasp
E732.1	E	7	32	1	G	Control
E732.3	E	7	32	2	Q	Fungal
E732.3	E	7	32	1	I	Wasp
F1742.1	F	17	42	1	G	Control
F1742.1	F	17	42	2	O	Fungal
F4217.1	F	42	17	1	H	Control
F4217.2	F	42	17	1	I	Wasp
G1214.1	G	12	14	1	K	Control
G1214.1	G	12	14	2	P	Wasp
G1412.1	G	14	12	1	J	Fungal
H1829.1	H	18	29	1	G	Control
H1829.2	H	18	29	1	J	Fungal
H1829.2	H	18	29	2	Q	Fungal
H2918.2	H	29	18	1	P	Wasp
I2734.1	I	27	34	1	G	Control
I2734.2	I	27	34	1	H	Control
I2734.2	I	27	34	2	P	Wasp
I3427.1	I	34	27	2	Q	Fungal
I3427.2	I	34	27	1	J	Fungal
I3427.1	I	34	27	1	I	Wasp
J938.1	J	9	38	1	H	Control
J938.2	J	9	38	2	Q	Fungal
J938.2	J	9	38	1	I	Wasp
J389.1	J	38	9	1	G	Control
K1920.1	K	19	20	1	G	Control
K1920.1	K	19	20	2	O	Fungal
K1920.3	K	19	20	1	I	Wasp
K2019.1	K	20	19	1	J	Fungal

L437.1	L	4	37	1	O	Fungal
L437.2	L	4	37	1	D	Wasp
L374.2	L	37	4	1	E	Control
L374.1	L	37	4	1	A	Wasp
M3849.1	M	38	49	1	E	Control
M4938.2	M	49	38	1	P	Fungal
M4938.1	M	49	38	1	A	Wasp
N4650.2	N	46	50	1	G	Control
N4650.1	N	46	50	1	O	Fungal
N5046.2	N	50	46	1	E	Control
N5046.1	N	50	46	1	D	Wasp
O229.1	O	2	29	1	G	Control
O229.3	O	2	29	1	I	Fungal
O229.2	O	2	29	1	H	Wasp
O292.1	O	29	2	1	J	Fungal
P331.1	P	3	31	1	I	Fungal
P313.1	P	31	3	1	G	Control
P313.2	P	31	3	1	H	Wasp
R1740.1	R	17	40	1	L	Wasp
R4017.2	R	40	17	1	K	Control
R4017.1	R	40	17	1	J	Fungal
S1845.1	S	18	45	1	M	Fungal
S1845.2	S	18	45	1	O	Fungal
S1845.1	S	18	45	2	Q	Wasp
S4518.1	S	45	18	1	N	Control
U418.1	U	41	8	2	Q	Control
U418.2	U	41	8	1	O	Fungal
U418.3	U	41	8	1	P	Fungal
U418.1	U	41	8	1	N	Wasp
V1654.3	V	16	54	1	C	Control
V1654.1	V	16	54	1	A	Fungal
V1654.2	V	16	54	1	T	Fungal
V1654.3	V	16	54	2	U	Wasp
V5416.1	V	54	16	1	D	Wasp
W3860.1	W	38	60	1	K	Control
W3860.3	W	38	60	1	F	Fungal
W3860.3	W	38	60	2	W	Wasp
W6038.3	W	60	38	1	C	Control
W6038.1	W	60	38	1	A	Fungal
W6038.2	W	60	38	1	T	Fungal
W6038.3	W	60	38	2	U	Wasp
X751.3	X	7	51	1	C	Control
X751.5	X	7	51	1	E	Control
X751.1	X	7	51	1	A	Fungal
X751.2	X	7	51	1	B	Fungal
X751.4	X	7	51	1	D	Wasp
X751.5	X	7	51	2	W	Wasp
Y2550.1	Y	25	50	1	C	Control

Y2550.2	Y	25	50	2	K	Control
Y2550.2	Y	25	50	1	D	Wasp
Y5025.1	Y	50	25	1	A	Fungal
Y5025.1	Y	50	25	2	W	Wasp
Z823.1	Z	8	23	1	A	Fungal
Z823.1	Z	8	23	2	W	Wasp
Z238.3	Z	23	8	1	E	Control
Z238.3	Z	23	8	2	S	Fungal

Table 8: The individuals crossed in the reciprocal crossing scheme for each individual line.

