# Genetic control methods for agricultural insect pests of global importance

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**“That was fun.  Let’s never do that again”**

- Chris Pontius

## Abstract

Insect pests of agricultural significance pose substantial risks for food security in an ever-growing global population. Conventional control measures used against these pests have had varying degrees of success and examples of pesticide resistance and off-target effects of pesticides highlight the urgent need for the development of new, environmentally benign control methods. Deployment of 'self-limiting' insects is a species-specific approach that can be used to combat many species, including two major agricultural insect pests, the Medfly, *Ceratitis capitata*, and the Diamondback moth (DBM), *Plutella xylostella*. In this thesis, I used transgenic 'self-limiting' strains of medfly and DBM to stress-test self-limiting technology in laboratory and field scenarios. In **Chapter 2**, I tested the effect of larval diet composition on the penetrance of a female-specific self-limiting system in the OX3864A strain of medfly under simulated control conditions. In **Chapter 3** I investigated the potential for resistance to self-limiting systems, using artificial selection for survival under a low dose of the transgene antidote, in the OX3864A medfly strain. In **Chapter 4** I used the OX4319L self-limiting strain of DBM and showed that its responses to an artificial pheromone source in wind tunnel flight trials were comparable to the wild type. I also described the field dispersal characteristics of a long-term, laboratory-reared wildtype DBM strain in a mark-release-recapture trial. In **Chapter 5** I demonstrated that the OX4319L DBM strain had comparable field longevity, but reduced mating competitiveness, in comparison to a wild-caught DBM strain. Finally, in **Chapter 6**, I discuss the broader context and address the practicalities, regulatory controls and implications of transgenic technologies for insect pest control under open field conditions.

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# 1 General Introduction

## 1.1 Summary

This thesis is a summary of my research into the control of insect pests of agricultural importance using the environmentally benign method of Release of Insects carrying a Dominant Lethal (RIDL; Thomas *et al.*, 2000). RIDL insects have been genetically engineered to contain a genetic construct with contains a self-limiting gene, causing lethality in all homo- and heterozygotes. RIDL constructs have been engineered by Oxitec Ltd into multiple insect pests including the focal species I investigated in this thesis: the Mediterranean fruit fly, *Ceratitis capitata* (Weidemann) and the Diamondback moth, *Plutella xylostella* (Linnaeus) (Fu *et al.*, 2007; Jin *et al.*, 2013). The thesis describes laboratory and field-testing of Oxitec Ltd lead strains of both species.

In this introductory chapter, I discuss the problems posed by insect pests globally and how they might be controlled. Traditional control methods, for example, the use of broad-spectrum pesticides and the sterile insect technique (SIT), are discussed at length, highlighting the advantages and disadvantages of each approach. Ultimately, traditional control methods often fall short of the level of control needed to secure global food supplies and to protect the public from insect-borne disease vectors (e.g. Campos *et al.*, 2015). They also often have significant negative environmental impacts. Collectively, this has led to an urgent requirement for new options for pest control approaches, which are highly species specific, effective and environmentally benign.

I discuss some of the newer measures being used and considered for control, for example, *Wolbachia*, CRISPR systems and RNA interference (RNAi). Each of these is discussed in the context of both self-limiting suppression and population replacement mechanisms. One of the newer genetic approaches to insect pest control is RIDL, which is generally thought to provide significant improvements over traditional techniques such as the Sterile Insect Technique (SIT). The potential for RIDL to offer an environmentally benign, efficient and economically viable method of pest control is then discussed.

## 1.2 Global insect pests

Insect pest species cost global economies billions of dollars each year. Broadly speaking, insect pests can be categorised into two groups: species of significance from a public health perspective (i.e. disease vectors) and those of significance from for agriculture (i.e. crop pests). These two distinct groups of pests incur costs to economies both in terms of mounting the pest control required and in countering the damage caused by the pest. Whilst control measures for these groups of pests may be similar, the effects or damage caused can vary dramatically, although all such pests may cause significant knock-on effects for human populations. The protection of people from insect-borne disease, as well as the safeguarding of global food supplies, are grand challenges and are of paramount importance to an ever-growing global population.

Insect pests come from a variety of orders across the Insecta, including Diptera and Lepidoptera. The Tephritid family, i.e. the 'true' fruit flies, is one of the most devastating globally, with over 1400 of 5000 known species known to oviposit in fleshy fruits, many of which have potential agricultural importance (Malacrida *et al.*, 2007). One such pest species of significant economic importance is the Mediterranean fruit fly *Ceratitis capitata* (Diptera: Tephritidae), commonly known as the medfly. Another globally significant pest of cruciferous vegetables is the Diamondback moth, *Plutella xylostella*, (Lepidoptera: Plutelidae). Due to increases in global fruit and vegetable trade and distribution, in recent years these two species have rapidly expanded their ranges. This has resulted in the damage and control costs of targeting these two species being amongst the highest for agricultural pests. Accordingly, there is an extensive and growing research base into the behaviour, ecology, control and distribution of these pests. In particular, the medfly has been a key model for understanding traits that underlie adaptation to new environments in new invading species (as discussed by Malacrida *et al.*, 2007; Lee, 2002; Lockwood et al., 2005). I introduce the major features of each of the two focal pests used in this thesis, below.

*1.2.1 Diamondback Moth*

Diamondback moths infest cruciferous vegetables across the globe, causing an estimated management cost of upwards of US $1 billion annually (Talekar *et al.*, 1992), with some estimates putting that figure at US $4-5 billion (Zalucki *et al.*, 2012). If untreated, DBM has the potential to cause losses of approximately US $70 million to the Texas cruciferous vegetable market alone. It is estimated that much of the New York and Californian cruciferous vegetable industries, worth over US $580 million, would produce unmarketable produce without such control measures being in place (Shelton, 2001). Cruciferous vegetables form a widespread global food source and include common species such as *Brassica oleracea*, cultivars of which include cabbage, broccoli, brussel sprouts, cauliflower, collard and kale; *B. napus*, rapeseed; and *B. rapa*, turnips, pak choi and Chinese cabbage. As well as many these cultivars, DBM will also infest large numbers of cruciferous weeds in the absence of more preferable host species (Talekar and Shelton, 1993). The cruciferous vegetable industry makes up US $26 billion of the global economy (FAO, 2012), from small-scale subsistence farming to large-scale farming industries. For this reason, it is widely reported that cruciferous vegetables make up the most commonly eaten vegetables across Asia, as well as forming a key part of diets across other cultures (e.g. Talekar and Shelton, 1993).

Female DBM begin to lay eggs on both the upper and lower surfaces of cruciferous foliage soon after mating and continue to lay for around four days, producing up to 188 eggs (as discussed in Talekar and Shelton, 1993). After hatching, first larval instars quickly begin consuming the foliage on which they were laid as eggs. Although temperatures ultimately dictate exact development times (Liu *et al.*, 2002), approximately 4-6 days is typically spent in each of four larval instars. Once feeding has been completed, fourth larval instars construct silk cocoons on the surface of leaves, in which individuals undergo pupation. The time of pupation is temperature dependent, with adults emerging between 4-15 days later (Talekar and Shelton, 1993). Emerging adults feed only on water droplets or dew and do not consume other nutrients.

DBM are thought to have originated in the Mediterranean basin, although they have now spread across the globe and can occur wherever a suitable host is grown. The

hosts inhabit both temperate and tropical regions globally. DBM are able to overwinter in areas with warmer climates, but must annually invade areas with colder winters (Talekar and Shelton, 1993). Reports of long-distance migration by DBM are common (e.g. Chapman *et al.*, 2002; Coulson *et al.*, 2002). However, there is no record of any migration of any of its parasitoids (Talekar and Shelton, 1993). This has led to the unchallenged spread of these naturally highly polyphagous pests.

### *1.2.2 Mediterranean fruit fly*

The Mediterranean fruit fly is a polyphagous species of Tephritid that can infest over 300 known species of fruit, vegetables and nuts (Hooper and Robinson, 1989; USDA, 2006). Many of these plant species form major global food sources. Mated females lay small batches of eggs just below the skin of these plant hosts, although they preferentially use existing cracks or wounds for egg laying, if present (Papaj *et al.*, 1989; Papaj *et al.*, 1992). Females will oviposit into a wide variety of hosts, even if those hosts do not support larval development (Carey, 1984), although there is also some evidence of host-choice learning in female medfly (Cooley *et al.*, 1986).

Once laid, eggs remain unhatched for approximately 48 hr at 25 °C (Messenger and Flitters, 1958; Shoukry and Hafez, 1979; Carey, 1984) before first instar larvae begin feeding on the flesh of the host. Females can lay upwards of 1000 eggs in a lifetime, depending on the strain, with survivorship of these eggs being host dependent (Carey, 1984). Three stages of larval instar develop whilst feeding on the host before pupation. The larval pupation time, i.e. time from hatching to pupation, is dependent on the nutrient content of the host, but typical values range between 7-14 days (Carey, 1984). Interestingly, eclosion time, i.e. the time between pupation and eclosion of an adult fly, is not host dependent and appears to remain constant at around 11-13 days (Carey, 1984).

Due to its large economic impact on global food markets, research into the medfly is widespread and extensive, encompassing a wide variety of different fields of study, from reproductive biology to gut microbiota and population control. Medfly has proven a useful tool for studying the evolution of life history traits in general, due to its

widespread distribution, well-studied dispersal pattern and well-documented population genetics (Diamantidis *et al.*, 2008; Diamantidis *et al.*, 2009).

Carey (1984) discusses the success of the medfly as an invasive species, and hypothesises four reasons for this: 1. multiple, highly overlapping generations; 2. high reproductive output in young individuals; 3. high larval fitness in certain hosts; and 4. lack of diapause. Other authors have also identified the ability of the medfly to cope with adverse weather and their polyphagous nature as key to its success (Dyck *et al.*, 2006). All of these factors combined help to show why the medfly is now the most globally economically significant of all the Tephritids and potentially of all insect pests (NASS-USDA, 2006).

### 1.3 Traditional insect control methods

Due to the economic impact on the plant hosts that both the medfly and DBM infest, pest control measures against these species have become an industry in themselves, with farmers and corporations willing to invest large sums of money in order to protect their crops from damage. Many approaches for control, each with varying degrees of success, have been used and are summarised below.

#### *1.3.1 Bait trapping*

Bait trapping involves the attraction, and subsequent killing of individuals in a pest population using substances attractive to the pest. This method can be used as both a control method (e.g. Haniotakis *et al.*, 1991) and a population tracking method (e.g. Brockerhoff *et al.*, 2006). The baits used may be pheromone-based (Haniotakis *et al.*, 1991), or food-based, e.g. sugar baits (e.g. Müller *et al.*, 2008). The traps used often contain an insecticidal chemical, which has the ability to kill the pest that is caught.

The potential for bait and mass trapping as control methods suitable for long-term, large-scale pest populations is discussed in depth by El-Sayed *et al.* (2006), who gathered metadata from studies involving these types of techniques. The authors concluded that bait trapping provides a viable population control method when used against isolated, low density pest populations. They found however, that all papers

examined contained at least one aspect that detracted from the efficacy of the control methods implemented. The key factors highlighted by El-Sayed *et al.* for successful implementation of these techniques include optimum pheromone blend, pheromone dose and the efficacy of the pheromone dispenser, as well as having an effective trap, and the process costing less than the economic limit, which may vary between pest species.

All in all, this means that whilst bait trapping may be viable for low-density, isolated pest populations, it is a hard process to make effective against large-scale, high-density agricultural pests. The species-specific problems such as optimum pheromone dose and the problem of producing an appropriate pheromone blend may also cause this type of method to lack transferability between pest species, and whilst effective against some species, may lack efficacy across the multitude of pest species seen globally.

*1.3.2 Trap and intercropping*

Trap cropping and intercropping are two traditional techniques designed to keep insect crop damage to a minimum. Trap cropping is the planting of less economically important crops, that are highly attractive to the pest species, in small numbers in areas around the main crop. The idea is that this will lure pest insects away from the main crop and reduce crop damage. Some trap crops even have the advantage of causing larval mortality and therefore reducing a pest population even further. For example, 1-10 % coverage of a trap crop in a soybean field can attract an estimated 70-85 % of the resident stinkbug population (McPherson and Newsom, 1984). Intercropping is a similar procedure by which interspersed crops are used as 'distractions', or physical barriers that stop insect pests from causing damage to the main crop.

Trap cropping and intercropping techniques lack the broad spectrum applicability of some of the other pest management approaches, which makes them less effective when used in agricultural scenarios where control is needed for more than one pest species. This is because they tend to be relatively species-specific, with trap crops only able to attract a certain species of pest. These techniques may also increase control

costs, with cheaper options available. Trap crops may require a different planting time, and different fertiliser and herbicide needs to the main trap crop.

Effective trap crops have been evaluated with regards to pest DBM populations, with a number of potential trap crops being tested (e.g. Badenes-Perez *et al.*, 2004), with yellow rocket being considered the most favourable trap crop. Yellow rocket is highly attractive to ovipositing DBM females, but also does not allow developing larvae to survive to pupation. Planting a cabbage field with 10 % yellow rocket is enough to significantly reduce DBM populations within that field (Badenes-Perez *et al.*, 2005). These examples of effective trap cropping approaches are rarely used in large-scale agriculture, with only a small number of trap crops being used globally as a long-term pest management system (Shelton and Badenes-Perez, 2006), although this number is rising.

As discussed for bait trapping, trap cropping may be most effective against isolated, low-density pest populations. At a larger scale, it may be economically less viable than alternative control approaches against some pest species. Shelton and Badenes-Perez (2006) discuss how these types of control methods are less attractive to some forms of research because they have no end product, as in the case of insecticide, and therefore have a lower commercial value. Therefore, it is essential that these types of control methods are explored fully, as they often carry a lesser environmental burden than the broad-spectrum insecticidal alternatives. Trap cropping is discussed in more detail in reviews by Shelton and Badenes-Perez (2006) and Hokkanen (1991).

### *1.3.3 Insecticides*

The use of synthetic insecticides against insect pest populations is widespread across the globe. Despite recent developments in non-pesticidal strategies, control of many insects is only possible due to the use of these chemicals. Unfortunately, in many parts of the developing world there is little or no regulation of the use of chemical control measures. Insecticides are often relatively cheap, sometimes subsidized and often overused or misused on the wrong crop type. This can lead to complete dependence on synthetic insecticides for control of some species of insect. This is perhaps most evident in the global crucifer industry and reliance on synthetic insecticides against

DBM, as discussed by Talekar and Shelton (1993). These authors describe situations in which insecticides are applied up to once every two days in some parts of the tropics, far above the intended level of use.

This culture of overuse has led to the rapid development of insecticide resistance across genera of insect pests, with the number of cases of resistance increasing rapidly. Some key insect pest species now show resistance to all major classes of insecticides (ARPD, 2012). Life history traits of insect pests often lead them to be predisposed to developing resistance traits in relatively short periods of time. For example, large numbers of progeny per generation and multiple generations per year can allow effective and rapid responses in insects to the strong and constant selection pressure for resistance alleles. The DBM is now reported to show resistance to 93 commonly used insecticides around the world (ARPD, 2012) meaning it is now the most resistant of all the Lepidoptera. Whilst the problem may not be so widespread in other pest species, cases of resistance are continuing to develop, making control increasingly difficult. For example, examples of resistance have now been reported in medfly to each of the three key insecticides, namely malathion (Magaña *et al.*, 2007), lindane (Couso-Ferrer *et al.*, 2011) and cyhalothrin-lambda (Arouri *et al.*, 2015).

The issue of resistance has a direct effect on the efficacy of insecticide applications in control. However, insecticides can also have other, off-target effects that make them damaging to parts of the ecosystem other than the intended pest insect. Many insecticides are broad-spectrum, and hence by their nature non-species-specific. This means that as well as lowering pest populations, insecticides also reduce in numbers the natural enemies of those pest species (e.g. Theiling and Croft, 1988) as well as key pollinators and other beneficial arthropods (Desneux *et al.*, 2007).

### *1.3.4 Integrated pest management (IPM)*

Due to the rapid spread of insecticide resistance through some insect pest populations, developments in control methods across a wide spectrum of different approaches are on-going and essential. The development of integrated pest management (IPM) techniques has seen an increase in the use of multiple control measures simultaneously, as well as the use of thresholds to determine when these measures

should be used. IPM aims to allow pest populations to occur at levels below those that cause economic injury to crops, but whilst using all suitable control methods that allow for considerations of population dynamics of the pest, and any associated environmental impact (Kogan, 1998). The term 'management' refers to a set of decision-making criteria based on the ecological system, as well as the economic and social consideration, which lead to the production of an economic injury level, i.e. a level of acceptable pest numbers, or damage to crops, above which action must be taken (Pedigo and Higley, 1992; Higley and Pedigo, 1993).

### *1.3.5 Sterile Insect Technique*

The sterile insect technique (SIT) is an important control method that was first developed in the 1950s by Knipling (1955). The premise of SIT is the use of factory-reared, sterile individuals, which are released into wild pest populations. Any matings with these released sterile individuals result in no viable offspring being produced. The overall reproductive output of the population as a whole is thus reduced. SIT has the ability to provide a species-specific approach to insect pest control (Hendrichs *et al.*, 1995; Krafsur, 1998) that is advantageous over the broad-spectrum approach of many classes of insecticide.

The idea for releasing individuals to cause sterility within a wild population was conceived by three separate individuals during the 1930s and 1940s - Knipling himself using ionising radiation in the US, Serebrovskii in the USSR using chromosome translocations, and Vanderplank in Tanzania using hybrid sterility (Dyck *et al.*, 2006). Previous work by Runner (1916) and Muller (1927) had shown that ionising radiation could cause visible mutations to *Drosophila spp.* and that large doses of X-rays caused a reduced reproductive capability in the cigarette beetle, *Lasioderma serricorne*.

One of the first applications of SIT, and perhaps the most notable, was against a wide-scale pest of livestock and humans, the New World screwworm, *Cochliomyia hominivorax*. This pest species is present in large numbers in the southern states of the US, as well as in Central and South America. In the larval form, it feeds on the living flesh of warm-blooded mammals. Knipling (1955) observed that males showed strong competition behaviours during mating and, once mated, females would not mate with

another male. This led to the hypothesis that if competitive, sterile individuals were released over consecutive generations and produced zero, or few, offspring, then the number of wildtype individuals would decline rapidly. New World screwworm individuals for release were first sterilized using 50 Gy of ionising radiation. This produced adults that appeared physically normal, but when mated to non-irradiated individuals produced very few viable offspring (Bushland and Hopkins, 1953).

Successful SIT trials of New World screwworm were carried out first in Curaçao (Baumhover *et al.*, 1955), an island off the coast of Venezuala, and then across the region. This led to the backing of SIT by the US government, with state-backed release programmes set up in Florida, spreading westwards across Texas, Arizona and California, where the screwworm was estimated to be causing US $100 million worth of damage annually (Novy, 1991). The spread of the use of SIT against the screwworm continued, with successful eradication programmes set up as far south as Panama (Wyss, 2000) as well as some programmes outside of the Americas, including in Libya (Lindquist *et al.*, 1992).

The success of SIT in the screwworm, and its reliance on simple biological mechanisms, made it applicable in a number of different species of economic importance. Subsequent, successful SIT projects have been set up against the Mexican fruit fly (Dyck *et al.*, 2006), the melon fly (Kuba *et al.*, 1996), the Queensland fruit fly in Eastern Australia (Dominiak *et al.*, 2000) and the medfly (Shelly *et al.*, 1994). However, whilst the success of SIT is evident, there have been some notable failures, particularly against mosquito pests (discussed by Benedict and Robinson, 2003).

SIT is generally considered to be more effective when only males are released, with some studies suggesting that unisex releases are 3-5 times more effective than bisex (Rendón *et al.*, 2004). The release of one sex only is advantageous for SIT programmes in two ways. Firstly, it removes the 'distraction' effect of assortative mating when irradiated males and irradiated females are co-released. SIT relies on sterile individuals mating with wild individuals in order to be effective, if sterile males are released with only other males, then they are forced to seek out wild females to mate with, and cannot mate with the co-released sterile females. Secondly, it is often females of insect pests that cause the majority of the damage. In agricultural pests, it is the

female that lays the eggs through the host epidermis that subsequently hatch into destructive crop pests. The male is often effectively harmless (Kender, 2004). Likewise, in pests of public health interest such as mosquitoes, it is the female that takes a blood meal after mating and therefore spreads insect-borne diseases by biting. Therefore, releasing large numbers of females into the environment, even if those females are sterile, is a potential health risk.

One obvious drawback in traditional SIT programmes is the use of ionising radiation to induce sterility. The irradiation treatment can be severely detrimental to the overall fitness of treated males (Briceño and Eberhard, 1998; Briceño *et al.*, 2002; Parker and Mehta, 2007). The mass-reared males may also have further reduced fitness due to their history of laboratory rearing and unnatural, artificial mating environments. In the medfly, sterilised males are generally thought to have a 4- to 10-fold reduction in overall fitness resulting from the sterilization and exposure to ionising radiation as well as from adaptation to factory and not wild environments (Lance *et al.*, 2000; Shelly *et al.*, 1994). The reduction in fitness of released, sterile individuals is usually alleviated, at least to some extent, by simply increasing the overflooding ratio at which sterile males are released. Therefore, it is common for SIT males to be periodically released in extremely high numbers into the wild resident population, in order to achieve the desired control. There is also evidence to suggest higher remating behaviours in females mated to sterile males (Kraaijeveld and Chapman, 2004), adding to the need for a high over-flooding ratio.

An additional problem is the rearing of large numbers of insects. Until irradiation occurs, a large number of the pest need to be reared, which may pose potential risk if escapes occur. The facilities that house the rearing facilities have to be safeguarded, and personnel and procedures kept under close supervision. There must be provisions in place to ensure the minimal escape of sterile or fertile pests into the environment. Rearing facilities are hence often expensive and difficult to maintain.

The ability to track and monitor the released individuals in the wild is essential in order to assess the success of a release programme. This can be difficult in traditional SIT scenarios. For example, the sterilised individuals are morphologically identical to their wildtype counterparts. Some methods of marking (e.g. fluorescent food dyes, Vail *et*

*al.*, 1966; fluorescent dusting, Stern and Mueller, 1968) have been used and provide some success at tracking over the short term. However, reliable, long-term marking of released individuals remains a challenge.

*1.3.6 Improvements to SIT using classical genetic techniques*

Although the drawbacks of SIT are well documented, the technique itself remains one of the most environmentally benign tools for insect pest control globally, due to its species-specific nature. This has led to the development of techniques that can improve the efficacy of SIT programmes. Classical genetic methods have been used to insert visible markers into mass-reared insects to enable them to be distinguished from wildtype individuals. Genetic sexing strains have also been developed by translocation of selectable markers onto the Y chromosome. This is commonly done using a mutant, white pupal colour and causes males to develop in the wildtype brown pupal colour, but female pupal casings to be white. This technique has proven successful in the melon fly (McInnis *et al.*, 2004), the oriental fruit fly (McCombs and Saul, 1995) and the medfly (Franz *et al.*, 1994). A similar method involves the use of a sex-specific, temperature sensitive lethal and was developed by the International Atomic Energy Agency (IAEA). This causes females exposed to a higher temperature during rearing to die, leaving a male only population for release (Robinson, 2002).

Unfortunately, the use of mutations and chromosome aberrations in these classical genetic techniques can have detrimental effects of overall fitness of the individuals involved, making them even less competitive in SIT programmes (Franz *et al.*, 1994; Lux *et al.*, 2002). The translocations may also be unstable (Franz *et al.*, 1994), especially when the numbers of these strains are increased to the levels needed for mass rearing. Despite much effort to fix such strains, their instability remains a problem (Franz *et al.*, 1994).

**1.4 New and emerging control technologies**

The use of transgenic approaches and other modern genetic techniques to ameliorate some of the problems encountered when using traditional control methods has long been a goal for insect pest control. Genetic engineering can allow the three major

issues of SIT to be improved with the insertion of, in some circumstances, just one transgenic construct. Genetic markers, usually fluorescent, can be used to mark transgenic insects, genetic sexing mechanisms can be incorporated to easily split males from females and radiation replacement mechanisms can be used to omit the need for ionising radiation. These methods are outlined here but discussed in more detail in reviews by Morrison *et al.* (2010) and Leftwich *et al.* (2015).

Generally speaking, methods of pest control using genetic mechanisms fall into two categories. The first aim to suppress pest populations using 'sterile' matings and are based around the principles of SIT, even if released males are not technically sterile. These mechanisms are self-limiting by design and after a small number of generations become extinct in the natural environment (e.g. Thomas *et al.*, 2000; Fu *et al.*, 2007; Gong *et al.*, 2005). The second category involves population replacement or enhancement. In these techniques, pest populations are replaced with a less damaging form of the pest. Alternatively, a gene is engineered in some way to persist and spread throughout the population in order to reduce damage caused by the pest (e.g. Ito *et al.*, 2002; Ghosh *et al.*, 2001). The benefits and drawbacks of these different approaches are discussed in depth by Alphey (2014).

*1.4.1 Release of insects carrying a dominant lethal (RIDL) – introduction*

One new approach, which falls into the first category described above, is the Release of Insects carrying a Dominant Lethal (RIDL). RIDL individuals have been genetically engineered to contain a genetic construct with a dominant, tetracycline-repressible lethal system (Thomas *et al.*, 2000). This construct causes mortality in both homo- and heterozygotes in the absence of a dietary supplement of tetracycline. RIDL individuals can be successfully reared in the laboratory in the presence of dietary tetracycline. However, when released into the wild, any resulting progeny of released male and wild females inherit one copy of the transgene. In the absence of tetracycline (which is unavailable in the environment) the transgene causes lethality. Hence the released individuals are not technically sterile, as in SIT programmes. However, both methods achieve the same final goal of lowering the reproductive output of the pest population. RIDL constructs have been developed in a variety of pest species in insects of significant health and agricultural significance (Thomas *et al.*, 2000; Alphey and Andreasen, 2002;

Gong *et al.*, 2005; Fu *et al.*, 2007; Morrison *et al.*, 2009). RIDL is a major topic of my thesis research and is discussed in further detail in Section **1.5 Insect control via Release of Insects carrying a Dominant Lethal**.

*1.4.2 Use of Wolbachia as a means of pest control*

The use of the arthropod endosymbiont *Wolbachia* is a particularly interesting example of novel population control. *Wolbachia* are inherited bacteria found throughout the arthropod phylum. They can be used to help fight against insect pests in two ways. Firstly, by using the phenomenon of cytoplasmic incompatibility (Werren, 1997; Bourtzis, 2008). Secondly, by driving refractoriness to pathogen transmission in its insect host (e.g. Blagrove *et al.*, 2012).

Research into *Wolbachia* infection in medfly is widespread, despite both laboratory and wild populations having no known naturally occurring infection by any *Wolbachia* species (Zabalou *et al.*, 2004; Bourtzis, 2008). Zabalou *et al.* (2004) used a closely related species to the medfly, *Rhagoletis cerasi* (Diptera: Tephritidae), which have naturally occurring *Wolbachia* infections, to introduce the bacterial symbionts into a medfly population at an embryonic stage. The males from the infected line were crossed to non-infected, wildtype female individuals and 100 % embryonic mortality was shown in cage experiments, as well as in single-pair matings, compared to that of just 12 % in wildtype-wildtype matings. When reciprocal crosses were performed, of the two newly infected strains, both showed increased embryonic lethality compared to wildtype matings (Zabalou *et al.*, 2004). Proof-of-principle trials demonstrate that this technique could potentially result in strong levels of suppression when used as a pest control measure.

The introduction of *Wolbachia* into populations has also been shown to cause refractoriness to vector transmission in multiple species of mosquito (Bourtzis, 2008). This has proven particularly useful in the fight against Dengue fever, which uses many species of mosquito as vectors. Infection by *Wolbachia* strains was not only shown as a method for control population numbers, but also in blocking transmission of dengue in *Aedes albopictus* (Blagrove *et al.*, 2012), and *A. aegypti* (Hoffman *et al.*, 2011; Walker *et*

*al.*, 2011). For a detailed review of the use of Wolbachia in this context, see Brelsfoard and Dobson (2009).

### *1.4.3 Homing Endonucleases*

Homing endonucleases (HEGs) are naturally occurring enzymes that potentially represent a means by which genes can be driven throughout a population to result in some form of insect control (Burt, 2003; Deredec *et al.*, 2008). The protein coded by HEG genes causes a break in double-stranded DNA in heterozygote individuals. When the break is repaired, using the HEG-containing gene, the HEG becomes homozygous. Therefore, HEGs offer huge potential as gene drive systems. The quick spread of resistance genes in disease vector control, or the spread of deleterious genes in agricultural pests, are just two ways that HEGs could be used to solve insect pest problems. Deredec *et al.* (2011) discuss in detail the requirements for implementing a practical HEG programme for controlling malaria transmission in *Anopheles* mosquitoes.

### *1.4.4 CRISPR and mutagenic chain reaction*

A new, novel method for gene drive is that offered by CRISPR, which is increasingly used as a gene-editing tool (Jinek *et al.*, 2012) with the potential to lead to mutagenic chain reaction. This technology has been introduced into the field of insect control, with theoretical descriptions of how genes promoting infertility, or reduced fecundity in females, or genes coding insecticide susceptibility could be introduced and rapidly spread throughout a wild-population. This could assist in insect management in a multitude of ways. The advantage of CRISPR gene editing over the naturally occurring HEG system is the speed and efficiency at which genes can be spread throughout a population. Studies in *Drosophila melanogaster* showed a 97 % transmission rate of a normally recessive gene, when a 25 % transmission rate would normally be expected by Mendelian principles (Gantz and Bier, 2015). Despite the high transmission rate, and seeming success of CRISPR gene drive systems, such mechanisms have been criticised for having few safeguards and no way of stopping the chain reaction once drive has been initiated. What is clear is that CRISPR systems are likely to be

increasingly used in the field of insect control and that the building in of additional safeguards will be an important part of the development of these tools.

### 1.5 Insect control via the Release of Insects carrying a Dominant Lethal

As discussed above, RIDL is an important and successful method for pest control involving the genetic engineering of laboratory-reared pest insects to contain a transgenic construct causing lethality in the absence of a tetracycline dietary additive (Thomas *et al.*, 2000). Because of this, RIDL can offer potentially significant improvements over traditional SIT. Large numbers of RIDL individuals can be reared in the mass rearing facilities in the presence of tetracycline allowing RIDL populations to survive and reproduce. However, when released, in the absence of tetracycline in the environment, the transgene is expressed, and lethality induced, killing individuals at a pre-adult stage.

The standout advantage of a RIDL system over traditional SIT is the removal of the need to use ionising radiation on mass-reared individuals for sterilisation. It removes the deleterious effects of irradiation as a whole. This avoids the associated loss in fitness and allows RIDL individuals to be more competitive for matings with wild individuals than their sterilised SIT counterparts.

As discussed in the context of SIT, single sex releases are often beneficial for control programmes and this has led to the development of female-specific RIDL (fsRIDL) strains, particularly in agricultural pest species (e.g. in medfly, Fu *et al.*, 2007; and DBM, Jin *et al.*, 2013). These strains use alternative splicing in order to cause lethality in only one sex, namely females, leaving males able to survive to adulthood even in the absence of dietary tetracycline (Fu *et al.*, 2007). This approach potentially has significant advantages, as it combines both an effective genetic sexing mechanism with a radiation replacement technique. In a control scenario, tetracycline can be removed from the larval diet of the pre-release generation causing female lethality and leaving a male-only population for release. These males can be released and will seek out wild females for matings without the attendant fitness loss caused by irradiation. Any resulting offspring will inherit a single copy of the dominant genetic RIDL construct. Therefore, all the female offspring will die before adulthood and all the male offspring

will pass on a copy of the transgene to 50 % of the subsequent $F_2$ generation, and so on. This reduction in the number of females within the population causes a decrease in the overall reproductive output of the population, leading to population reduction overall.

The life stage at which lethality is caused by RIDL constructs depends on the specific insect in question. It may be that for insects of agricultural interest, where developing larvae are the main damage-causing life stage, an early acting lethality is preferable, as it minimises damage to crops (Leftwich *et al.*, 2014). On the other hand, in pest mosquito populations, late acting lethality is beneficial as it increases density dependent mortality resulting from increased competition (de Valdez *et al.*, 2011).

In RIDL systems, the transgenic construct is usually inserted using a type of transposable element, a *piggybac* vector. This allows the construct to be incorporated into the genome in the presence of a transposase (Thomas *et al.*, 2000). The insertion can then be stabilised further by removal of the flanking sequences. This then stops the transposable element from being mobile within the genome (Dafa'alla *et al.*, 2006). This insertion technique has a relatively 'random' aspect to it, with the construct being incorporated into a non-specified TTAA insertion site, which are common throughout the genome. For this reason, when creating RIDL constructs is it commonplace to create a selection of parallel strains, each containing the same construct but with a different insertion point. These lines are then stringently tested against one another, and against wildtype and control progenitor strains, to investigate their suitability and competitiveness. In such tests, insertion site effects, transgene expression effects, level of lethality, life stage of lethality, and other factors can all be assessed and the most preferential strain chosen for further testing (e.g. Jin *et al.*, 2013).

Homozygous colonies are founded with a large number of individuals (frequently greater than 50), with the colony number being rapidly increased thereafter. Homozygous strains are then maintained in isolation from one another in large numbers and any subsequent bottlenecks are avoided in order to reduce the risk of inbreeding and any subsequent effects on fitness. Leading RIDL strains in each species (including those discussed in this thesis) are quality control checked regularly to ensure a high level of fitness, using standard life history traits, such as development times and

competitiveness. These measures can be used as a proxy to ensure the absence of high levels of inbreeding within a homozygous RIDL strain. RIDL strains are reared under the same conditions as the wildtype progenitor strains from which they originate, with the exception of the addition of dietary tetracycline where necessary. This reduces any selection for divergence between strains, which can be used for comparison in any subsequent testing, and allows for adequate testing of construct and insertion site effects in transgenic lines.

The stage of current testing of RIDL technology varies between species. There is an example of successful open-field releases in RIDL using the mosquito *Aedes aegypti*, a global disease vector. The RIDL transgenic strain of *A. aegypti* (Phuc *et al.*, 2007) has shown promising results in the Cayman Islands (Harris *et al.*, 2012), Malaysia (Lacroix *et al.*, 2012), Panama (Gorman *et al.*, 2015) and Brazil (Carvalho *et al.*, 2015). Medfly RIDL strains have shown positive results both in laboratory experiments (Fu *et al.*, 2007) and in field cage trials (Leftwich *et al.*, 2014). A DBM RIDL strain has undergone extensive laboratory (Harvey-Samuel *et al.*, 2014) and multiple generation glasshouse experiments. These studies indicated that RIDL represents a highly effective method for both population suppression and the slowing the spread of resistance to insecticides simultaneously (Harvey-Samuel *et al.*, 2015).

Whilst RIDL as a technology is at a more advanced stage of development than other genetic control technologies, there are major aspects of RIDL implementation that are left untested. The potential for breakdown of RIDL effectiveness under stressors is relatively untested, especially in 'lead' medfly and DBM strains. Different RIDL strains also pose species-specific questions. For example, in the medfly, the question of how varying larval diet compositions affect the efficacy of construct functionality might be of particular interest due to the highly varied host range. For similar reasons, the effect of temperature on RIDL efficacy in medfly might be of interest. The possibility for development of resistance has yet to be fully investigated, despite efforts to render RIDL technology as robust to resistance as possible. Resistance could come in the form of behavioural resistance, with wild females being able to discriminate against matings with RIDL males. Alternatively, there could be biochemical resistance, where genotype changes negate the action of the lethal construct. It is these gaps in knowledge that are my focus of investigation into RIDL technologies and their applications.

## 1.6 Outline of thesis

This thesis focuses on the RIDL method of pest control developed by Oxitec Ltd. Promising results in both laboratory and early-stage, field testing of RIDL strains have shown that highly competitive transgenic strains have been produced, with the ability to control pest population numbers in tightly controlled settings. However, as it stands, many RIDL strains are relatively untested in key aspects of field performance. Studies into agricultural pests have yet to simulate the full range of adverse conditions that RIDL males may encounter in a wild release. In addition, how susceptible will RIDL males be to problems encountered by other pest-control measures such as the evolution of resistance and reductions in male competitiveness in a mass release scenario. The aim of this thesis is to test the efficacy of RIDL systems for environmentally benign control of agricultural insect pests in these testing scenarios, in the Mediterranean fruit fly, *Ceratitis capitata*, and the Diamondback moth, *Plutella xylostella*.

In **Chapter 2** I investigated the effect of larval diet composition on penetrance of the fsRIDL construct lethality in heterozygous offspring of the OX3864A strain of medfly, as well as their key developmental times. Experimental crosses were set up to mimic that of a control programme release of OX3864A males, where the OX3864A male-only population was crossed to wildtype females to produce offspring heterozygous for the fsRIDL transgene. These offspring were then seeded onto larval diets with varying nutritional content. I varied both the carbohydrate source as well as protein level, as might be the case when RIDL-mated, wild females oviposit heterozygous offspring into a variety of host fruits in the wild. The results showed no female survival in heterozygotes when supplied with high levels of protein. However, female escape from lethality was observed in heterozygotes seeded onto low protein diets.

Having determined that female survival was possible if the OX3864A fsRIDL system was sufficiently stressed, I then assessed whether it was possible to select for resistance traits within the OX3864A strain. In **Chapter 3**, tetracycline dose response curves were used to inform the starting point for the artificial selection lines, i.e. to select the base line starting level of tetracycline at which there was an appropriate level of female

survival. The number of female offspring surviving to adulthood across generations was recorded, and rt-qPCR was used to investigate any changes in transgene expression across generations. After multiple generations of selection, dose response curves were performed again to assess if the selection had caused notable change in the lines. Dose response curves had shifted over the course of 9 generations, with females surviving in larval diets with lower concentrations of tetracycline. Throughout generations of selection lines, the sex ratios in reduced tetracycline selection lines showed some sign of rebalancing from an original, highly male-biased ratio.

In **Chapter 4** I tested the ability of fsRIDL DBM to respond to artificial pheromone sources using wind tunnel experiments. The OX4319L strain of DBM was used in wind tunnels at the New York State Agricultural Experiment Station (NYSAES), Cornell University. Males from the OX4319L strain showed the ability to fly upwind towards the artificial, female sex pheromone source, comparable to that of wildtype counterparts. This is important for control programmes, as males in RIDL releases are expected to seek out and mate with wild females, who call using sex pheromone signalling. It is also an important finding for monitoring, as I found that pheromone-baited traps can be significantly more effective at recapturing males than non-baited traps. Also discussed in **Chapter 4**, is the ability of a laboratory-reared, wildtype strain to disperse in the open field. Dispersal capabilities of the long-term, laboratory-reared Vero Beach strain were investigated, with the strain showing good levels of dispersal within a host crop. This is encouraging for the study of the OX4319L strain, as the Vero Beach strain is the progenitor strain from which the OX4319L strain was transformed.

In **Chapter 5**, the comparative mating competitiveness and longevity of the OX4319L strain of DBM was assessed against that of a wild-caught strain of DBM, in field cage studies at NYSAES. Males from the OX4319L and wild-caught, Georgia strain were co-released to compete for matings with females from the Georgia strain, to simulate a release-style scenario. Mated females were then collected and allowed to lay eggs. These progeny were reared and PCR analysis was used to determine the paternity of the progeny. The levels of paternity were used to determine the mating competitiveness of each male strain, and the number of males recaptured from the cage after 3 days was used as a measure of male survival in the field, and therefore a proxy for longevity. Whilst longevity of OX4319L males was not significantly lower than

the Georgia counterparts, their mating competitiveness was significantly lower. However, competitiveness was within acceptable limits for RIDL strains.

In **Chapter 6**, I discuss the wider implications of the findings of this thesis to pest control programmes. The use of RIDL technologies is just one method of improving pest control, but its success and cost-effectiveness may be context-dependent. It may be that the incorporation of such technologies into already existing control programmes is the best way forward, rather than as a blanket technology, as the latter has been a weakness in traditional control approaches. The development of control methods involving a genetically engineered element also requires high levels of public and government approval, which is currently not always the case. The future of such technologies, including RIDL, is discussed.

**Appendix 1** contains a review paper titled 'Evolutionary biology and genetic techniques for insect control', which was published in the journal *Evolutionary Applications* in 2015. The paper discusses various genetic approaches to insect pest management, and include commonly used approaches, as well as new, underutilised approaches which may hold promise in the future.

The work in this thesis was part of an industrial "collaborative awards in science and engineering" (CASE) studentship with Oxitec Ltd, funded by the Natural Environment Research Council (NERC). Professor Tracey Chapman (UEA – primary supervisor), Professor Matt Gage (UEA – secondary supervisor), Dr Phil Leftwich (UEA – supervisory team) and Dr Neil Morrison (Oxitec Ltd – supervisor) supervised the project.

The author carried out all work unless stated below. Tracey Chapman and Phil Leftwich helped devise experiments in Chapters 2 and 3. Lucy Friend (UEA) helped with maintaining of selection lines and counting of progeny in Chapter 3. Neil Morrison, Anthony Shelton (Cornell University) and I devised protocols for Chapters 4 and 5. Hilda Collins (Cornell University) reared wildtype stock lines of DBM lines at the New York State Agricultural Experiment Station (NYSAES), Cornell University used in Chapters 4 and 5. In Chapter 4, Tim Harvey-Samuel (Oxitec Ltd/University of Oxford) and Devan George (Cornell University) helped with rearing of DBM and field releases; DG also helped with subsequent trapping. Dan Olmstead (Cornell University) and Masanori Seto (Cornell University) set up field cages in Chapter 4 and 5, and DO helped

maintain the field throughout the season. In Chapter 5, Adam Walker (Oxitec Ltd) helped with rearing of transgenic insects at NYSAES, and carrying out of experiments. NM, AS, DO and MS helped with moth recaptures in Chapter 5. Oxitec Ltd carried out PCR analysis in Chapter 5, with the help of the author. Appendix 1 was co-authored by PL, TC and MB, as is laid out in the author contributions in the appendix.

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## 2 The effect of larval dietary components on the penetrance of a female-specific RIDL strain of the Mediterranean fruit fly, *Ceratitis capitata*

## 2.1 Abstract

The Release of Insects carrying a Dominant Lethal (RIDL) is a species-specific, transgenic control method used against the globally problematic agricultural pest, the Mediterranean fruit fly, *Ceratitis capitata*. RIDL control systems involve the release of large numbers of male medfly containing a transgene, which induces female-specific lethality (fsRIDL) in both the homo- and heterozygote condition. The released males mate with wildtype females and any female progeny resulting from such matings die before reaching adulthood, hence reducing the pest population size. The medfly is noted for its extreme polyphagy and hence mated females may have a wide choice of host fruits in which to oviposit, each of which may represent a contrasting nutritional environment. The potential effectiveness of RIDL under such nutritional variation has not previously been investigated and was the subject of the tests conducted in this chapter. Wildtype females were mated to RIDL OX3864A males and the resulting heterozygous progeny were seeded onto 4 larval diets: sucrose, fructose, glucose and low protein, sucrose-based. 100% female lethality was observed in the sucrose, fructose and glucose-based diets. However, a low level of female survival was evident when progeny were seeded onto the low protein, sucrose-based diet. The level of survival was low, below the threshold of fertility that is accepted for other RIDL strains, and indeed for the sterile insect technique (SIT) itself. Hence, the frequency of female escapes is not thought to represent a significant issue for the prospect of medfly population control using the OX3864A strain in field trials. Tests of the effect of the larval diets on development showed that progeny developing on low protein diets had significantly longer pupation times than progeny developing on diets with a standard level of protein.