Replicate ID	Line	Female adult ID	Male adult ID	Clutch ID	Plant ID	Treatment
A3033.1	A	30	33	1	A	Control
A3033.2	A	30	33	1	D	Fungal
A3033.2	A	30	33	2	D	Fungal
A3330.1	A	33	30	1	L	Wasp
AA2934.1	AA	29	34	1	F	Fungal
AA2934.1	AA	29	34	2	O	Wasp
AA2934.3	AA	34	29	1	J	Control
AA2934.3	AA	34	29	2	P	Control
AB1448.2	AB	14	48	1	P	Control
AB1448.1	AB	14	48	1	F	Fungal
AB4814.1	AB	48	14	1	R	Wasp
AC26.2	AC	2	6	1	J	Control
AC26.3	AC	2	6	1	H	Fungal
AC26.2	AC	2	6	2	O	Wasp
AC26.3	AC	2	6	2	R	Wasp
AD1147.1	AD	11	47	1	F	Fungal
AD1147.1	AD	11	47	2	L	Wasp
AD4711.3	AD	47	11	1	J	Control
AD4711.1	AD	47	11	1	S	Fungal
AE623.2	AE	62	3	1	J	Control
AE623.1	AE	62	3	1	G	Fungal
AE623.2	AE	62	3	2	O	Wasp
AF3582.1	AF	35	82	1	K	Control
AF3582.1	AF	35	82	2	AG	Fungal
AF3582.2	AF	35	82	1	L	Wasp
AG2075.1	AG	20	75	1	K	Control
AG2075.1	AG	20	75	2	AG	Fungal
AG2075.2	AG	20	75	1	L	Wasp
AH6145.1	AH	61	45	1	K	Control
AH6145.2	AH	61	45	2	AC	Fungal
AH6145.2	AH	61	45	1	L	Wasp
AJ6668.1	AJ	66	68	2	AH	Control
AJ6668.2	AJ	66	68	2	AI	Fungal

AJ6668.1	AJ	66	68	1	X	Wasp
AJ6668.2	AJ	66	68	1	Y	Wasp
AK1170.1	AK	11	70	1	Z	Fungal
AK7011.1	AK	70	11	2	AH	Control
AK7011.2	AK	70	11	2	AI	Fungal
AK7011.1	AK	70	11	1	X	Wasp
AK7011.2	AK	70	11	1	Y	Wasp
AL6487.1	AL	64	87	1	AA	Control
AL8764.1	AL	87	64	1	AH	Control
AL8764.3	AL	87	64	2	AJ	Fungal
AL8764.3	AL	87	64	1	Z	Fungal
AL8764.2	AL	87	64	1	Y	Wasp
B82.1	B	8	2	1	A	Control
B82.1	B	8	2	2	E	Fungal
B82.2	B	8	2	1	D	Fungal
B82.3	B	8	2	1	L	Wasp
C510.1	C	5	10	1	A	Control
C510.2	C	5	10	1	E	Fungal
C105.1	C	10	5	1	I	Wasp
D2513.3	D	25	13	1	H	Control
D2513.2	D	25	13	2	E	Fungal
D2513.2	D	25	13	1	B	Wasp
E732.1	E	7	32	1	G	Control
E732.3	E	7	32	2	Q	Fungal
E732.3	E	7	32	1	I	Wasp
F1742.1	F	17	42	1	G	Control
F1742.1	F	17	42	2	O	Fungal
F4217.1	F	42	17	1	H	Control
F4217.2	F	42	17	1	I	Wasp
G1214.1	G	12	14	1	K	Control
G1214.1	G	12	14	2	P	Wasp
G1412.1	G	14	12	1	J	Fungal
H1829.1	H	18	29	1	G	Control
H1829.2	H	18	29	1	J	Fungal
H1829.2	H	18	29	2	Q	Fungal
H2918.2	H	29	18	1	P	Wasp
I2734.1	I	27	34	1	G	Control
I2734.2	I	27	34	1	H	Control
I2734.2	I	27	34	2	P	Wasp
I3427.1	I	34	27	2	Q	Fungal
I3427.2	I	34	27	1	J	Fungal
I3427.1	I	34	27	1	I	Wasp
J938.1	J	9	38	1	H	Control
J938.2	J	9	38	2	Q	Fungal
J938.2	J	9	38	1	I	Wasp
J389.1	J	38	9	1	G	Control