## 2.2 Introduction

The Mediterranean fruit fly, or medfly (Weidemann: *Ceratitis capitata*) is one of the most damaging agricultural insect pests within the Tephritid family, globally. Medflies are known to infest over 300 known species of cultivar across several continents, including many fruits, vegetables and nuts (Hooper and Robinson, 1989; USDA, 2006). Females lay eggs into these plant hosts, which then hatch into larvae that feed on the fleshy tissues of the fruit, causing enormous damage. These infestations result in millions of dollars of damage and control costs.

### *2.2.1 Control methods – SIT and SIT-like approaches*

Since the 1970s many control methods, such as bait trapping (Burns *et al.*, 2001; Chueca *et al.*, 2007), biological control (Headrick and Goeden, 1996) and pesticide application (Braham *et al.*, 2007), have been used against medfly pest populations with varying degrees of success. The most commonly used control methods are the sterile insect technique (SIT), or SIT-like technologies. As efforts are made to move away from large-scale, broad-spectrum insecticide application, the advantages of SIT and SIT-like programmes are becoming more evident. SIT programs involve rearing large numbers of the pest species in mass rearing facilities followed by sterilization using ionizing radiation and finally release of sterilized individuals (usually only males) into the area to be targeted. Control is achieved when the released individuals seek out wild individuals of the opposite sex with which to mate. Such matings produce few, but usually no, viable offspring, which lowers the size of the pest population as a whole. Such releases can be carried out in such a way that maximizes sustained population eradication or suppression (Knipling, 1955).

SIT programmes were first used to target the New World Screwworm, *Cochliomyia hominivorax*, in Central and North America. The effect of this pest on livestock farming was significant (Knipling, 1955). Since then, SIT has been used to successfully combat pest populations of codling moth (*Cydia pomonella*, Linnaeus) in Canada (IAEA, 2001) as well as being used with some success against the medfly in suppression and prevention contexts (Hendrichs *et al.*, 1995). In spite of the relative success of SIT programs as a whole, the technique has some potentially serious drawbacks. For

example, the use of ionizing radiation as a source of sterilization has major fitness effects. Sterilized individuals have a reduced lifespan, competitiveness and flying ability than non-irradiated individuals (Wong *et al.*, 1982). Overall, a 4- to 10-fold decrease in fitness has been reported for irradiated versus wildtype males (Lance *et al.*, 2000; Shelly *et al.*, 1994). The selection pressures placed on laboratory-reared individuals in the mass-rearing context, likewise, can reduce the control efficiency of the released individuals. These pressures are very different from those experienced by the wildtype pest populations. The evolution of assortative mating within mass-reared individuals may also lead to a reduced number of 'control' (i.e. mass-reared with wild) matings in a mass release control programme scenario (McInnis *et al.*, 1996).

Various methods have been used to try and improve the effectiveness of SIT programs. For example, male-only releases are usually regarded as more effective than bi-sex releases (Hendrichs *et al.*, 1995; Meats *et al.*, 2002; Robinson *et al.*, 1999). This is certainly the case for medfly, because it avoids the release of 'fruit-stinging' females. It is the female medflies that lays eggs in host cultivars, resulting in the infestation of the fruit by larvae that feed on the plant tissue, leading to destruction and spoiling of crops. In contrast, males do not lay eggs or feed on crops, and so are effectively harmless. Released females may also not be completely sterile, as irradiation in most SIT trials is not complete, owing to the need to strike balance between the degree of sterilization achieved versus overall fitness costs of the irradiation treatment. Any released fertile females will be able to lay fertile eggs and contribute to crop damage rather than reduce it. The avoidance of matings within the release population is another key advantage of male-only releases. Released males are forced to seek out wildtype females in order to mate, and cannot now mate with co-released females. Male-only releases result in a several-fold increase in induced sterility within a pest population in comparison to bi-sex releases (Rendon *et al.*, 2004).

The advantages in using single sex releases have led to the development of genetic sexing strains that allow for ease of sorting of males from females and hence facilitate the selection of a male-only release population. These strains usually contain a sex-specific visual marker, inserted by chromosomal translocation, allowing separation of males and females (Robinson, 2002). An alternative to the above is the use of strains

that express a sex-specific lethality, resulting in the production of a unisex population ready for release (Fu *et al.*, 2007; Ant *et al.*, 2012).

The Release of Insects carrying a Dominant Lethal (RIDL) is a genetic control technique designed to provide additional benefits over and above traditional SIT programs. RIDL systems involve the insertion of a genetic construct containing a tetracycline-repressible, dominant lethal gene system, into laboratory-reared insect species. These constructs, when expressed, induce lethality in homozygous and heterozygous individuals. RIDL constructs have been successfully introduced to several insect pest species (Thomas *et al.*, 2000; Gong *et al.*, 2005; Fu *et al.*, 2007; Jin *et al.*, 2013). RIDL stocks can be reared with a dietary tetracycline supplement that suppresses the lethal effect of the construct and allows survival. In a pest management scenario, RIDL individuals are released, and any resulting offspring from wildtype matings are non-viable before reaching reproductive maturity due to the inheritance of one copy of the RIDL transgene and the lack of tetracycline in the environment (hence no opportunity for suppression of the lethality).

Female-specific RIDL (fsRIDL) constructs have been developed and introduced into agricultural pests in which unisex releases are particularly useful (Fu *et al.*, 2007). Sex-specific RIDL systems only express lethality in members of a population from one sex, usually females. This approach can be used to generate a genetic sexing strain and a radiation replacement technique, hence alleviating two drawbacks of traditional SIT. In the pre-release generation, tetracycline is removed from the diet, leading to female mortality and the production of a male-only population. This male-only population can be released into the environment to seek out wildtype females. Any offspring from matings between the release males and wild females will inherit a single copy of the fsRIDL construct. Thus, any female offspring will express lethality, and any male offspring will survive but have a 50% chance of passing on an fsRIDL transgene to $F_2$ offspring.

*2.2.2 Pest control and host oviposition choice*

Both SIT programs and RIDL systems rely on released males seeking out wildtype females for matings, and some or all of the resulting offspring being non-viable. Mated

females from both wildtype and RIDL matings will have a choice of host plant species into which to oviposit. Choice of plant host can have significant effects on developmental traits of the offspring (Krainacker *et al.*, 1987). The type of host plant that is chosen for oviposition may therefore have a significant influence on the efficacy of this kind of pest control method in the $F_1$ generation. Pests of global significance, such as the medfly, may have vastly different hosts across continents, providing very different nutritional environments into which fsRIDL males could be released. Therefore, it is key to the success of an fsRIDL programme that the penetrance of fsRIDL constructs remains complete, even under nutritional stress or variation.

The >300 host species that can be utilized by the medfly for oviposition represent a striking variety of different nutritional environments in which larval development can successfully occur. The two major nutritional components that vary are the quality and quantity of proteins and carbohydrates, with variation being seen between host species as well as in different strains or cultivars of the same species. Various carbohydrates are present across the host range, however sucrose, fructose and glucose are the major three, being found in various combinations across fruit. For example, apple and pear strains have predominantly fructose-based carbohydrates in their flesh; grapes and other berries have approximately equal contents of fructose and glucose; and citrus fruits, including oranges have predominantly sucrose-based carbohydrates (Department of Health, 2013). Protein levels can also vary in a similar way both between and within species, depending on strain, with 3-fold differences in protein levels seen between some strains of apple and some berry species (Department of Health, 2013).

Proteins in the larval diet, specifically amino acids, are crucial for survival - insects are reported to need to gain 10 essential amino acids from their diets (House, 1961). Protein quantity and quality can have significant effects on key fitness traits such as lifespan (Dussutour and Simpson, 2012; Grandison *et al.*, 2009) and immune responses (Lee *et al.*, 2008). Carbohydrates are also essential for providing the energy needed for growth during development and adulthood. Both protein and carbohydrate availability during development can have long-lasting implications for the overall fitness of an individual in holometabolous species (Boggs, 1981).

The use of different host fruits can have significant effects on medfly survival rate, body size, development time and fecundity (Kaspi *et al.*, 2002; Krainacker *et al.*, 1987) as well as tolerance to several environmental stress factors such as heat and desiccation (Anderson *et al.*, 2010). In addition, the constituent make up of larval diets has been shown to have significant effects on gene expression at multiple loci in other holometabolous insect species (Chang *et al.*, 2010). There are potentially long lasting, carry-over effects of larval development on adult life history traits of individuals. For example, there can be a trade-off between extended development and adult life history. Slower development may produce larger adults with more resources, but with a higher likelihood of predation and loss of habitat. In insects such as medfly in which the adult stage also feeds, this situation may generate a greater reliance on foraging success in adults (Kaspi *et al.*, 2002).

Such stresses could impact on the success of RIDL, potentially by the reduction in gene expression of the transgene causing lethality, or in the slowing of development to a rate at which the necessary build-up of the transgene product does not get to the toxicity at which lethality can occur. However, it is currently unknown what effect any variation in larval carbohydrate sources and protein levels will have on the functionality and penetrance of fsRIDL in heterozygous individuals in a release style scenario, or on developmental characteristics themselves.

In this chapter, I investigated this phenomenon by testing the effects of larval diet composition on penetrance of an fsRIDL genetic construct within the OX3864A medfly strain in a release-style scenario. Experimental crosses were used to simulate a pest control release, using OX3864A populations reared in the absence of tetracycline, resulting in male-only cohorts, crossed with wildtype females. The resulting offspring are heterozygous for the fsRIDL transgene. Experimental larval diets that differed in both carbohydrate source and protein level were used to stress these heterozygote offspring. The sex ratio of eclosing adult offspring was used to assess the penetrance of the transgene within the heterozygous cross. Key developmental traits were also measured across all the experimental diets in fsRIDL heterozygotes, namely the number of days until pupation, eclosion and total development time.

## 2.3 Materials and Methods

### *2.3.1 Medfly stocks and culturing*

The OX3864A medfly strain developed by Oxitec Ltd was used to measure the penetrance of the lethality in a female-specific RIDL strain (fsRIDL) in response to different larval diets. The Toliman strain, originating in Guatemala and reared at Oxitec Ltd (Oxford, UK) since 2004, and maintained at UEA (Norwich, UK) since 2009, was used as the wildtype (wt) control strain in all experiments. Toliman is the genetic progenitor strain from which OX3864A was derived. All adult flies were reared in a constant environment room held at 25 °C, 50-60 % relative humidity and on a 12h:12h light:dark cycle.

Adults were maintained in population cages and fed *ad libitum* on a diet of 3:1 sugar:hydrolysed yeast throughout, unless otherwise stated. Damp cotton wicks were inserted into the cages to provide a continual water source. In order to suppress, under standard propagation conditions, the female lethality of the OX3864A strain, the water supplied to the OX3864A adults contained a tetracycline hydrochloride (Sigma-Aldrich) antibiotic solution in deionised water (100 µg/ml). Wt adults received only deionised water, unless otherwise stated.

Standard 'ASG' larval diets were used for stock rearing and comprised sugar (147 g), maize (134 g), Brewer's yeast (95 g), nipagin (10% in ethanol; 50 ml), propionic acid (4 ml), agar (25 g) and water (1700 ml). Larvae were raised in 300 ml bottles each containing 100 ml of ASG medium. Stock populations of OX3864A were maintained on ASG diet supplemented with tetracycline hydrochloride (100 µg/ml) to suppress the expression of the RIDL genetic construct and maintain female survival. Toliman stock populations were maintained on the standard ASG diet, with no tetracycline supplementation. During experimentation, larval diets of varying carbohydrate and protein levels were used, both with and without tetracycline supplementation, as outlined below.

*2.3.2 Parental generation*

Eggs were collected from the OX3864A stock and seeded onto ASG larval diet without tetracycline supplementation in order to activate the female-specific lethal effect of the construct. Flies were allowed to develop until eclosion, at which point the resulting male-only population was used to set up parental crosses. Simultaneously, eggs were collected from Toliman stocks and seeded onto the ASG larval diet, and allowed to develop until eclosion. Within 24 hours of eclosion, adults were sorted by sex, with males and females being separated, to generate cohorts of single sex virgins.

Two parental population crosses were set up. The first comprised OX3864A males and Toliman females, resulting in offspring that were heterozygous for the dominant 3864A construct. The second cross was Toliman males and females. Populations were set up to contain approximately 500 individuals with a 2:3 male:female sex ratio. These groups were then allowed to mate and females allowed to produce eggs that were collected in deionized water.

*2.3.3 Experimental larval diets and seeding*

Four larval diets were used (Table 2.1). Three diets varied the carbohydrate source (sucrose, glucose and fructose) and a fourth the amount of protein available (Low Protein diet). All diets were tested both in the presence and absence of tetracycline hydrochloride (Sigma-Aldrich), resulting in a total of eight experimental diet treatments. The diets were devised based on the experimental design of Nash and Chapman (2014) where the use of simple carbohydrate sources of mono- and disaccharides, as well as reduced protein content in the larval diet caused an increased egg to adult mortality, thus representing a significant stressor for developing larvae.

Table 2.1 – Components of four experimental diets, Sucrose (Suc), Glucose (Gl), Fructose (Fr), and Low Protein (LP). Shown are the constituent ingredients needed to make 1 L of diet.

| Ingredient | Sucrose | Glucose | Fructose | Low Protein | |
|---|---|---|---|---|---|
| Water | 1000 | 1000 | 1000 | 1000 | ml |
| Agar | 15 | 15 | 15 | 15 | g |
| Sucrose | 30 | - | - | 30 | g |
| Glucose | - | 30 | - | - | g |
| Fructose | - | - | 30 | - | g |
| Yeast | 50 | 50 | 50 | 30 | g |
| Propionic acid | 10 | 10 | 10 | 10 | ml |

All larval diets were placed in 300 ml bottles each containing 100 ml of the desired larval diet. Eggs from each experimental cross were collected daily and approximately 500 eggs added to each bottle of larval diet. Egg number was estimated using a volumetric approximation of 500 eggs. Multiple replicate bottles of each experimental larval diet were seeded for each experimental cross.

*2.3.4 Heterozygote fsRIDL penetrance in varying larval diets*

In order to test the penetrance level of the lethality of the OX3864A construct in heterozygotes, individuals from each bottle were allowed to freely eclose. Once all adult individuals had emerged the number of males and females (hence sex ratio of offspring) was scored. Complete penetrance of the OX3864A construct was indicated by the absence of female offspring.

*2.3.5 Developmental life history traits of heterozygous fsRIDL medfly*

After seeding, all eggs were allowed to develop in the diets contained within the larval bottles and then laid in pupal boxes on their side to allow the larvae to exit the food medium and pupate within sand. The date of the first pupation was recorded and the number of days between seeding and pupation was used to determine the pupation time. The date of the first fly to eclose was documented, and the number of days between pupation and eclosion was used to record eclosion time. These two values

were combined to represent total development time from egg seeding to adult eclosion.

*2.3.6 Data analysis*

Data analysis was performed using R v.3.2.4 (R Core Team, 2016). Total development time was calculated as a count of the number of days between seeding of eggs and eclosion of the first adult. Pupation time was the number of days between seeding of eggs and the pupation of the first pupa. Eclosion time was the number of days between the first pupation and the eclosion of the first adult. Development time, pupation time, and eclosion time were all analysed using generalised linear models (GLMs) using a Poisson distribution. Data were checked for overdispersion using a dispersion test ("AER" package, R); no overdispersion was found. Models were fitted and post-hoc analysis was carried out using Tukey contrasts ("multicomp" package, R; Hothorn *et al.*, 2008).

**2.4 Results**

*2.4.1 Heterozygote fsRIDL penetrance of lethality across different larval diets*

In the Toliman crosses, a sex ratio of 1:1 males:females was observed in the offspring across all eight experimental diets (Fig. 2.1). In RIDL x Toliman crosses, resulting in offspring heterozygous for the RIDL transgene, 1:1 sex ratios were observed in all diets containing tetracycline hydrochloride (SuTet, FrTet, GlTet and LPTet), as expected. As expected, male only offspring were obtained from RIDL x Toliman crosses seeded onto Suc, Gl and Fr larval diets. However, female survival was observed when eggs from RIDL x Toliman crosses were seeded onto the LP larval diet in the absence of a tetracycline hydrochloride supplement (Fig. 2.2). This female survival (i.e. lethality 'escape') was evident in 6.4 % of RIDL x Toli LP sets of egg seedings, resulting in approximate 1 in 1000 adults from these cultures being female.

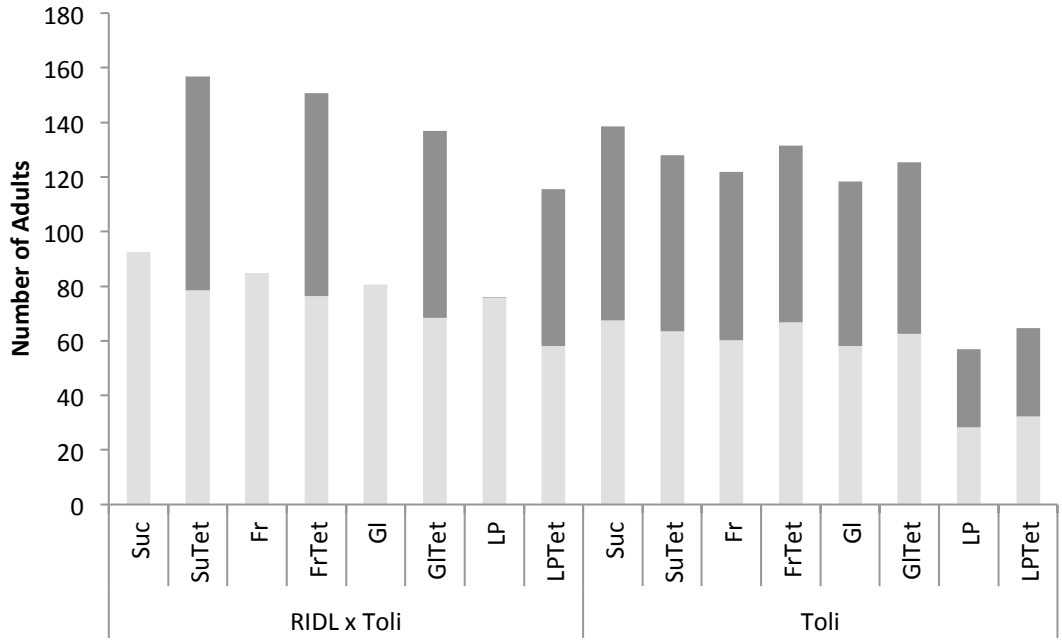


Figure 2.1 – Mean number of adults per larval bottle in offspring of two experimental crosses: a RIDL male plus wildtype female cross (RIDL x Toli) resulting in offspring heterozygous for the RIDL construct and a wildtype male plus wildtype female cross (Toli). Each cross was tested on eight different larval diets: sucrose-based (Suc), fructose-based (Fr), glucose-based (Gl) and low protein, sucrose-based (LP), all in the presence or absence of tetracycline hydrochloride supplement (Tet). The total number of adults is split into males (light grey) and females (dark grey).

Table 2.2 – Sample sizes. Shown in the table are the numbers of replicate egg seedings (each of 500 eggs) placed onto each of the experimental larval diets, for the two experimental treatment crosses: RIDL male x wildtype female (RIDL x Toli), resulting in offspring heterozygous for the RIDL construct and a control wildtype male x wildtype female cross (Toli x Toli).

| | Experimental cross | |
|---|---|---|
| Diet | RIDL x Toli | Toli x Toli |
| Sucrose | 53 | 45 |
| Sucrose Tet | 66 | 47 |
| Fructose | 54 | 45 |
| Fructose Tet | 64 | 56 |
| Glucose | 49 | 35 |
| Glucose Tet | 57 | 52 |
| Low Protein | 78 | 45 |
| Low Protein Tet | 64 | 33 |

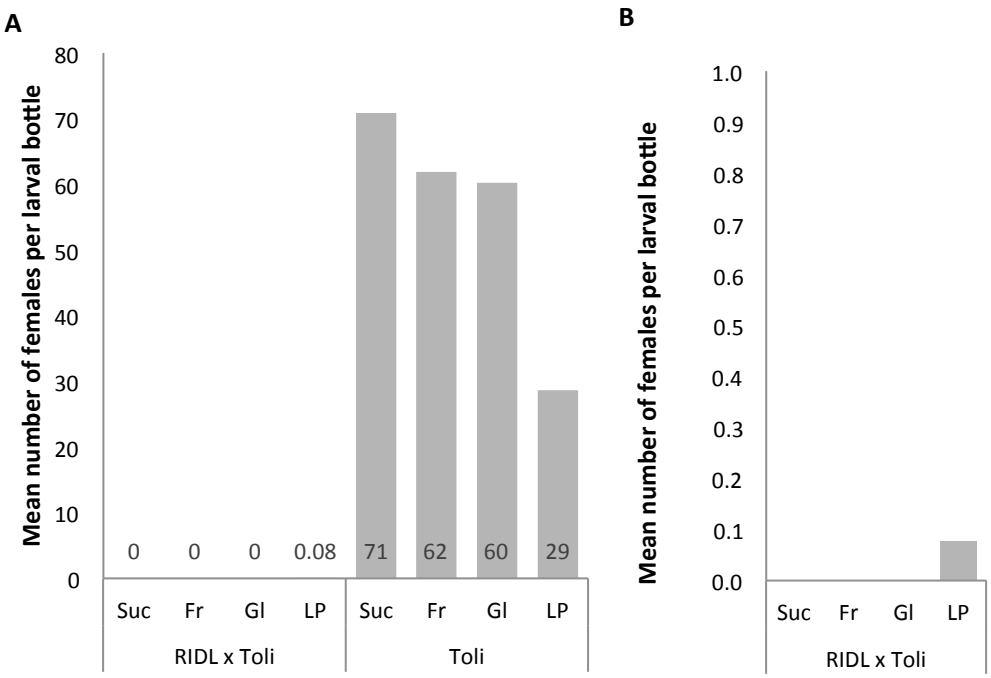


Figure 2.2 – Mean number of females present in the total adult population per larval bottle in offspring of two experimental crosses: a RIDL male plus wildtype female cross (RIDL x Toli) resulting in offspring heterozygous for the RIDL construct and a wildtype male plus wildtype female cross (Toli). Each cross was tested across four experimental larval diets: sucrose-based (Suc), fructose-based (Fr), glucose-based (Gl) and low protein, sucrose-based (LP). Panel A shows offspring from the two experimental crosses. Panel B is an inset to show offspring, and specifically female 'escapes' from the RIDL x Toli cross, at higher resolution of the Y-axis.

### *2.4.2 Developmental life history traits of heterozygous fsRIDL medfly*

*Pupation time* – A GLM model was used to analyse differences in pupation time between strains and across experimental larval diets. No significant interaction was seen between strain and larval diet ($\chi_{7,823}$=8.534, P=0.288) and this term was therefore removed from the model. The resulting reduced model, excluding the interaction term, revealed a significant effect of strain on pupation time ($\chi_{1,837}$=79.124, P<0.01), with RIDL heterozygotes having a significantly shorter pupation time than the homozygous Toliman control (Fig. 2.3). A significant effect of larval diet on pupation time was also observed ($\chi_{7,830}$=112.601, P<0.01). Post-hoc Tukey tests on larval diet showed that the LP and LPTet diets had significantly longer pupation times than all other diets (p<0.05). None of the other diets showed significant differences.

*Eclosion time* – I tested for differences in eclosion times between strains and across larval diets using a GLM. There was no significant interaction between strain and larval diet ($\chi_{7,823}$=0.709, P=0.998), and this term was removed from the model. The resulting model showed no effect of diet ($\chi_{7,830}$=0.670, P=0.999) or strain ($\chi_{1,837}$=3.5299, P=0.060) with the eclosion time of RIDL heterozygotes not being significantly shorter than for the Toliman wildtype on any diet (Fig. 2.4).

*Development time* – Differences in development time between strains and across experimental larval diets were tested using a GLM. No significant interaction was seen between strain and larval diet ($\chi_{7,823}$=4.916, P=0.670) and this term was removed. The resulting model showed a significant effect of strain on development time ($\chi_{1,837}$=64.703, P<0.01), with RIDL heterozygotes having a significantly shorter development time than the Toliman control (Fig. 2.5). A significant effect of larval diet on development time was also observed ($\chi_{7,830}$=73.549, P<0.01). The LP diet in the absence of tetracycline was significantly different from the Su, Fr, FrTet and Gl diets (p<0.05). The LPTet diet showed a significantly longer development time in comparison to all other diets (p<0.01) except LP (p=0.108).

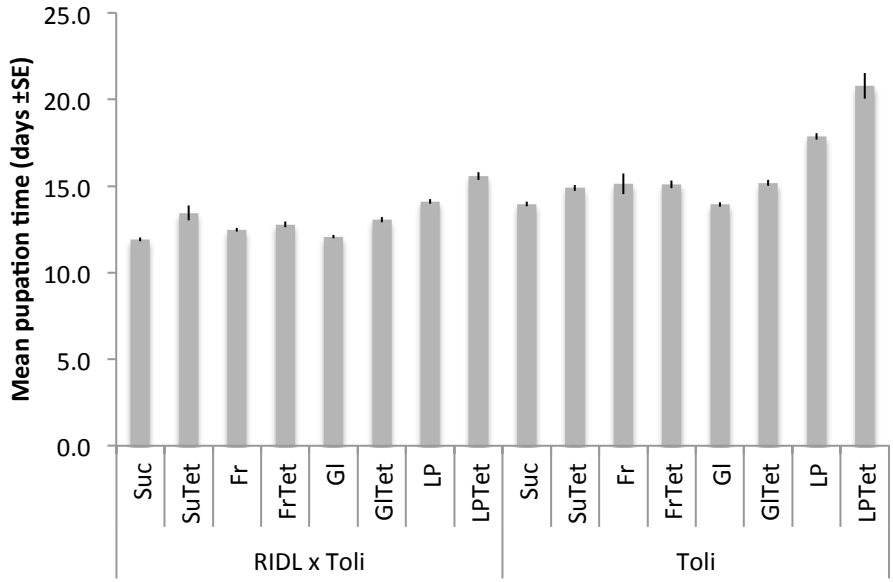


Figure 2.3 – Mean pupation time (±SE) of offspring from two experimental crosses: a RIDL male plus wildtype female cross (RIDL x Toli) resulting in offspring heterozygous for the RIDL construct, and a wildtype male plus wildtype female control cross (Toli). There were 8 experimental larval diets: sucrose-based (Suc), a fructose-based (Fr), a glucose-based (Gl) and low protein, sucrose-based (LP), either in the presence or absence of tetracycline hydrochloride supplement (Tet).

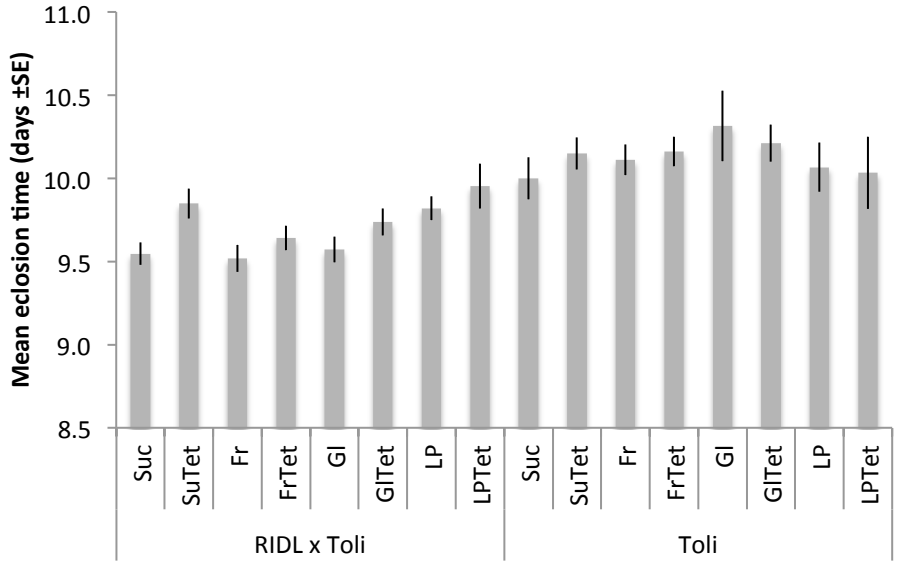


Figure 2.4 – Mean eclosion time (±SE) of offspring from two experimental crosses: a RIDL male plus wildtype female cross (RIDL x Toli) resulting in offspring heterozygous for the RIDL construct, and a wildtype male plus wildtype female control cross (Toli); tested across 8 experimental larval diets: sucrose-based (Suc), a fructose-based (Fr), a glucose-based (Gl) and low protein, sucrose-based (LP), either in the presence or absence of tetracycline hydrochloride supplement (Tet).

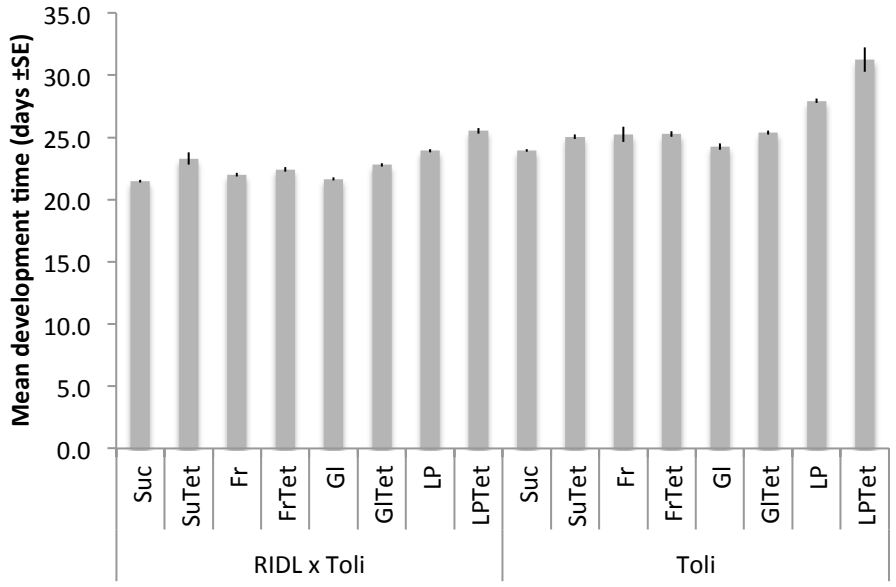


Figure 2.5 – Mean total development time (±SE) of offspring from two experimental crosses: a RIDL male plus wildtype female cross (RIDL x Toli) resulting in offspring heterozygous for the RIDL construct, and a wildtype male plus wildtype female control cross (Toli); tested across 8 experimental larval diets: a sucrose-based (Suc), a fructose-based (Fr), a glucose-based (Gl) and a low protein, sucrose-based (LP), all in the presence and absence of a tetracycline hydrochloride supplement (Tet).

## 2.5 Discussion

In this chapter I used the OX3864A medfly strain, which contains a female-specific RIDL construct, to investigate the penetrance of female lethality in fsRIDL heterozygotes in response to larval diets with differing nutritional contents. Key developmental traits were also recorded in the RIDL heterozygote individuals across the same experimental larval diets.

Heterozygote penetrance of the expression of the fsRIDL construct in the OX3864A strain was measured counting the sex ratio of offspring from crosses involving OX3864A males and wildtype, Toliman females. These heterozygous RIDL offspring were seeded onto eight larval diets: sucrose, fructose, glucose and low protein sucrose, in the presence or absence of tetracycline hydrochloride supplementation. In all experimental diets with added tetracycline supplementation, sex ratios that were not

significantly different to 1:1 male to female were observed (Fig. 2.1), as expected. The addition of tetracycline to these larval diets is expected to result in the repression of the female-specific lethal RIDL transgene expression (Fu *et al.*, 2007). This allows, unlike in the absence of tetracycline, female offspring to survive until eclosion and onto reproductive maturity. This is the diet used in the stock rearing of fsRIDL strains, when normal bi-sex populations need to be propagated.

Heterozygous OX3864A progeny seeded on sucrose, fructose and glucose larval diets in the absence of tetracycline showed no female survival to adulthood. This was the expected result as, in the absence of tetracycline, the female-specific lethality was expressed. However, I observed that low numbers of adult females emerged from the cultures seeded onto the low protein sucrose diet in the absence of tetracycline, (6 females from a total of 5910 total adults; Fig. 2.2). The number of females surviving, although likely not statistically significant, is biologically significant, given that this is the first case to the authors knowledge of female escape being seen in this RIDL line. The fitness of these females is, as yet, untested. Hence it is not yet known whether these females were fertile.

These females represent potential 'escapes' from lethality. As outlined above, all heterozygous progeny from fsRIDL male and Toliman female crosses inherit a single copy of the transgene, which codes for female-specific mortality. Without the addition of tetracycline to the diet, all female progeny should exhibit this lethality and die before reaching adulthood. The low level of female survival on this diet could be caused by the slower developmental time exhibited by progeny developing on low protein diets (Fig. 2.5). RIDL constructs rely on the overexpression of a fusion protein, tTAV, at key developmental stages, leading to apoptosis, and eventual death (Thomas *et al.*, 2000). It is possible that during development in larval conditions in which there are only low levels of nitrogenous compounds, that development is slowed to a level at which the required level of overexpression of the lethal effector tTAV simply does not occur, and therefore no lethality is exhibited even in the absence of a tetracycline supplement. To explore this mechanism further, qPCR analysis of the transgene expression at differing levels of dietary protein availability could be employed. This would allow tests for correlations between the level of protein in the larval diet and the expression of the construct.

This potential explanation is supported by the findings from the other life history data, i.e. the development times of heterozygote individuals across the eight experimental diets. My data suggest that progeny from heterozygous crosses and wildtype crosses seeded onto the low protein larval diets in this experiment experienced significantly longer pupation times than did individuals seeded onto larval diets with the standard protein level (Fig. 2.3). In many cases, these differences observed at a pupation time level were translated into similar differences at the total development time (Fig. 2.5). RIDL heterozygotes also appeared to have shorter developmental times, in general, in comparison to wildtype, Toliman controls (Fig. 2.3; Fig. 2.4; Fig. 2.5). These differences could potentially arise due to an insertional effect of the construct itself. However, this has not yet been tested in heterozygotes under similar nutritionally stressed conditions and could usefully be investigated further.

The escape of females from an fsRIDL system could theoretically pose concerns for the use of this system as an agricultural pest control method. However, it is important to note that the level of female survival observed is extremely low and within current accepted thresholds, as discussed below. It is females of the species that directly and indirectly cause damage to the host crop, by puncturing the surface of the crop with their ovipositor (allowing sources of infection to enter the host tissues), and by laying eggs that develop into larvae within the host fruit on which the larvae feed. However, the percentage of heterozygote females surviving to adulthood on a low protein sucrose diet in the absence of tetracycline in this experiment was 0.1%. This level of female production appears to be within operational limits for such systems. For example, the OX4319L transgenic strain of Diamondback Moth, which also contains a fsRIDL construct, shows a 9% survival of female heterozygotes to pupation, and 1% to adulthood, even when raised on a standard, non-tetracycline based diet (Jin *et al.*, 2013); the RIDL strain of *Aedes aegypti*, OX513A, shows a penetrance of 95-97% (Phuc *et al.*, 2007) but has still been used in highly effective population suppression trials in the field (e.g. Carvalho *et al.*, 2015; Gorman *et al.*, 2015; Harris *et al.*, 2012). In addition, during irradiation-based SIT procedures, the need to balance the trade-off between complete sterility and fitness costs associated with ionizing radiation leads to the application of ionizing radiation at a level that does not result in complete sterility in released males. The Food and Agriculture Organization of the United Nations (FAO),

the International Atomic Energy Agency (IAEA) and the United States Department of Agriculture (USDA) all set their maximum hatch rate of eggs from matings with sterile males at 0.5% (FAO/IAEA/USDA, 2003). This is a higher level than the observed lethality shown in OX3864A heterozygotes on the low protein larval diet.

As discussed by Gong *et al.* (2005), technologies such as RIDL are not a "one size fits all" approach. The level of female emergence observed in my experiments was a replicable effect across different cultures, yet was very low (at ~1 in 1000). The existence of such females, even if fertile, could be acceptable in some circumstances but not others. For example, in the control of a pre-existing pest population, the release or survival of small numbers of females would likely be insignificant, or even negligible, due to the presence of wildtype females in the natural field population. However, in preventative releases that are adopted pre-infestation (in which no pests are yet present in the environment), the addition of any females where they are not present represents a potential risk that should be minimized or avoided.

A potential issue with low levels of female escape in a genetic system designed specifically to cause lethality in females, is the development of resistance traits or alleles in the pest population. The nature of the RIDL approach is that it has the potential to place strong selection pressure for any females able to escape the lethal effects of the expression of the genetic construct. In the event of female survival, this strong selection could cause the rapid spread of resistance alleles. This kind of resistance development can already be a feature of pesticide-based control methods used against Tephritid pests (Vontas *et al.*, 2011). Such resistance is also evident and spreads rapidly in many other taxa (e.g. Shelton *et al.*, 1993). In this chapter, protein levels were manipulated in the laboratory, and it is as yet unclear how these manipulated protein levels might compare to those likely to be encountered in the field. Hence, it is not yet possible to estimate the precise risks of the observed level of female emergence. It is possible to compare the relative levels of protein in field fruits, but the levels of protein available to individual larvae themselves is difficult to estimate accurately. For example, larva may show taxis towards and away from local gradients of protein within fruits as they ripen and then decay. Therefore, the conditions simulated in the laboratory may not be representative of those encountered by RIDL heterozygotes in the field and further tests would be useful here. The potential for the

development of resistance against fsRIDL systems itself is discussed in more detail in **Chapter 3**.

A 1:1 male to female sex ratio was observed in the control crosses involving wildtype, Toliman males crossed with wildtype, Toliman females, across all experimental larval diets, both with and without tetracycline. The progeny from these crosses did not possess any copies of the transgene in their genome. Hence I conclude that the addition of tetracycline to the larval diet does not have an impact on female survival.

The data described in this chapter show the potential for breakdown of the OX3864A fsRIDL strain, as well as changes in development times for fsRIDL heterozygotes, under dietary stress in a release-style scenario. During fsRIDL heterozygote development in environments with low protein, a low level of female survival was evident. The findings are relevant in the context of control programmes involving fsRIDL technologies, in which RIDL-mated, wild females are free to oviposit into a range of host fruits with varying levels of protein. Whether the field conditions for larvae ever approximate the protein levels tested here in the laboratory, and at which some females emerged, requires further testing. Further investigation of female escapes could also be carried out on real host fruit, with a focus being on key cultivars that the medfly uses as a host and on which control methods are frequently currently used. The data thus far do not provide evidence for the potential mechanisms underlying female escape and this would be an interesting avenue for further research.

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## 3 Experimental evolution of responses to tetracycline hydrochloride

### 3.1 Abstract

The Mediterranean fruit fly, *Ceratitis capitata*, is one of the world's most damaging agricultural insect pests. Multiple measures have been used to tackle this pest, and a significant contributor to control efforts has been the sterile insect technique (SIT). The Release of Insects carrying a Dominant Lethal (RIDL) is a newer, species-specific method for insect pest control based on SIT. RIDL individuals contain a tetracycline-repressible, genetic lethal system and are genetically 'sterile'. RIDL systems in the medfly are female-specific (fs); hence lethality is only expressed in transgenic RIDL females in the absence of tetracycline. It is of key importance that the lethality shows high penetrance under field conditions, in order to prevent the propagation of fertile females from the released population. My research results from the previous chapter highlighted that a low level of female escapes were possible under a low protein diet in the absence of tetracycline. The aim of this chapter was to assess the potential for such escapes to lead to the evolution of resistance. I measured tetracycline dose response curves for the OX3864A RIDL strain of medfly. The lowest tetracycline concentration at which female survival was observed was 1 µg/ml tetracycline, with females making up 4.5 % of the total adult population at this level. To investigate the potential for the development of resistance against RIDL lethality at low levels of tetracycline, and to investigate any underlying mechanisms involved, I initiated artificial selection lines. In these, I reared replicated populations of OX3864A on larval diet containing 2 µg/ml tetracycline and replicated OX3864A controls at the standard high tetracycline dose (100 µg/ml) as well as wild type controls at 100 µg/ml tetracycline. Any females that survived in the low dose of tetracycline lines had a strong selective advantage. There was a replicated, significant increase in the proportion of females produced from the low tetracycline selected treatment over the 9 generations that was not observed in either set of control lines. This suggests that the frequency of female resistance had evolved. After 9 generations of selection, I also repeated the dose response curve measurements on the low and high dose OX3864A lines. This revealed a replicated shift in the shape of the response curves between the low and high dose control OX3864A lines at low level of tetracycline, with evidence of higher female survival in the low dose lines at lower doses of tetracycline (0.3 µg/ml). However, there was also a significant and replicated difference between the shape of the pre- and post-selection dose response curves for the control high dose OX3864A lines. The

explanation is unknown but may be due to husbandry differences. The implications of these data for control programmes are discussed.

### 3.2 Introduction

Insect pest species are among the biggest threats to global food supply, having devastating effects on farming outputs across several continents. Tephritid fruit fly species account for some of the most damaging species, with the Mediterranean fruit fly, or medfly (Weidemann: *Ceratitis capitata*), infesting over 300 known species of fruits, vegetables and nuts (USDA, 2005). Current control methods for the medfly include pesticide application (Braham *et al.*, 2007), biological control (Headrick and Goeden, 1996), bait trapping (Burns *et al.*, 2001; Chueca *et al.*, 2007) and the sterile insect technique (SIT; Hendrichs *et al.*, 2002).

Of the techniques mentioned, SIT and SIT-based methods, represent approaches with good potential for additional future development, as efforts are made to move away from wide-scale pesticide dependency. SIT involves the mass rearing of large numbers of the pest insect, which are then sterilized using ionizing radiation, before being released into the pest population. Sterile individuals seek out matings with the opposite sex, often with individuals from the pest population. Such matings are sterile, which results in a lowered reproductive potential for the population as a whole. If such releases are continued over successive generations, the target pest population can be suppressed, if not eradicated (Knipling, 1955).