K1920.1	K	19	20	1	G	Control
K1920.1	K	19	20	2	O	Fungal
K1920.3	K	19	20	1	I	Wasp
K2019.1	K	20	19	1	J	Fungal
L437.1	L	4	37	1	O	Fungal
L437.2	L	4	37	1	D	Wasp
L374.2	L	37	4	1	E	Control
L374.1	L	37	4	1	A	Wasp
M3849.1	M	38	49	1	E	Control
M4938.2	M	49	38	1	P	Fungal
M4938.1	M	49	38	1	A	Wasp
N4650.2	N	46	50	1	G	Control
N4650.1	N	46	50	1	O	Fungal
N5046.2	N	50	46	1	E	Control
N5046.1	N	50	46	1	D	Wasp
O229.1	O	2	29	1	G	Control
O229.3	O	2	29	1	I	Fungal
O229.2	O	2	29	1	H	Wasp
O292.1	O	29	2	1	J	Fungal
P331.1	P	3	31	1	I	Fungal
P313.1	P	31	3	1	G	Control
P313.2	P	31	3	1	H	Wasp
R1740.1	R	17	40	1	L	Wasp
R4017.2	R	40	17	1	K	Control
R4017.1	R	40	17	1	J	Fungal
S1845.1	S	18	45	1	M	Fungal
S1845.2	S	18	45	1	O	Fungal
S1845.1	S	18	45	2	Q	Wasp
S4518.1	S	45	18	1	N	Control
U418.1	U	41	8	2	Q	Control
U418.2	U	41	8	1	O	Fungal
U418.3	U	41	8	1	P	Fungal
U418.1	U	41	8	1	N	Wasp
V1654.3	V	16	54	1	C	Control
V1654.1	V	16	54	1	A	Fungal
V1654.2	V	16	54	1	T	Fungal
V1654.3	V	16	54	2	U	Wasp
V5416.1	V	54	16	1	D	Wasp
W3860.1	W	38	60	1	K	Control
W3860.3	W	38	60	1	F	Fungal
W3860.3	W	38	60	2	W	Wasp
W6038.3	W	60	38	1	C	Control
W6038.1	W	60	38	1	A	Fungal
W6038.2	W	60	38	1	T	Fungal
W6038.3	W	60	38	2	U	Wasp
X751.3	X	7	51	1	C	Control

X751.5	X	7	51	1	E	Control
X751.1	X	7	51	1	A	Fungal
X751.2	X	7	51	1	B	Fungal
X751.4	X	7	51	1	D	Wasp
X751.5	X	7	51	2	W	Wasp
Y2550.1	Y	25	50	1	C	Control
Y2550.2	Y	25	50	2	K	Control
Y2550.2	Y	25	50	1	D	Wasp
Y5025.1	Y	50	25	1	A	Fungal
Y5025.1	Y	50	25	2	W	Wasp
Z823.1	Z	8	23	1	A	Fungal
Z823.1	Z	8	23	2	W	Wasp
Z238.3	Z	23	8	1	E	Control
Z238.3	Z	23	8	2	S	Fungal

8.2 The potential for resistance evolution to biological control agents over six generations data

8.2.1 Set up dates generation 1-6

The selection experiment from generation 1 to generation 6 took 6 months to complete from start to finish. Each generation took approximately 26 days from set up to mortality counts.

Table 9: The start days of each generation in the selection experiment

Generation	Set up date
1	12/10/2022
2	07/11/2022
3	03/12/2022
4	29/12/2022
5	24/01/2023
6	19/02/2023

8.2.2 The founding size of each generation in the experiment

For the initial set up generation one lines were established from a single cage containing two plants with 100 whitefly with a 0.5 sex ratio. The adult whitefly that emerged from generation one were counted, sexed and used to establish the next generation (generation 2). I took the first 76-100 adults (due to variation in the total number of adults emerged) to initiate the next generation, with the total number added remaining the same for all lines in the same generation (based on the replicate line with the fewest emerging individuals, i.e. all replicates in generation 1 used N=76 whitefly to found generation 2. I added these whitefly to a new cage to start the next generation adding equal numbers of males and females to keep a sex ratio of 0.5.

Table 10: The founding number of adults for generations 1-6 in the selection experiment

Generation	Founding number of adults	Number of males	Number of females
1	100	50	50
2	76	38	38
3	84	42	42
4	78	39	39
5	64	32	32
6	72	36	36

8.3 Assessing the impacts of tank-mixing on biopesticide efficacy

Chapter six Assessing the impacts of tank-mixing on biopesticide efficacy was undertaken as part of an AHDB/BBSRC partnership grant that aimed to support the agricultural transition to net zero, this project was titled best-practice for tank-mixing biopesticides. The grant was led by Dr Matthew Tinsley, with a meta-analysis conducted by Dr Lucy Nevard. The empirical data experiment was designed by myself alongside Dr Matthew Tinsley. I conducted all data collection alongside the laboratory technician Pauline Blakie. I conducted all data analysis and the write up of the results that fed into a wider report.

8.4 R code for thesis statistics

<https://github.com/MiaGraham24>