Male-only releases are generally preferred to bi-sex simultaneous release of males and females (Hendrichs *et al.*, 1995; Meats *et al.*, 2002; Robinson *et al.*, 1999). One key reason is that, for many pests, it is the female that causes the greater problem in comparison to the male. In agricultural pests, the females lay eggs in fruits and nuts, which then hatch into destructive and crop-spoiling larvae. Males are often effectively harmless. Likewise, in insects of public health interest, for example, mosquitoes, it is often only the female that bites humans and livestock. Hence only females pose a risk of disease transmission. Therefore, releasing more females into a population can exacerbate a pest problem if ionizing radiation fails to produce complete sterility in all released insects. Single sex releases offer additional advantages in the avoidance of matings within the release population. Male-only SIT releases can induce a several-fold higher level of induced sterility in comparison to bi-sex releases (Rendon *et al.*, 2004) due to the lack of a 'distraction effect'. SIT relies on sterile individuals seeking out wild

individuals for matings, and if sterile males and females are released together, then the chances of matings with wild flies can be greatly reduced.

SIT has enjoyed much success across many species over the past 60 years. Originally used against the New World screwworm, *Cochliomyia hominivorax*, in Central and North America, it has since been used against the codling moth (*Cydia pomonella*, Linnaeus), in Canada (IAEA, 2001) as well as with relative success against the medfly (Hendrichs *et al.*, 1995). Despite its multiple success stories, SIT has some notable drawbacks. SIT relies on the ability of released individuals to seek out, and successfully mate with a wild member of the opposite sex. Laboratory raised populations are subject to vastly different selection pressures than their wild counterparts, which could lead to assortative mating within these different populations and hence fewer 'control' matings between them. Additionally, the use of ionizing radiation to induce sterility causes a reduced lifespan and competitiveness in laboratory-reared, sterile flies, as well as reducing flight ability (Wong *et al.*, 1982). The result of this is a 4- to 10-fold reduction in fitness in laboratory-reared, sterile males versus their wild equivalents (Lance *et al.*, 2000; Shelly *et al.*, 1994).

Since the drawbacks of classical SIT are well described, many approaches have been developed to try to combat the drawbacks described above and to development improvements so as to increase the production of high quality, competitive male populations from laboratory / factory strains. Genetic sexing strains (GSSs) to allow easier separation of males and females are one way to improve the efficiency of an SIT program. GSSs usually contain a sex-specific, visually evident marker allowing effective separation of males from females (Robinson, 2002). Others employ mechanisms for inducing sex-specific lethality, where mortality is expressed in one of the sexes, leaving a healthy unisex population for potential release (Fu *et al.*, 2007; Ant *et al.*, 2012).

One control technology that can provide significant improvements over classical SIT is the Release of Insects with a Dominant Lethal (RIDL). RIDL is a genetic control method in which insects contain repressible, dominant lethal genetic systems to induce lethality in homozygous and heterozygous individuals. It has been successfully implemented in several insect pests (Thomas *et al.*, 2000; Gong *et al.*, 2005; Fu *et al.*, 2007; Jin *et al.*, 2013). Laboratory reared individuals can be reared with a tetracycline supplement in

their diet, alleviating the lethal effects of the transgene. When released, no tetracycline can be accessed in the environment. Therefore, all progeny from wildtype matings (which will be heterozygous for the dominant lethal transgene) will die prior to reaching reproductive age. Female-specific RIDL (fsRIDL), where lethality is only experienced in the females of a population, can be used to combine GSSs and a radiation replacement technique. In the pre-release generation, tetracycline is removed from the diet, leading to female-specific mortality. The resulting male-only population can be released to seek out and mate with wild females. The OX3864A strain of medfly, developed by Oxitec Ltd, is a transgenic medfly strain containing a fsRIDL system, capable of causing female lethality in the absence of a tetracycline supplement in the diet (Fu *et al.*, 2007).

RIDL systems have been successful in glasshouse, field cage and open release tests (Harvey-Samuel *et al.*, 2015; Carvalho *et al.*, 2015; Gorman *et al.*, 2015; Harris *et al.*, 2012). An important component of the testing of such strains is to examine how robust they are to environmental perturbations and to 'scaling up', to assess their effectiveness when reared in mass rearing environments and released into open field conditions. For example, in many of the GSSs mentioned previously, ionizing radiation was used to induce mutations and chromosome aberrations to produce visible markers for sexing. These induced translocations remained effective when reared in small numbers, but when production was increased to a mass rearing level (Franz *et al.*, 1994), the translocations became increasingly unstable, and this only became more apparent over longer periods of time. Therefore, it is important to stress test and investigate the stability of all new technologies, including RIDL, in comparison to existing technologies.

In previous research (**Chapter 2**) I assessed the possibility of RIDL breakdown and 'female escape' in response to nutritional stress. In this chapter I investigate the relationship between female escape and dietary tetracycline. In the OX3864A strain used is this investigation, tetracycline is used in laboratory diets to suppress the expression of female-specific lethality. Therefore, it is important that tetracycline is not available in the release environment at doses that could allow females to escape the lethality and contribute to the pest problem. If the heterozygous progeny of RIDL released individuals were able to access sufficient tetracycline, then survival to

adulthood might be possible. Although the concentration of tetracycline used to maintain RIDL stocks at an approximate 1:1 sex ratio is well known, it is currently unclear how close is the stock concentration to the threshold of female mortality, or at what tetracycline concentrations females might be able to survive to adulthood. There would be extremely strong selection pressures on any genes contributing to female survival. Hence understanding the relationships between female survival and minimal tetracycline thresholds is important to maintain RIDL effectiveness and to prevent the evolution of resistance.

Although the effectiveness of synthetic pesticides in safeguarding global food security is clear, the number of instances of resistance that is detected is ever increasing, with some insect species now having some form of resistance against all major classes of pesticides (ARPD, 2012). Large numbers of progeny per generation, and multiple generations per crop cycle set up ideal conditions for the rapid evolution of resistance in many arthropod pests (strong, constant selection pressure, large populations sizes in the selected population, etc). Whilst the medfly has only shown resistance to three key insecticides, malathion (Magaña *et al.*, 2007), lindane (Couso-Ferrer *et al.*, 2011) and cyhalothrin-lambda (Arouri *et al.*, 2015), other key insects that have been targeted by RIDL technologies, such as the Diamondback moth, have shown resistance to up to 93 commonly used insecticides (ARPD, 2012).

One positive aspect to come from the rapidly accelerating development of insecticide resistance is increased research into, and understanding of, this area. Over the past 30 years, knowledge of the extent and mechanisms of resistance, as well as techniques to reduce the spread of resistance alleles has grown rapidly (Denholm and Rowland, 1992; Popp *et al.*, 2013; Tabashnik *et al.*, 2013).

In this chapter, I explored the possibility of development of resistance to the tetracycline-repressible female-lethal gene in OX3864A medfly. Baseline tetracycline dose response curves were first determined, to assess the tetracycline concentration at which females are able to survive to adulthood. Based on these findings, I conducted experimental evolution to select for resistance to the lethality in the transgene. Concentrations of tetracycline within the larval diet were varied at two levels to create two selection regimes for OX3864A populations, one at the standard stock rearing

concentration of tetracycline, and one 50 times lower, at a level that induced 75% female mortality.

Three selection lines for each regime were maintained for nine generations, with the sex ratio of each adult population being maintained at a 1:1 ratio of males to females, to equalize the effective population size. Counts of the number of males and female progeny present at each generation were taken, as well as female samples for quantitative PCR analysis of the transgene. This kind of qPCR analysis of the expression in the transgene allowed us to investigate potential mechanisms (e.g. variation in the expression of the lethal transgene) underlying resistance. Finally, after nine generations of selection, tetracycline dose response curves were repeated, to determine whether the strong selection imposed had caused a shift in the dose response profile of the selected population in comparison to the control.

## 3.3 Materials and Methods

### *3.3.1 Medfly stocks and culturing*

The OX3864A medfly strain developed by Oxitec Ltd was used to measure the dose response curve of the female-killing effect of tetracycline, both before and after selection in the artificial selection regimes described here. The Toliman strain, originating in Guatemala and reared at Oxitec Ltd (Oxford, UK) since 2004, and maintained at UEA (Norwich, UK) since 2009, was used as the wildtype (wt) control strain in all experiments. The Toliman strain is the genetic background from which the OX3864A strain was derived. All adult fly colonies were reared at 25 °C, 50-60 % relative humidity and on a 12 h:12 h light:dark cycle.

Adults were maintained in population cages and fed *ad libitum* on a diet of 3:1 sugar:hydrolysed yeast throughout, unless otherwise stated. Damp cotton wicks were inserted into the cages to provide a continual water source. In order to suppress, under standard propagation conditions, the female lethality of the OX3864A strain, the water supplied to the OX3864A stocks contained a tetracycline hydrochloride (Sigma-Aldrich) antibiotic solution in deionised water (100 μg/ml). Wildtype stocks received only deionised water, unless otherwise stated.

Standard 'ASG' larval diets were used throughout this chapter and comprised sugar (147 g), maize (134 g), Brewer's yeast (95 g), nipagin (10% in ethanol; 50 ml), propionic acid (4 ml), agar (25 g) and water (1700 ml). Larvae were raised in 300 ml bottles each containing 100 ml of ASG medium. Stock populations of OX3864A were maintained on ASG diet supplemented with tetracycline hydrochloride (100 µg/ml) to suppress the RIDL lethality. Toliman stock populations were maintained on the standard ASG diet, with no tetracycline supplementation. During the dose response and artificial selection experiments varying levels of tetracycline hydrochloride supplementation were used in the larval diets of both OX3864A and Toliman strains, as outlined below.

### *3.3.2 Determination of baseline tetracycline hydrochloride dose response curves prior to selection*

In order to establish the baseline dose responses of sex ratio in the OX3864A and the Toliman strains to tetracycline hydrochloride, I set up 4 replicate cages of each and measured sex ratio variation in each population as a function of tetracycline concentration. Four small population cages (20 x 20 x 20 cm) containing 300 OX3864A pupae each were set up, with adult diet and a tetracycline hydrochloride-treated water source (100 µg/ml). Simultaneously, three control wt cages, each containing 300 unsexed Toliman pupae, were set up, using the same diet and tetracycline hydrochloride source.

Egg collections were made from each cage 5-9 days post-eclosion. Egg trays were cleared at lights on, and collections made every 4 h, throughout the subsequent 12 h light period. This allowed the maximum number of eggs to be collected whilst maintaining synchronicity of individual egg samples. Eggs from each egg laying period were filtered through filter paper, and placed in petri dishes, subsequently sealed with Parafilm and left to hatch for 48 h.

Once hatched, L1 larvae were collected from each egg sample and 200 larvae seeded onto ASG diet containing 0, 0.1, 0.3, 1, and 3 µg/ml of tetracycline hydrochloride. Each seeded batch of 200 L1 larvae came from the same four-hour collection window. Two complete sets of seedings were performed for each cage of adults.

Each set of larvae was allowed to develop fully until eclosion, at which point the number of pupae, as well as the number of adult males and females was recorded. Samples of three virgin females were collected from each seeding where possible, flash frozen in liquid nitrogen, and stored at -80 °C for subsequent qRT-PCR tests to measure the expression of the transgenic construct in the OX384A lines.

*3.3.3 Artificial selection for resistance to the female-suppression effects of tTAV*

To test for the evolution of resistance in the OX3864A strain to the female killing effects of the tetracycline-driven expression of tTAV I conducted an artificial selection experiment. In this I initiated the experiments at a low dose of tetracycline in which some female 'escape' occurred. Tests for resistance to female killing were then conducted by recording whether there were increasing numbers of female escapes over generations as well as tests for changes in the shape of the baseline dose response curves over time.

Three selection regimes were set up: two involving OX3864A flies, and a third using the Toliman wt strain. The first selection treatment regime comprised 3 x replicated populations of OX3864A flies seeded onto ASG larval diets containing a reduced level of tetracycline supplementation ('low' 2 µg/ml tetracycline hydrochloride). The other two treatments comprised 3 x replicated controls: the first was populations of OX3864A flies seeded onto ASG larval diet containing the standard 'high' 100 µg/ml tetracycline hydrochloride dose. The second was populations of Toliman wt flies reared on an ASG larval diet containing the 'low' 2 µg/ml tetracycline hydrochloride. These treatments therefore allowed the evolution of resistance, separate from the effects of tetracycline per se, to be measured. Each regime had three replicate cages, resulting in a total of nine artificial selection lines. The variation in tetracycline dose occurred only in the larval diet. During the adult life stages, no additional tetracycline was provided (as is standard culturing of this strain), and all adults were maintained on standard 3:1 sugar:yeast diet and a deionised water source.

To initiate the selection lines, eggs were collected from the stock populations of OX3864A and Toliman and seeded onto appropriate larval diets to supply adults for the

F1 generation of the artificial selection lines. Males and females from the same strain and larval diet were sorted and randomly allocated to each of the three replicate lines, until each cage contained 50 males and 50 females.

From each of the nine selection lines, three egg collections were made and seeded onto the appropriate larval diet for that selection line. The first collection was taken approximately 2-3 days after initial egg production, the second 2-3 days later and the third collection approximately 7 days after the first collection. Each egg collection consisted of a seeding of ~500 eggs using a standard protocol that I developed. 500 egg aliquots were obtained by allowing eggs to settle to the bottom of an Eppendorf tube. Then, using a 200 µl micropipette tip from which the last 10mm had been removed, 25 µl of the egg solution was then taken up and expelled in two lots of 12.5 µl. This method provided replicable collections of 500 ± 50 eggs. Therefore, the method facilitated the rapid set up of replicated egg samples and cultures.

All larvae arising from the egg collections were allowed to develop freely until eclosion. From each of the first collections, 50 adult males and 50 adult females were collected and placed into cages to set up the next generation. Supplementary adults from the second collection were used when necessary to boost the numbers to 100. After eclosion, the fly collections from all lines were scored for number of pupal casings, and the number of adult males and females present. Each generation, a sample of three virgin females was also isolated from each selection line, flash frozen in liquid nitrogen, and stored at -80 °C for subsequent qRT-PCR to determine tTAV expression levels.

*3.3.4 Determination of post-selection tetracycline hydrochloride dose response curves*

For post-selection tetracycline dose response tests, the two OX3864A selection regimes were analysed. One cage per selection line was set up containing 300 pupae from generation F9, and maintained on a 3:1 sugar:yeast *ad libitum* diet and a deionised water source. The procedure outlined above for testing the baseline tetracycline dose response was followed, with the exception that four complete eggs seedings were made per cage.

*3.3.5 Molecular analysis – qRT-PCR*

To provide an additional measure of variation in the activity of the female-killing construct as selection progressed, I also measured the level of expression of the tTAV using qRT-PCR and employing the TaqMan probe set method for increased accuracy. For each of the samples of three virgin females collected during both dose response experiments and during the artificial selection experiment, RNA extraction was performed (mirVANA, ThermoFisher). cDNA was then produced using Revertaid kits (Thermo-Fisher Scientific), and qRT-PCR carried out using the primers and probes found in Table 3.1.

Table 3.1 – Primers and probes used for tTAV expression quantification using qRT-PCR.

| Primer/Probe No. and Name | Sequence |
|---|---|
| 1693) Cc17STaqF2 | TCGCAAGTTCGTGGTATTTCTATC |
| 1694) Cc17STaqR | CAAGCAATTTCAACATCTCCTTTG |
| 1695) Cc17Sprobe | FAM-CCGGTACGTAGTTATCACGACGTTCACG-BHQ1 |
| 1557) Taqvp16F3 | CGATGCCCCGGAGGAAGCC |
| 1558) Taqvp16R3 | CTCGTCGCCCAGGCTCACATC |
| 1559) Vp16probe3 | HEX-CGGACACACGCGCCGCCTGAGC-BHQ1 |

Primers 1693, 1694 and probe 1695 correspond to a 17S ribosomal RNA endogenous control gene (as used by Fu *et al.*, 2007) and were marked with a FAM-labelled probe. Primers 1557, 1558 and probe 1559 correspond to VP16 expression from the *tetO* enhancer, and were marked with a HEX-labelled probe.

Relative expression was calculated using the formula:

$$\text{Relative expression} = 2^{(-\Delta CT)}$$

Where –ΔCT = CT target gene – CT endogenous gene, as used by Schmittgen and Livak (2008).

*3.3.6 Data Analysis*

To test for the interaction between proportions of females within the adult populations over consecutive generations during artificial selection, a generalised linear mixed model (GLMM) was fitted and used to analyse female proportion data, using regime and generation as fixed effects, and selection line as a random effect. The number of females as a function of total adults was treated as proportional data. An observational level random effect was added to the model to account for overdispersion of data.

To test for an interaction between the *VP16* gene expression across generations between the 'high' and 'low' tetracycline treatments, a GLMM was fitted using the lme4 library (Bates *et al.*, 2014). Selection line was included as a random effect to account for variation between lines within the same treatment. P-values were approximated using the Satterthwaite approximation found in the lmerTest library (Kuznetsova *et al.*, 2014). All data were analysed using R (R Core Team, 2016).

**3.4 Results**

The baseline tetracycline dose response curve allowed me to determine the lowest tetracycline concentration at which OX3864A females could survive to adulthood, as well as the concentration at which the resulting female progeny survived to a comparable level to that of their male counterparts. OX3864A females were able to survive to adulthood at 1 μg/ml tetracycline in the ASG larval diet, and at this dose approximately 4.5 % of the adult progeny were females. On ASG larval diet containing 3 μg/ml tetracycline, female survival to adulthood was much higher, with 38 % of the total adult population being female (Fig. 3.1). As expected, the proportion of females found in the Toliman control assays remained constant across all the tetracycline concentrations, indicating that there were no effects of tetracycline on female survival *per se*.

The same tetracycline dose response curve assay was then repeated after 8 generations of selection regimes in order to examine whether the pattern of dose responses had changed.

I observed significant changes in the number of females emerging at 0.3 µg/ml tetracycline due to the selection treatment ('low' versus 'high' tetracycline dose OX treatments, BLUE versus RED on Fig. 3.1). These differences were subtle and only occurred at low doses of tetracycline. The percentage female survival for each of the three selection lines in the two treatments at 0.3 µg/ml was 1.8 %, 1.5 %, 1.4 % and 1.0 %, 0.2 %, 0.5 % for the 'low' and 'high' tetracycline regimes, respectively; at 1 µg/ml tetracycline 43 %, 40 %, 51 % and 41 %, 45 %, 43 % for 'low' and 'high' tetracycline regimes respectively; at 3 µg/ml tetracycline 49 %, 48 %, 45 % and 47 %, 47 %, 46 % for 'low' and 'high' tetracycline regimes respectively.

In addition, there were replicated, but unexplained, differences in the shape of the curves in the pre- (baseline) and post-selection 'high' tetracycline dose curves (GREY versus BLUE in Fig. 3.1) at the higher tetracycline doses. Further investigation of these differences, for example the addition of an extra non-selected, $F_0$ generation alongside the post-selection response curves, would be useful to fully attribute effects due to husbandry versus tetracycline treatment.

The proportion of females in the total adult population was also measured at each generation throughout the 9 generations of selection. Using data from the baseline does response curves, I estimated that a selection coefficient of $S$=0.75 had been created, so that approximately 25 % of the total number of female progeny would survive to adulthood. In the F1 generation, the proportion of females was not measured directly, but was assumed to be 0.25 for OX3864A flies in the lowered tetracycline regime, and 0.5 in both the OX3864A high tetracycline and Toliman low tetracycline regimes (Fig. 3.2). To analyse changes in the proportion of females in the total population between different regimes, a GLMM was fitted, including generation and regime as fixed effects, and selection line and observation as random effects. However, there was no significant interaction between regime and generation, so this interaction term was removed from the model. No significant effect of generation was found in the model, so this term was also removed. The resulting model showed the proportion of females in the population to be significantly higher in the Toliman regime in contrast to either the 'high' tetracycline or the 'low' tetracycline regimes (Table 3.2).

qRT-PCR analysis of the *VP16* transgene expression was used to determine expression levels of tTAV in OX3864A females in each of the two selection regimes derived from the OX3864A strain, across generations. qRT-PCR showed consistently low levels of *VP16* expression in OX3864A females in the 'high' tetracycline regime across the generations tested (Fig. 3.3). In the 'low' tetracycline regime, expression of *VP16* in OX3864A females varied across generations (Fig. 3.3 and 3.4). A GLMM was fitted to analyse changes in *VP16* expression across generation between the regimes. No significant interaction was seen between generation and regime, so this term was removed from the model. No significant effect of generation was seen; therefore this term was also removed from the model. The resulting model showed a significantly higher level of *VP16* expression in 'low' tetracycline selection lines than in 'high' tetracycline selection lines. This suggests that any changes in the proportion of females emerging in the 'low' tetracycline regime were not associated with changes in the expression of the lethal gene itself.

Table 3.2 – Results of a GLMM of the association between regime and proportion of females in the difference selection lines. Selection line and observation were included as random effects to control for variation between selection lines and overdispersion of data, respectively.

| Fixed effects | Estimate (SE) | *z*-value | *p*-value |
|---|---|---|---|
| Intercept | -0.174 (0.045) | -3.839 | 0.000* |
| 'Low' 2 µg/ml ASG-TET larval diet | -0.071 (0.065) | -1.088 | 0.276 |
| Toliman | 0.179 (0.065) | 2.759 | 0.006* |
| Random effects | Variance (SD) | | |
| Selection line | 0.002 (0.046) | | |
| Observation | 0.020 (0.143) | | |

Table 3.3 – Results of a GLMM of the association between regime and *VP16* gene expression for different selection lines. Selection line was included as a random effect to control for variation in gene expression between different selection lines.

| Fixed effects | Estimate (SE) | *t*-value | *p*-value |
|---|---|---|---|
| Intercept | 0.157 (0.196) | 0.801 | 0.468 |
| 'Low' 2 µg/ml ASG-TET larval diet | 1.185 (0.277) | 4.278 | 0.013* |
| Random effects | Variance (SD) | | |
| Selection line | 0.084 (0.290) | | |

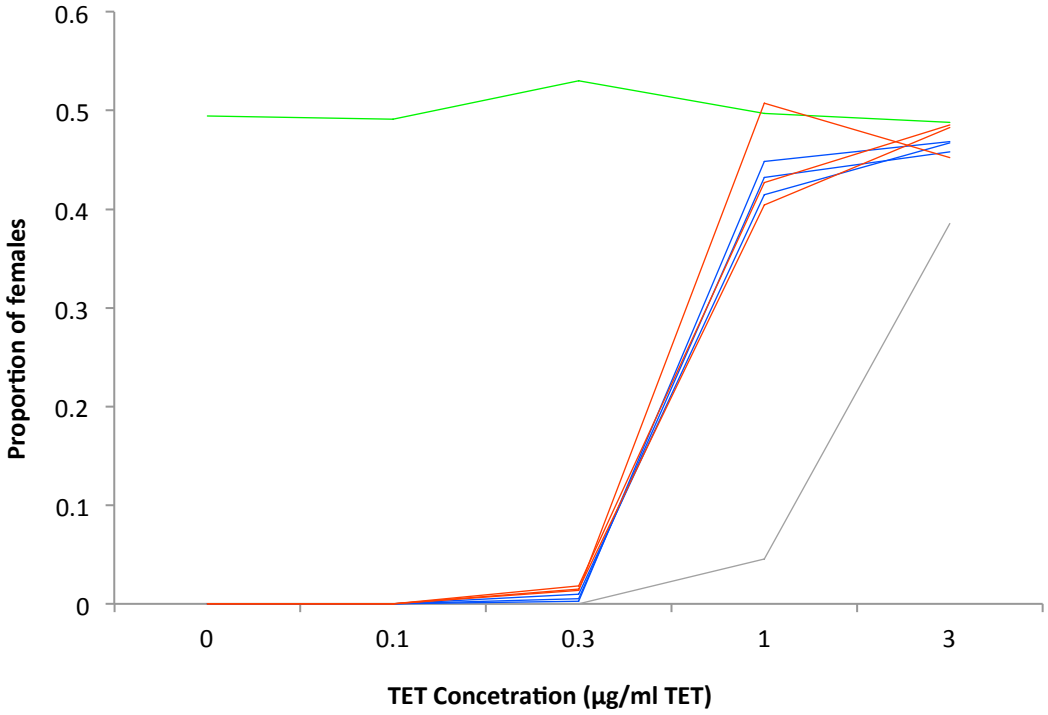


Figure 3.1 – Before and after selection, dose response of proportion of females to varying tetracycline concentrations. GREY – pre-selection, baseline tetracycline dose response of homozygous OX3864A flies from colonies maintained on 100 µg/ml ASG-TET larval diet; GREEN – Toliman, wildtype flies OFF-TET. GREY and GREEN lines both represent mean values comprised of three biological replicates. RED – 3 replicate population of OX3864A flies after 8 generations of selection on 'low' 2 µg/ml ASG-TET larval diet; BLUE - 3 replicates of OX3864A flies after 8 generations of rearing on 'high' 100 µg/ml ASG-TET larval diet. RED and BLUE lines each represent replicated independently evolving populations, with each point being a mean value of four biological replicates.

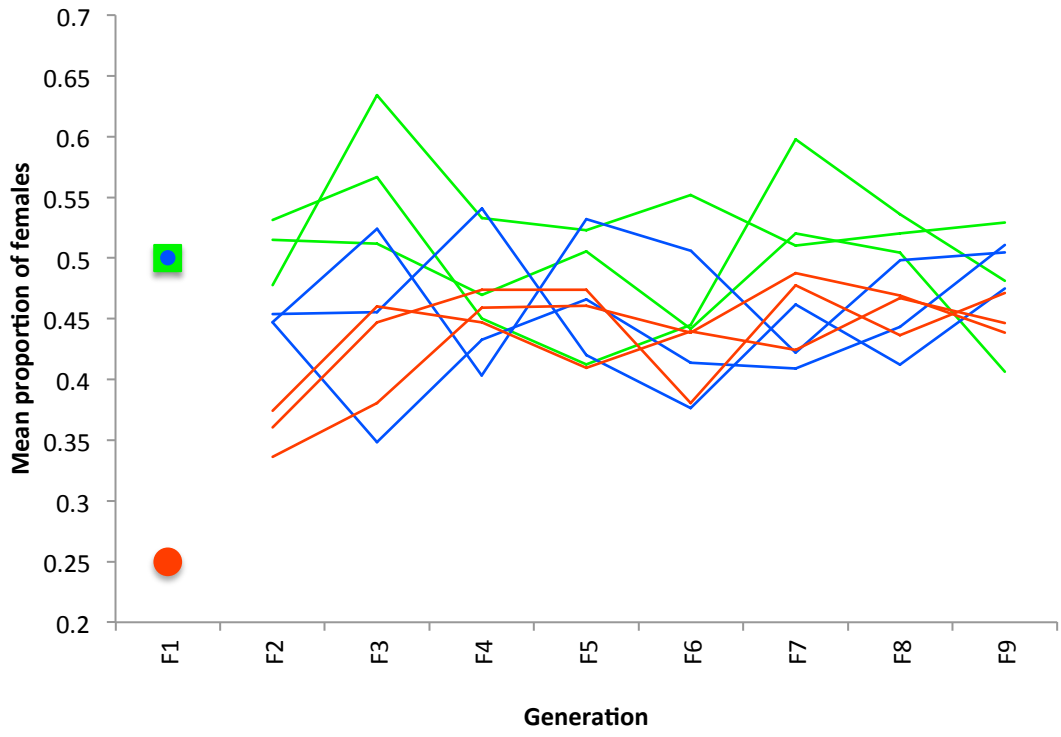


Figure 3.2 – Proportion of females in adult population across 8 generations (F1-F8). Colours represent different selection regimes (GREEN – Toliman, wildtype flies on 'low' 2 µg/ml ASG-TET larval diet; BLUE – OX3864A flies on 'high' 100 µg/ml ASG-TET larval diet; RED – OX3864A flies on 'low' 2 µg/ml ASG-TET larval diet), with three independent selection lines per regime. The F1 generation point represents the initial, expected proportion of females in each population, given the selection pressures introduced to each regime.

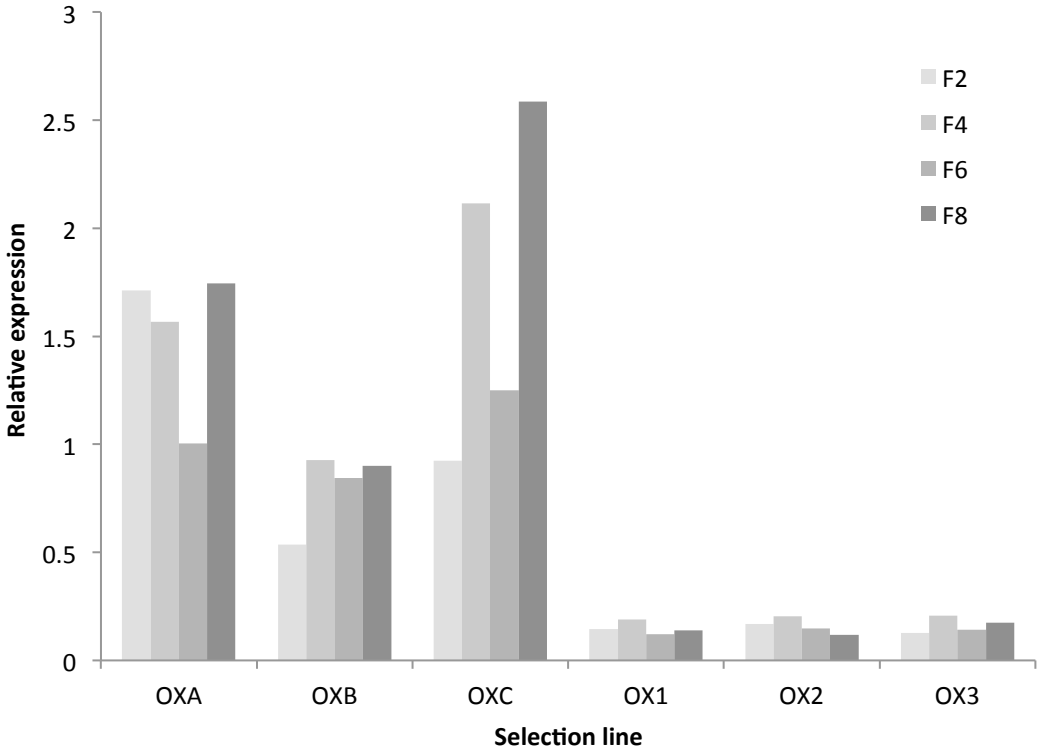


Figure 3.3 – Relative expression of *VP16* transgene, normalised to the 17S ribosomal RNA gene, across generations (F2-F8), across two selection regimes. OXA, OXB and OXC represent three selection lines from the 'low' 2 µg/ml ASG-TET larval diet regime; OX1, OX2 and OX3 represent three selection lines from the 'high' 100 µg/ml ASG-TET larval diet regime.

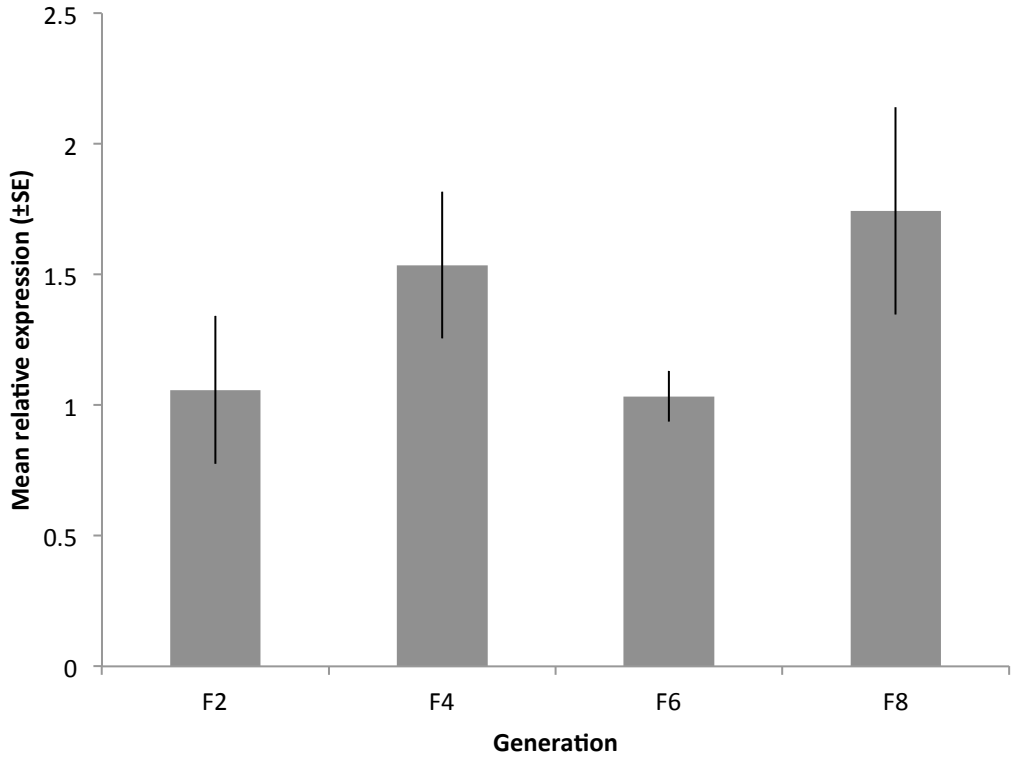


Fig. 3.4 – Mean (±SE) relative expression of *VP16* transgene in pooled cohorts of three OX3864A females under the 'low' 2 µg/ml ASG-TET larval diet regime, across generations (F2-F8). *VP16 g*ene expression was normalised to the 17S ribosomal RNA gene.

### 3.5 Discussion

In this chapter I measured the dose response of sex ratio to tetracycline in a female-specific RIDL line in which female lethality is suppressed by the addition of dietary tetracycline, both before and after selection for decrease female killing in the presence of tetracycline.

The initial dose response curves measured before selection were used to gain a baseline tetracycline response from the OX3864A strain. Under normal stock rearing conditions, the OX3864A strain is maintained on an ASG larval diet containing 100 µg/ml tetracycline, but it was unclear at what tetracycline concentration all female progeny were able to survive to adulthood. Dose response experiments revealed approximately 9% of female progeny were able to survive at 1 µg/ml tetracycline, with up to 80% surviving at 3 µg/ml tetracycline (Fig. 3.1). No females survived below 1 µg/ml tetracycline. This information was then used to define an appropriate starting

concentration of tetracycline for the selection experiment of 2 µg/ml, a dose at which approximately 25 % females emerged.

After 8 generations of the selection regimes, tetracycline dose response experiments revealed that there had been a shift in the response curves, with detectable female survival evident at a concentration of 0.3 µg/ml tetracycline within the larval diet, approximately three times lower than pre-selection response curves. 0.5% and 1% of females in the total number of adults were present at 0.3 µg/ml tetracycline, in the high and low tetracycline regimes respectively, and almost 50 % females in both regimes at 3 µg/ml tetracycline (Fig. 3.1). This shift in response curves was seen in both the 'low' and 'high' tetracycline selection lines in comparison to the pre-selection base line curve. Hence the control lines also showed a significant change in dose responses to tetracycline, mostly at doses > 1 µg/ml tetracycline.

The reason for this unexpected effect is unclear, and - viewed in isolation - the similarity in dose response of the two treatments implies no selection took place. The shift could potentially be caused by selection pressures added by the husbandry techniques used in the selection lines, which are in slight variation to the standard stock-rearing husbandry. Selection for first laid eggs, and therefore first eclosed adults, over multiple generations, as found in the selection lines described here, could be investigated as a source of this shift. There is also the potential of genetic drift in the selection lines which may help to explain the changes in dose response observed, although this is unlikely given the repeated observation across three replicate selection lines. Batch differences in the larval diet could also be a possible source of this shift. Variations in ingredients, including tetracycline supplements, are one possible explanation of the variation observed here. Further tetracycline dose-response tests using a non-selected, $F_0$ generation control group, alongside selected 'high' tetracycline lines, would enable the extent of batch variation in the larval diet and the effects of the selection regimes on the dose response curves to be determined.

Analysis of the proportion of females in the adult population across consecutive generations suggested that OX3864A populations reared on a reduced level of tetracycline, in this case a 50x reduction from the stock level of 100 µg/ml, had started to rebalance their sex ratio (Fig. 3.2). Baseline dose response curves were used to

create a low tetracycline selection regime in which there was a selection coefficient of *S*=0.75, ensuring that only 25% of females survived to adulthood (at a tetracycline concentration of 2 μg/ml within the larval diet). Within the selection lines in the reduced tetracycline regime, the proportion of females in the total adult population appeared to increase across early generations, and by the F9 generation the number of females was comparable to that of the OX3864A selection lines maintained at 'high' 100 μg/ml tetracycline (Fig. 3.2). Although the analysis described here showed no significant interaction between regime and generation, there is some evidence to say the proportion of females in the 'low' tetracycline regimes increased in the early generations, and it may be that we do not have the statistical power to show that increase with the current dataset. The overdispersion in the dataset, and the need to include an observational level random effect, reduces by the power of the statistical test and may be covering up a possible significant interaction between these two variables. The proportion of females witnessed in the 'low' tetracycline regimes shows a higher level of female survival than would have been expected, as shown in the dose response curves, and therefore, some form of evolutionary change cannot be completely ruled out.

In the OX3864A selection lines in the 'high' tetracycline regime, the proportion of females in the total adult population appeared to remain relatively constant across consecutive generations, at a level just below 50 %, with the same trend being true for the Toliman control lines reared in the low tetracycline regime, with there proportion of females being significantly higher throughout at just over 50 %.

Relative expression of *VP16* and therefore, tTAV was consistently low across test generations in the OX3864A 'high' tetracycline regime, as would be expected in a TET-OFF genetic system at the level of tetracycline used to maintain bi-sex stock populations. Elevated levels of *VP16* expression were seen in all three selection lines in the OX3864A 'low' tetracycline regime, however no interaction was seen between relative expression and generation. The increase in the proportion of females within the total number of adults in the OX3864A 'low' tetracycline regime suggested that that had been a rapid emergence and spread of some form of resistance to the lethal effects of the transgenic construct within the OX3864A strain under the strong selection pressure applied. There was no evidence from my data that associated

changes in the expression of the lethal construct was the underlying mechanism involved.

Molecular analysis of the low dose tetracycline regimes indicated a relatively flat response of tTAV expression over consecutive generations, suggesting that lower expression of the transgene was probably not the primary mechanism for the increased numbers of females surviving. Second site effectors could potentially provide an explanation, with these effectors having knock-on effects and potentially causing disruption to the lethal capabilities of the overexpression of tTAV. However, the exact mechanism for the rapid changes in the proportion of females awaits further exploration.

The problem of resistance is a major issue for current methods of insect pest control, particularly using pesticides, with numerous examples of resistance being evident across the Tephritid family (Vontas *et al.*, 2011) as well as in the order Lepidoptera (Tabashnik *et al.*, 1990; Forrester *et al.*, 1993). The introduction of novel genetic technologies to help combat insect pests provides a key new resource in the pursuit of pest control, however, knowing the limitations of such technologies may be key to implementing them in a way that maximizes their efficacy, as well as potentially slowing the development of resistance to other, more conventional techniques.

One way in which to improve the efficacy, and often reduce resistance, of pest control systems is using combinations of different strategies simultaneously against a target species, often referred to as integrated pest management (IPM). IPM is a broad approach to pest control covering biological, ecological and economical approaches, which can include arthropod pests. IPM can include the use of a broad range of methods for control, including trap cropping (Shelton and Badenes-Perez, 2006), bait trapping using pheromone lures (Smit *et al.*, 2001) and pesticides (Epstein and Bassein, 2003), as well as trying to investigate the economic impact that IPM can have on farm profits (Fernandez-Cornejo, 1996).

Despite the success of many IPM programs globally, in many cases there is still a strong reliance on the use of pesticides in cyclic patterns as the primary control method (Kogan, 1998). As a consequence, many cases of resistance to commonly used

chemical controls have been reported across insect orders, including Dipterans (Magaña *et al.*, 2008) and Lepidopterans (Zhao *et al.*, 2002). The reduction of pesticide reliance, in combination with other techniques to reduce pesticide resistance, is already a commonly used practice. The use of transgenic insects to slow down the development of resistance to pesticides has already been shown in the Diamondback moth (Harvey-Samuel *et al.*, 2015) by the introgression of insecticide susceptibility alleles from the released populations into the wild population. Similarly, dilution and slowing of resistance by the release of susceptible, non-resistant, insects could also be useful if resistance to RIDL constructs were ever detected in the field. This could also hold promise for the control of other economically important insect pests including the medfly. However, as with any control method, genetic control is also not immune to the potential for resistance.

The results of this chapter show the potential for development of resistance against the lethal effect of the transgene, such as carried by the OX3864A strain, given strong enough selection pressures. It is unlikely that the conditions under which this strain was selected in the laboratory translate to any conditions that a released strain might encounter in the field, and with this kind of experimental design, it is important to distinguish between proof-of-principle testing, and realistic field conditions when discussing the results. In mass-rearing settings, selection pressures of this kind are kept at an absolute minimum so ensure this kind of resistance does not develop. But these data do highlight the importance of understanding precisely the threshold concentrations of tetracycline needed to maintain very low selection pressures for resistance during stock rearing and in addition, ensuring that stock husbandry is carried out in such a way that these thresholds are never reached. More investigation could also be made into the mechanisms behind such resistance. My data so far indicate that changes in the expression of the lethal construct per se are not responsible. Exploration of the mechanisms in more detail could give a greater understanding of the problem of resistance to transgenic technologies, as well as how to combat it.

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## 4 Response to artificial pheromone sources by OX4319L transgenic Diamondback Moth and dispersal characteristics of a laboratory-reared, wildtype strain

Photograph: Dan Olmstead
http://nault.entomology.cornell.edu/people/olmstead/

**4.1 Abstract**

The Diamondback Moth, *Plutella xylostella*, is an agricultural insect pest of global significance, infesting cruciferous vegetables throughout the *Brassica* family. A transgenic strain of DBM that can be used as a method of population control in the DBM has been developed containing a female-specific version of the Release of Insects carrying a Dominant Lethal (RIDL) system. Female DBM release a sex pheromone in order to attract potential mates in the wild. In this chapter, response of the OX4319L RIDL strain of DBM to an artificial female sex pheromone source was tested in wind tunnel flight trials. I found that OX4319L males were able to respond to the pheromone stimulus as well as did the wildtype and wild-caught males, all of which flew upwind towards the pheromone source. Therefore, the RIDL strain shows comparable pheromone responses in comparison to the wild type. Mark, release, recapture studies of a wildtype DBM strain, from which the OX4319L strain was developed, were also carried out to access the dispersal abilities of a long term, laboratory-reared strain. Released males were recaptured using both pheromone-baited and passive traps within a ~10 acre circular cabbage field, with a recapture rate of 7.93 %. Males were recaptured at all distances up to the boundary of the field at 95 m from the central release point. The median dispersal of males was 14 m. These results show the ability of laboratory-reared strains to disperse within a host field in natural conditions. I discuss the results of these experiments in relation to the potential for the effective use of RIDL DBM strains under open field conditions.

## 4.2 Introduction

Of the many agricultural pests seen across the globe, the Diamondback Moth (DBM), *Plutella xylostella*, is one of the most devastating. Its polyphagous nature allows it to feed on a multitude of economically important host plants within the *Brassica* family, for example, broccoli, cauliflower, mustard, and cabbage. The *Brassica* family forms a major part of the global food economy, with figures estimating the value of these crops at over $26 billion per annum (FAO, 2012). Damage and control methods to combat DBM are estimated to cost between US $1-5 billion dollars annually (Talekar and Shelton, 1993; Shelton *et al.*, 1993; Zalucki *et al.*, 2012). These issues make the control of DBM an issue of importance both in terms of the global economy and food security.

A significant body of research work has described the different methods of control of DBM that are used across the globe. Trap cropping (Shelton and Nault, 2004) and bait trapping (Chisholm *et al.*, 1983) have both been used with some success and sterile insect-like approaches have been demonstrated in small-scale cage studies (Sutrisno *et al.*, 1991). Despite the availability of these more targeted techniques, much of the control effort currently relies on the use of synthetic and natural pesticides. However, the widespread use of pesticides is well known to select for resistance. In this respect DBM represents a significant problem, with resistance to 93 commonly used insecticides being reported to date (APRD, 2012).

The multiple reports of broad-spectrum pesticide resistance in DBM led to the development and introduction of integrated pest management (IPM) schemes across the globe (Guan-Soon, 1990). These schemes aim to use a combination of control approaches and use them in the context of specific pest outbreaks, for example, with reference to measures of outbreak size and location. The use of combinatorial approaches can slow down the development of resistance in comparison to single-method control approaches (Metz *et al.*, 1995). A key breakthrough for IPM programmes has been the development of *Bacillus thuringiensis* (*Bt*) gene incorporation into the genome of crop plant species. *Bt* toxins can be used as insecticides against a large number of insect species (Höfte and Whitely, 1989). The genes for these toxins have now been introduced into a wide variety of popular cultivars to allow them to express insecticidal proteins. Examples of these cultivars

transformed in this manner include *Brassica oleracea* (Cao *et al.*, 1999), *B. napus* (Stewart Jr *et al.*, 1996) and *B. rapa* (Cho *et al.*, 2001).

Another control method that can circumvent the need for insecticides is the use of artificial pheromones, to induce a form of mating disruption. Releasing large amounts of sex pheromones into a targeted area can cause a reduction in the reproductive output of the pest insects due to the disruption of normal mating behaviours. In some cases, this method can be highly trait and species-specific (e.g. Chen *et al.*, 2007; McLaughlin *et al.*, 1994; Schroeder *et al.*, 2000). Pheromones can be used directly for pest control, but also for monitoring population and infestation levels by using pheromone baited traps (Sulifoa and Ebenebe, 2008; Walker *et al.*, 2003). Such trapping techniques are frequently used to monitor the success in implementing other control methods, such as sterile insect technique (SIT) programmes.

Whilst the incorporation of a range of control methods has undoubtedly helped to slow the spread of resistance, new cases of resistance continue to appear rapidly. The ability of agriculturalists to control DBM are becoming increasingly restricted. This has led to the need for new, more efficient control measures against the DBM and other pest insects, as well as the delivery of increased efficacy from current control methods.

The Release of Insects carrying a Dominant Lethal (RIDL) is an insect control technique that involves the manipulation of the DBM genome. RIDL for DBM is based upon the incorporation into the DBM genome of a tetracycline-repressible, dominant genetic system, which can cause lethality in individuals both homozygous and heterozygous for the insertion. RIDL constructs have been successfully transformed into a number of insect species (Thomas *et al.*, 2000; Gong *et al.*, 2005; Fu *et al.*, 2007; Jin *et al.*, 2013) including the DBM, medfly (*Ceratitis capitata*), and olive fly (*Bactrocera oleae*). The type of RIDL construct transformed into the DBM and other agricultural pests is female specific (fsRIDL), meaning that lethality is only expressed in females within a population (Jin *et al.*, 2013; Fu *et al.*, 2007). The lead RIDL strain for DBM used here is referred to as OX4319L. The tetracycline-repressibility of the RIDL construct allows for viable, bi-sex populations to be reared in the laboratory in the presence of a dietary additive of tetracycline. This technology exploits the benefits of effecting male-only releases and concentrating the lethal effects in females.

The OX4319L DBM strain has undergone extensive laboratory and glasshouse testing to assess many aspects of its fitness, such as mating competitiveness, suppression capability and associated fitness costs of transgene insertion (Harvey-Samuel *et al.*, 2014). These experiments have shown that this strain can effect population suppression of wildtype individuals and has the potential to reduce the rate of spread of insecticide resistance (Harvey-Samuel *et al.*, 2015). As for SIT releases, it is reasonable to assume that any future RIDL releases for DBM would use trapping in order to track field releases of moths and to assess the suppression performance of the strain in the field. This is most likely to be achieved using pheromone baited traps. Likewise, the ability of RIDL males to seek out and mate with wild females may be largely influenced on their ability to respond to sex pheromones. However, the ability of OX4319L males to respond to pheromone sources, both natural female sex pheromone and artificial pheromone sources, is as yet untested.

The role of sex pheromone communication in Lepidopteran species is highly complex and species-specific (Roelofs and Comeau, 1969). It is thought to play a key role in mating success, with an important influence on reproductive isolation and potentially speciation. In laboratory experiments, changes in sex pheromone communication represent the basis for divergence in closely related species (Tabata and Ishikawa, 2005). Assortative mating within strains of the same insect species with different pheromone components, or different ratios of those pheromone components, has also been described (Zhu *et al.*, 1997; Pélozuelo *et al.*, 2004; Pélozuelo *et al.*, 2007). Recent work shows that environmental factors can have a large influence on changes to pheromone-based systems (Svensson *et al.*, 2002; Yang and Du, 2003; Pélozuelo *et al.*, 2004; Robbins *et al.*, 2008). It is possible that RIDL strains of DBM reared in very specific laboratory conditions have distinct pheromone profiles in comparison to their wild counterparts, or indeed other laboratory reared strains. This could have negative effects on their ability to provide effective and efficient control because their interactions with wild individuals within a pest population could be compromised.

One aspect of DBM ecology that facilitates its status as one of the globe's most devastating agricultural pests is its capacity for dispersal and migration. Movement of DBM can be divided into two broad categories: long distance migration and short

distance dispersal. The DBM is thought to have originated in Asia Minor, modern day Turkey, but with the spread of agriculture of cruciferous vegetables, it has moved across the globe (Chu, 1986). Its long distance migratory capacity of upwards of 3000 km is a key factor in its global spread (Thygesen, 1968; Bretherton, 1982).

It is perhaps the shorter distance dispersal that poses more of a challenge for the successful development of RIDL technologies in DBM. An important aspect of any RIDL release program is the ability of released RIDL males to disperse a sufficient distance and seek out wild females for matings. The number and location of releases is dependent upon the flight ability of the RIDL males. The average dispersal distance of wildtype DBM in mark-release-recapture trials is 21-35 m when capturing males with 'active' pheromone-baited traps and 14-18 m and 13-24 m when using yellow, sticky bucket 'passive' traps to capture males and females, respectively (Mo *et al.*, 2001). Furthermore, 95% of males were recaptured within 102 m using pheromone-baited traps and within 54 m using yellow, sticky bucket traps (Mo *et al.*, 2001). Therefore, it is of key importance to understand the field dispersal characteristics of strains released into the host environment and to assess the effects on dispersal of long term laboratory rearing. However, it is as yet unknown how effectively males of the OX4319L strain or its wild type laboratory reared progenitor strains will perform in this respect.

In this chapter, I first evaluated the likely success of using pheromone-baited traps for monitoring or trapping during control programs. I did this by testing the response of OX4319L males to an artificial pheromone source in a wind tunnel in comparison to the responses of males from two laboratory-reared strains and males from one wild-caught strain of DBM. I then explored the dispersal characteristics of a long term, laboratory-reared strain in a host field using a mark-release-recapture technique. The laboratory-reared strain used, Vero Beach, is the progenitor strain of the OX4319L RIDL DBM. In order to directly compare the efficacy of different trap types, I compared passive versus pheromone-baited trapping methods in this release scenario.

## 4.3 Materials and Methods

### *4.3.1 Diamondback moth strains and rearing*

The transgenic OX4319L strain was used for experiments involving pheromone performance assessment. OX4319L stocks were maintained at the New York State Agricultural Experiment Station (NYSAES), Cornell University, New York. The OX4319L strain has been genetically engineered to incorporate a tetracycline-repressible, male selecting (female lethal) genetic construct into the DBM genome (Jin *et al.*, 2013). The introduction of this transgenic construct causes suppression of the male selecting (female lethal) gene in the presence of chlortetracycline hydrochloride (100 µg/ml) within the larval diet media, allowing a normal bi-sex stock population to be propagated. In the experimental generation, eggs were seeded onto larval media containing no tetracycline, leading to the expression of the male-selecting (female lethal) gene and hence the generation of a male-only experimental cohort. Adult moths were housed in Bugdorm cages (30 x 30 x 30 cm) containing approximately 2000 individuals and fed with tetracycline sugar water solution.

The laboratory DBM strains used were Geneva 88 and Vero Beach. The Geneva 88 strain was first colonized in the laboratory in 1988 after being wild-caught in Geneva, NY, USA. It has been maintained in the NYSAES laboratories since that date. The Vero Beach strain was acquired by Oxitec Ltd from Syngenta plc, Jealott's Hill, UK in 2008, and was reared at Oxitec Ltd facilities until being sent to NYSAES laboratories in May 2014. These populations were reared using the same non-tetracycline larval diet as for the OX4319L populations. Adult moths from these strains were housed in smaller, cylindrical cages (20 Ø x 30 cm) of approximately 500 individuals and fed with sugar water solution. Eggs were collected using sections of tin foil painted with cabbage water residue as an attractant, before seeding onto larval diet. All laboratory strains were housed at 25 °C with 50 % humidity, on a 16:8 light to dark cycle, in controlled environment facilities.

A wild-caught strain, captured from Omega, GA, USA in May 2014, hereafter referred to as the Georgia strain, was also reared in the NYSAES facility. This population was reared on broccoli plants in large cages within glasshouses with a semi-natural light

cycle of 16 h:8 h light to dark, at 25 °C. Cages were approximately 2 $m^3$ and contained 8-10 potted broccoli plants at any one time. The moth population fluctuated across generations and was not routinely monitored. Fresh broccoli plants were introduced to the cages weekly to maintain a constant source of diet to support larval development.

*4.3.2 Response to artificial pheromone source in a wind tunnel*

Pupae were collected from all four strains, de-cased by hand and kept individually in 1 oz pots. De-casing of males was performed to allow accurate measurement of pupal mass, across strains that may have differing levels of silk production. All pots were moved to a constant temperature chamber at 25 °C and 50 % humidity, with an adjusted 16 h:8 h light to dark cycle, so that "dusk" occurred in the afternoon.

*Pupal mass:* Male OX4319L pupae, reared on non-tetracycline larval diet, were weighed individually and their masses recorded (n=30). Unsexed pupae from the Geneva 88, Vero Beach and Georgia strains were similarly weighed individually. Upon eclosion, adult moths were sexed. Females, as well as the masses of the pupae from which those females emerged, were discarded in order to gather data on male mass only (n=30 males per strain).

*Wind tunnel facility:* Observations were made in a wind tunnel using the set up described by Miller and Roelofs (1978). Briefly, the wind tunnel was approximately 2 m in length and generated an air speed of 0.5 m/s throughout. The tunnels floor was static, but supplemented with artificial leaf-like shapes to provide males with visual stimuli of landmarks whilst they were moving.

*Flight trials:* Males were housed in the constant temperature chamber for 24-48 h post-eclosion before any flight trials. Male moths were moved to the wind tunnel facility 1 h before lights out and transferred into mesh cages from which flight trials could be carried out. Males were left for 1 h in the wind tunnel prior to experimentation to acclimatize to the facility. After lights out, males were given another 15 min to acclimatize to dark conditions before any trials were initiated. During this time, the artificial pheromone lure (ISCAlure-Xylostella, ISCA Technologies, CA, USA) was added to the upwind end of the wind tunnel and placed on a septum stand.

Male moths were added to the downstream end of the wind tunnel individually, and allowed to exit from their mesh cages freely. These males were monitored and assessed against a number of behavioural checkpoints. These checkpoints were: *Active*, where a male was active within the mesh cage before the flight trial; *Take Flight*, whether a male took flight during the flight trial; *Orientation*, whether a male detected the pheromone and orientated towards it; *Upwind*, whether a male moved upwind towards the artificial pheromone source; *Midway*, whether a male reached the midway point of the wind tunnel; *Close*, whether a male reached within 10 cm of the pheromone lure; and *Land*, whether a male landed on the pheromone lure or the septum stand on which the lure was placed.

Multiples males were used in the flight trials per day, with each male only being used once. The procedure was carried out on multiple days over a period of three weeks. The number of males used from each strain varied slightly between days due to time constraints of "dusk" and logistical constraints of rearing sufficient numbers of males from laboratory and wild-caught strains. The trials were continued until a minimum of 30 males had been tested for each strain.

*4.3.3 Field dispersal of a laboratory-reared, wildtype DBM strain*

The field site used for releases was the NYSAES Research Park North, Geneva, NY, USA. Within a 10-acre circular cabbage-planted field, 48 sticky traps (Fig. 4.1) coated in Tanglefoot (Tanglefoot, USA) were positioned in eight concentric circles around a central release point (Fig. 4.2). The inner five circles were positioned at 7 m intervals from the release point up to 35 m and contained the highest density of traps. Within these inner five rings, the number of traps increased with each increase in distance in order to normalise the trap density throughout all rings. The purpose of the inner circles was to provide data on the directional movement of released moths. The outer three rings of traps were positioned at 20 m intervals from the 35 m ring (55 m, 75 m and 95 m respectively) and each contained four traps. The purpose of these rings was to provide data on the upper limit of moth dispersal within the circular field.

Half of the sticky traps placed in the field were also baited with a pheromone lure in order to assess the attractiveness to laboratory-reared moths of this kind of baited trap. As each ring contained an even number of traps, pheromone lures were added in an "on-off" formation around each ring, with alternative start points per ring, i.e. if Trap 1 in the 7 m ring was baited with a pheromone lure, then in the 14 m ring Trap 1 was not be baited, Trap 2 was baited and so on.

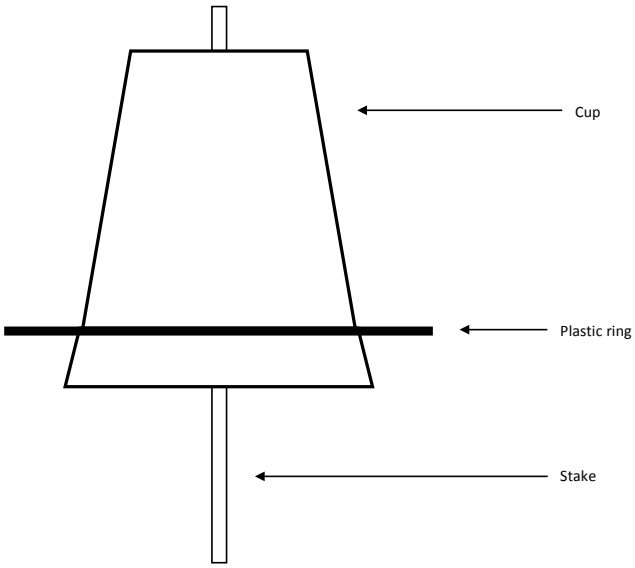


Figure 4.1 – "Pilgrim hat" trap design comprising an upturned cup with plastic ring at its base, all of which was coated with Tanglefoot (based on Musser *et al.*, 2005). Where appropriate, pheromone lures were attached at the top of the cup, secured to the stake with a bulldog clip.

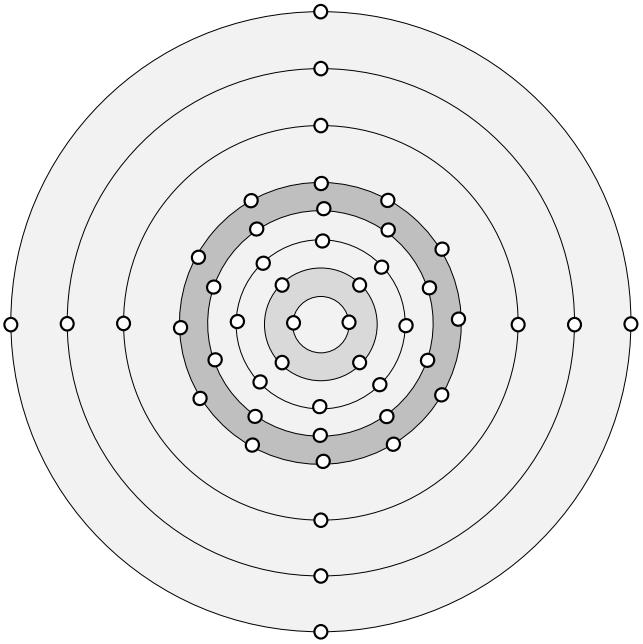

Figure 4.2 – Schematic diagram of trap layout in the circular cabbage-planted field. White circles illustrate trap positions in concentric rings around a central release point. The inner five rings, up to and including the grey ring, were spaced 7 m apart, encompassing a total radius of 35 m from the central release point (7 m, 14 m, 21 m, 28 m, and 35 m from the release point respectively). The outer three rings were spaced 20 m apart (55 m, 75 m, and 95 m from the release point respectively).

The Vero Beach DBM strain was used for the field dispersal trials, to assess the effects on dispersal of a using a laboratory-reared DBM strain. Vero Beach is the genetic background strain from which Oxitec Ltd derived the OX4319L transgenic line of DBM. Therefore, the use of this strain gives a good indication of the likely dispersal characteristics of the Oxitec RIDL strain. Mixed populations of Vero Beach were maintained in cylindrical cages (20 Ø x 30 cm) containing approximately 500 adult moths. Eggs were collected from these populations on tin foil painted with cabbage water as an attractant. These eggs were seeded onto non-tetracycline larval diet, and allowed to develop until pupation. Pupae were then transferred into a Bugdorm cage (30 x 30 x 30 cm) and allowed to eclose.

Upon eclosion, males were isolated and aspirated out of the Bugdorm containers and moved to cylindrical release cages each containing up to 250 male moths and a sugar water source. All females were discarded. Collections were carried out every three hours over a period of 48 h to ensure that the collections were of virgin males. The collected male cohorts were grouped into 24 h windows, resulting in a pair of releases on consecutive days.

Males were dusted with a fluorescent powder (Day-Glo, Day-Glo Color Corp), with a different colour used for each day of release to facilitate recognition of the release day in the recaptured individuals. Although some studies suggest fluorescent dusting has no ill effects on insect behaviour (Holbrook *et al.*, 1970; Naranjo, 1990; Moth and Barker, 1975), others have suggested dusting causes diminished field longevity and dispersal in some species (Moffitt and Albano, 1972; Williams *et al.*, 1977). Fluorescent dusting is the industry standard for mark-release-recapture trials involving many insect species including DBM, and has been shown to be highly efficient compared to other marking methods used with DBM (Cameron *et al.*, 2002). To try to reduce any adverse effects of dusting, minimal fluorescent powder was used and applied with minimal agitation of the adult moths. Once collected and dusted, males were left for between 24 and 48 h before release, to ensure all males were at least 24 h old.

Release cages were transported to the field site an hour before dusk and left to acclimatize after the short journey by car in the centre of the cabbage field. At dusk, the lids and bases were removed from the cylindrical release cages and moths were allowed to freely disperse into the cabbage field. Cages were left at the release point until all moths had exited. This release procedure was repeated the next day with the second cohort of collected males.

Traps were checked the morning after any releases took place and every two days thereafter. Any traps containing moths were removed, taken back to the laboratory for moth identification via scoring of the fluorescent dust and replaced with a fresh trap. The number of males of each dusted colour was scored for each collected trap. The presence and colour of fluorescent powder indicated the whether it was a released male and, if so, the release day. Trapping was continued until no dusted moths were recaptured.

This release experiment protocol was carried out in two blocks. One set of releases in July and the second in August 2014. A total of 1993 male moths were released (July n=993, August n=1000).

*4.3.4 Data Analysis*

Data analysis was carried out using R (R Core Team, 2013). Pupal mass data from wind tunnel experiments were analysed using a Kruskal-Wallis test and subsequent Wilcoxon Rank Sum tests. Analysis of behaviour in the wind tunnel experiments was performed using Fisher's exact tests.

**4.4 Results**

*4.4.1 Body mass and response to artificial pheromone source in a wind tunnel*

Prior to the initiation of the wind tunnel trials, the pupal masses of the four strains (OX4319L, Georgia, Vero Beach and Geneva 88) were measured and analysed (Fig. 4.3). There was a significant difference in pupal mass between the strains ($\chi^2$ = 49.7043, df = 3, $p<0.001$). The OX4319L strain was then tested against the wildtype strains to determine where the differences in pupal mass lay. Wilcoxon rank sum tests showed the OX4319L strain to have a significantly smaller pupal mass than the three wildtype strains: Vero Beach (W = 27.5, $p<0.001$), Geneva 88 (W = 114.5, $p<0.001$) and Georgia (W = 95, $p<0.001$).

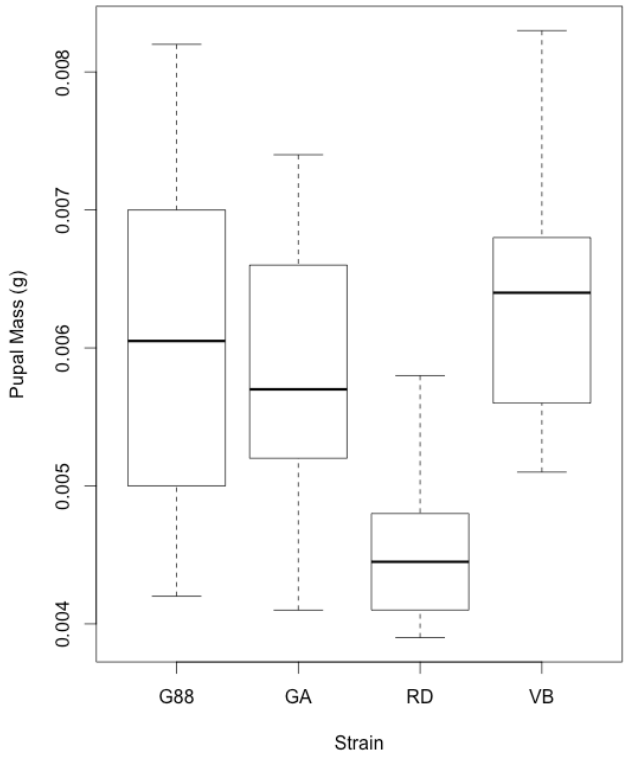


Figure 4.3 – Pupal mass of four DBM strains used in the wind tunnel experiments. G88 and VB refer to the Geneva 88 and Vero Beach laboratory-reared strains respectively; RD refers to the OX4319L transgenic RIDL strain; GA refers to the wild-caught, broccoli-fed Georgia strain. Boxplot shows mean, upper and lower quartiles, and upper and lower bounds (n=30 per strain).

Wind tunnel behaviours were then analysed independently, by assessing the proportion of males completing each behaviour in the different DBM strains (Fig. 4.4). No significant differences were observed between strains in any of the behaviours: *Active* (p=0.056), *Take flight* (p=0.754), *Orientation* (p=0.216), *Upwind* (p=0.263), *Close* (p=0.806), and *Land* (p=1). These results suggest that the different strains did not respond significantly differently in comparison to one another in response to the artificial pheromone lure placed in the wind tunnel experiment.

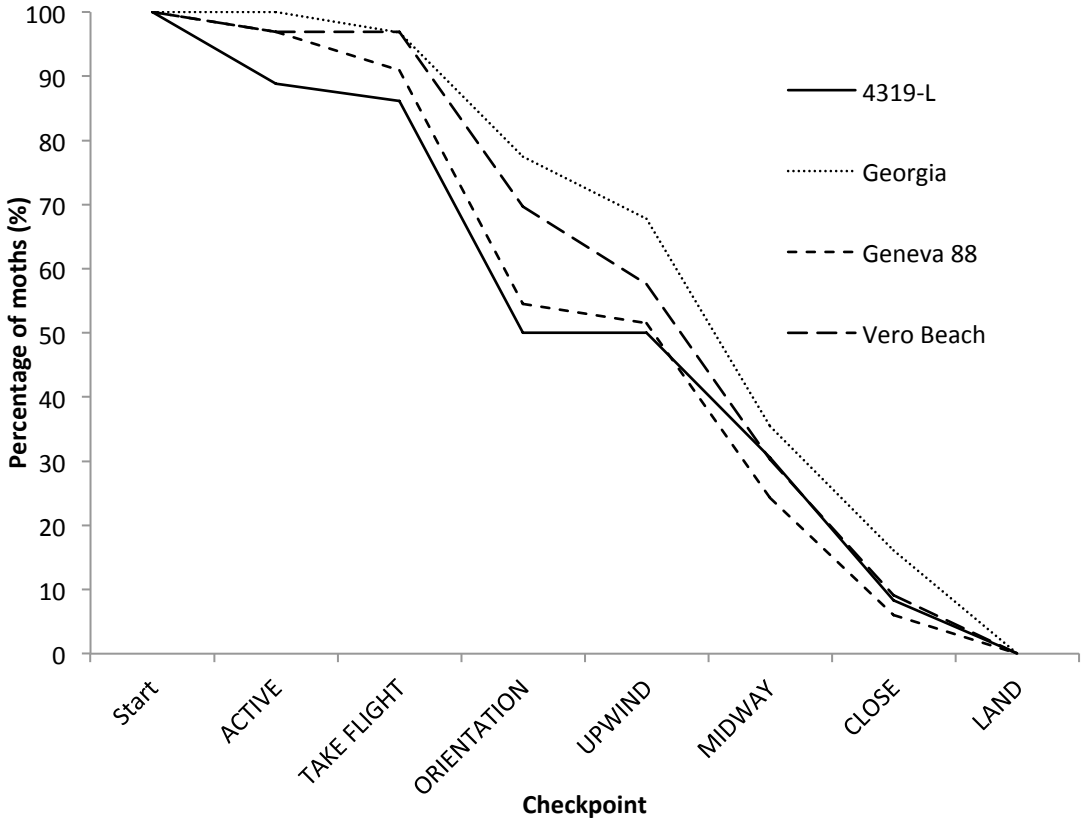


Figure 4.4 – Behavioural responses of different male DBM strains to an artificial pheromone lure in a wind tunnel. The data shown are the percentage of each strain completing each of the behaviours listed on the X-axis (OX4319L, n=36; Vero Beach, n=33; Geneva 88, n=33; Georgia, n=31).

### *4.4.2 Field dispersal of a laboratory-reared, wildtype DBM strain*

Of the total number of Vero Beach male moths released (n=1993), a recapture rate of 7.93 % was recorded across all releases. The median distance travelled by male moths was 14 m, with the mean 26.8 m. Males were recaptured at all distances from the central release point, including the maximum of 95 m. No trapping was carried out outside of the circular cabbage-planted field, so the maximum dispersal of the released moths outside of the host range tested is unknown. No released moths were recaptured more than 8 days after release.

Of those males recaptured, 75 % (n=119) were recaptured using traps baited with artificial pheromone lures. Passive traps were only effective up until a distance of 28 m, after which recapture of moths was observed only on the pheromone-baited traps. A number of non-dusted moths (n=363) were captured on both passive and

pheromone-baited traps across the two releases, indicating the presence of a background population of wild DBM present within the cabbage-planted field.

**4.5 Discussion**

I found no significant difference in response of OX4319L males to an artificial pheromone source when compared to laboratory-reared strains and males from a wild-caught strain. This suggests that OX4319L males were able to respond to pheromone sources in a comparable way to their wildtype counterparts (Fig. 4.4). Males were assessed in a wind tunnel against a number of behavioural checkpoints. Each behaviour was analysed independently using the proportion of males successfully carrying out the behaviour and there was no significant difference between these proportions per strain at any of the checkpoints tested. Most importantly, the key checkpoints of orientation towards to the pheromone plume and movement upwind showed no significant differences between the RIDL and other strains. The data illustrate the ability of OX4319L males to show an initial response to a pheromone source and show that the transgene insertion does not appear to have a strong effect on these behaviours.

Very few males from any of the strains managed to get close to the pheromone lure in the wind tunnel. No males landed on, or made contact with, the pheromone lure (Fig. 4.4). This might be due to the pheromone communication system acting as a long but not short distance system for locating a mate. Hence when a male has located a potential mate, or in this case, an artificial pheromone source, it is other sensory systems (e.g. sight or touch) that then take over. Therefore, at close range the pheromone lures may not continue to act as an adequate stimulus for attraction. Another explanation could be that the artificial pheromone used in the lure is more simplistic than the natural pheromones used by this species and may lack the key components needed for short-range communication. The artificial pheromone source used was a three-component system containing only the main active ingredients of the true DBM female sex pheromone, which supports this idea. The concentration of sex pheromone used may also not have been optimal. No tests on the concentration or rate of pheromone release from the septum were carried out, and therefore it is possible that the pheromone concentration in the wind tunnel was enough to induce

pheromone disruption. The optimal component ratios and blend optima have been debated for different outcomes, whether it be trapping purposes or mating disruption purposes (Chisholm *et al.*, 1984; Minks and Cardé, 1988). Whilst this experiment suggests that the ability of OX4319L males to react to pheromone sources is not diminished in comparison to other laboratory strains, it does not answer the question of whether OX4319L males can gain matings or paternity once they have actively responded to a true pheromone source emitted by wild females.

My results showed that the OX4319L males were significantly smaller than males from the laboratory-reared wildtype and wild-caught strains (Fig. 4.3). There are several explanations, including the possibility of an effect of the insertion or expression of transgene construct itself. The insertion site of the transgene could alter the expression of genes that influence body size. Alternatively, the expression of the transgene itself could be responsible through a trade off by diverting resources within cells that might otherwise be used for the maintenance of body mass.

Smaller moths tend to have shorter longevity and lowered flight ability over the first three weeks of life (Shirai, 1995), so this reduced pupal mass could be a concern in releases of OX4319L males for population control. It may however be possible to produce larger moths by slowing down development, typically lowering the temperature at which development takes place. It has been suggested that moths developing at lower temperatures develop into larger adult moths, with greater flight capacity (Shirai, 1993). This change in body size caused by temperature is due to seasonal variation in behavioural needs of the species. Larger moths in winter and spring have an increased body size and flight capability that enables long range migration to areas where they could not overwinter, whereas summer moths exhibit a smaller body size and lowered flight ability (Shirai, 1991). This natural variation in body size throughout the year may be advantageous to the OX4319L strain, as it could mean that body size is not an important factor in mate choice or reproductive success, an area that has not been extensively researched in the DBM.

Using a larval rearing diet with improved nutritional content or quality could also circumvent the effects observed. Nutritional content could be improved by adding increased levels of dietary protein. The quality of the diets might be augmented by

adding additional vitamin supplements. There is also a need for a more standardized rearing protocol for the OX4319L strain, to maintain more stable densities both between and within generations.

During the field dispersal experiments, I found that males from a long term, laboratory-reared strain were capable of flight and of dispersal in a field release scenario. Males were recaptured at all distances where traps were placed within the circular field up to the maximum distance of 95 m. The mean and median recapture distances for Vero Beach males were 26.8 m and 14 m. These values are consistent with Mo *et al.* (2001) who reported the average dispersal distance of male DBM to be 21-35 m and 14-18 m when using pheromone-baited and passive traps, respectively.

Males were only recaptured at distances greater than 28 m using traps baited with an artificial pheromone lure. This is consistent with findings from the literature, and the finding that pheromone traps can actively draw in males from outside the release site (Mo *et al.*, 2001; Mo *et al.*, 2003). This suggests that the presence of pheromone-baited traps may affect the behaviour of male moths and thus may not be the best way of tracking steady state dispersal characteristics. The use of passive traps might give a better representation of the base line dispersal characteristics of males of any given strain. However, when trapping methods are being used to monitor a RIDL release style scenario, it may be beneficial to recapture as many individuals as possible. If this is the case, then the pheromone-baited traps might be advantageous. Overall, my experiment demonstrated that laboratory-reared strains can be readily tracked after release using mark-release-recapture techniques, using fluorescent dusts as well as passive and active trapping methods.

The data presented in this chapter give valuable information about the ability of the OX4319L transgenic strain of DBM to be used as a method of suppressive pest control against wild populations. The data highlight the ability of OX4319L males to respond to artificial pheromone sources, suggesting the same capabilities would be in present upon exposure to wild type females, and also show an easy tracking method for released moths in the field. Both pheromone-baited and passive traps are effective at capturing male DBM in the field. It may be the case that during dispersal trials, a trade off needs to be made between high recapture rates of pheromone baited traps and the

potential for behavioural manipulation of these pheromones. Passive traps may result in a more natural picture of moth behaviour, but the total number of recaptures may be significantly lower, especially over longer distances. Pheromone traps change moth behaviour by attracting male moths. They may capture higher numbers of males, but may also elevate estimates of moth density, particularly over longer distances. The trap choice during MRR trials, should therefore be dependent upon the number, and density of moths being released.

The data here also show the ability of a long term, laboratory-reared strain to disperse when released in a male-only, SIT based scenario. Data on the average distance dispersed is an important factor when planning an SIT style release, as gaining sufficient coverage of sterile, or transgenic males is key to gaining a sufficient suppressing effect on the pest population. In general, for mating-based control, long distance dispersal is good as it allows for fewer release points, providing more economical control of the target species. However, in scenarios of localised outbreaks, a localised treatment might be seen as a better option, with lower levels of dispersal being advantageous.

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## 5 Reproductive competitiveness and longevity of OX4319L DBM males in field cage trials

Photograph: Dan Olmstead
http://nault.entomology.cornell.edu/people/olmstead/

## 5.1 Abstract

The Release of Insects carrying a Dominant Lethal (RIDL) is a genetic control system developed for control of insect pest populations. RIDL individuals are genetically 'sterile' and produce no, or few, offspring when mated to wildtype individuals. The Diamondback moth, *Plutella xylostella*, is a major pest of cruciferous vegetables from the *Brassica* family, for which a RIDL system has been developed. The DBM RIDL system is female-specific, meaning that female offspring die before adulthood, leaving only males to survive. In order for the RIDL technique to be successful, it is crucial that the released RIDL males survive and are competitive in obtaining matings under field conditions. I tested this in the experiments presented in this chapter. Field cage experiments were used to compare the relative field longevity of males from the OX4319L RIDL strain of DBM against wild-caught males. The recapture rates of the two strains after three days in the field were similar suggesting that the field longevity of the transgenic strain was similar to that of controls under these conditions. The reproductive competitiveness of OX4319L males was also tested against wild-caught males. I found that OX4319L were significantly less competitive than wild-caught males and achieved a mating competitiveness score of 0.29. Although their competitiveness was significantly lower than their wild-caught counterparts, it was higher than for other transgenic and irradiated males used in successful population control trials. The data suggest that the field competitiveness of the OX4319L RIDL strain was within acceptable limits for use in control programmes.

## 5.2 Introduction

The Diamondback moth, *Plutella xylostella* (DBM), is a polyphagous pest of cruciferous vegetables of global agricultural significance. *Brassica* vegetables now contribute over US $26 billion to the global economy annually (FAO, 2012). Hence, pest damage to these crops if of importance both economically and in terms of food security. It is predicted that current control methods of the DBM alone have an annual global cost of US $1-5 billion (Talekar and Shelton, 1993; Shelton *et al.*, 1993; Zalucki *et al.*, 2012).

To attempt to minimize the negative effects of such an economically damaging pest, many methods have been developed and employed, including bait trapping (Chisholm *et al.*, 1983), trap cropping (Shelton and Nault, 2004), mating disruption via pheromones (McLaughlin *et al.*, 1994; Schroeder *et al.*, 2000) and sterile insect technique-like approaches (Sutrisno *et al.*, 1991). All these have all shown some success in controlling DBM populations and the successes and limitations of the currently used techniques have been discussed extensively in review articles (Furlong *et al.*, 2013; Talekar and Shelton, 1993; Verkerk and Wright, 1996).

The primary focus of many control programs for DBM remains the use of synthetic and natural pesticides, as indeed is the case for many of the world's most damaging pest insects. This has led to increasing problems with insecticide resistance. Indeed, since the introduction of broad-spectrum pesticides, cases of insecticide resistance have been reported across many insect families, including in key pests of economic significance such as the Mediterranean fruit fly, *Ceratitis capitata* (Magaña *et al.*, 2007; Couso-Ferrer *et al.*, 2011; Arouri *et al.*, 2015) and the Olive fly, *Bactrocera oleae* (Vontas *et al.*, 2002). There are widespread reports of resistance in the DBM (e.g. Sun *et al.*, 2010; Tabashnik *et al.*, 1990). Large numbers of progeny and high numbers of generations per year allow for the quick appearance and spread of insecticide resistance traits across insect species. DBM is the most insecticide-resistant of any of the Lepidopteran pest species, with evidence of resistance to 93 commonly used insecticides (APRD, 2012).

The discovery of resistance to broad-spectrum pesticides in the DBM led to the development and introduction of integrated pest management (IPM) strategies against

this pest (Sivapragasam *et al.*, 1985; Guan-Soon, 1990). IPM programs use a combination of multiple control strategies according to the specific context of the outbreak, e.g. location and population size. This potentially allows IPM a higher efficacy than broad-spectrum insecticides, if rigorously applied. In addition, the use of a combination of control techniques can slow the development of resistance to each individual component of IPM methods (Metz *et al.*, 1995). One key breakthrough in control has been the use of toxins isolated from the bacteria *Bacillus thuringiensis* (*Bt)*, which can be isolated and used as natural pesticides against a wide variety of insects (Höfte and Whiteley, 1989). *Bt* sprays are often used by organic growers on cruciferous crops, however it degrades quickly in the field, and so multiple applications are necessary.

Since the discovery of *Bt*, the genes that express the insecticidal proteins have been transformed into multiple species of the *Brassica* genus to render them insect 'pest proof', for example, *B. oleracea* (Cao *et al.*, 1999), *B. napus* (Stewart Jr *et al.*, 1996), and *B. rapa* (Cho *et al.*, 2001); although these have not been commercialised at present. However, many studies have reported the evolution of resistance to *Bt* toxins (Zhao *et al.*, 2001; Sarfraz *et al.*, 2005) and have revealed the underlying mechanisms involved (Guo *et al.*, 2015). Hence increasing efforts are necessary in order to combat the spread and impact of this resistance (Shelton *et al.*, 2000). All these factors make DBM an excellent model organism for studying Lepidopteran pests of *Bt* crops.

The numerous cases of pesticide resistance, and the difficulties involved in controlling the development and spread of resistance has led to a need for more efficient application of current control strategies and the development of new tools for wide-scale pest management.

The Release of Insects carrying a Dominant Lethal (RIDL) is an insect control method that could hold some promise in this context. RIDL insects contain a tetracycline-repressible, dominant lethal genetic system, which can cause lethality in both homozygotes and heterozygotes. RIDL constructs have been successfully transformed into several invertebrate species (Thomas *et al.*, 2000; Gong *et al.*, 2005; Fu *et al.*, 2007; Jin *et al.*, 2013). Viable populations can be reared in the laboratory by the addition of tetracycline to the larval diet, which suppresses the expression of the lethal

transgene. In agricultural pests, such as DBM, and medfly, a female-specific RIDL (fsRIDL) has been developed in which lethality is restricted to females (Jin *et al.*, 2013; Fu *et al.*, 2007). This system offers advantages in terms of effecting male-only release and in concentrating the lethal effect in females.

Successful use of RIDL insects has been observed in open-field trials of RIDL *Aedes aegypti* (an important vector of viral disease such as dengue, chikingunya and zika) in multiple locations (Harris *et al.*, 2012; Gorman *et al.*, 2015; Carvalho *et al.*, 2015). RIDL use in other insects has been limited to laboratory testing, glasshouse and field-cage testing, but has also yielded promising results (Ant *et al.*, 2012; Leftwich *et al.*, 2014; Harvey-Samuel *et al.*, 2014).

An important aspect in the success of RIDL-based population control methods is the ability for released individuals to win matings with wild individuals. This becomes more difficult when these types of technology are applied to species that have mating systems involving polygamy. DBM, like a wide variety of other Lepidopteran species, are polygamous, displaying both polygyny and polyandry (Drummond, 1984; Wang *et al.*, 2005). Polyandry has been shown to increase female fecundity (Wang *et al.*, 2005), genetic diversity in progeny and overall female fitness (e.g. Taylor *et al.*, 2008) across insect species. The occurrence of polyandry brings into question sperm competition of mating males. RIDL males must not only be able to compete for matings against other males, but their sperm must be able to compete for fertilisations against the sperm of wild males in order for effective population control to occur.

A RIDL construct was successfully transformed into DBM, resulting in the expression of female specific lethality in the absence of tetracycline (Jin *et al.*, 2013). The resulting strain was designated OX4319L. Laboratory and glasshouse testing of OX4319L has shown effective population suppression of wildtype individuals, as well as the possibility to slow the rate of the spread of insecticide resistance by the addition of susceptibility alleles into wild populations (Harvey-Samuel *et al.*, 2015). Extensive laboratory and glasshouse studies have provided vital insight into the possible capabilities of the OX4319L strain for use in the suppression of pest DBM populations. The next logical step for testing this pest control tool is to take this transgenic strain into the field and stress it under field conditions. In this chapter, field cage studies to

assess the longevity of OX4319L males versus males from a wild-caught, recently colonized wildtype strain of DBM (Georgia; GA) are described. The study also analysed the mating competitive capability of the OX4319L versus GA males, to measure the relative success in gaining paternity in matings with GA females in field cage studies.

## 5.3 Materials and methods

### *5.3.1 Diamondback moth strains and rearing*

The OX4319L strain of diamondback moth was used for all experiments described here. OX4319L stocks were maintained at the New York State Agricultural Experiment Station (NYSAES), Cornell University from July 2015. The OX4319L strain contains a tetracycline-repressible, male selecting (female lethal) transgene. Hence, the expression of the male selecting gene is suppressed in larvae reared in the presence of chlortetracycline hydrochloride (100 µg/ml), allowing the propagation of a bi-sex population. In the release generation, eggs were seeded onto non-tetracycline diet, leading to female lethality and resulting in a male-only release cohort. Adult moths were housed in Bugdorm cages (30 x 30 x 30 cm), and supplemented with tetracycline sugar water solution.

The Georgia (GA) wildtype strain was also maintained at NYSAES insectaries. This strain was wild-caught in early 2014, housed at NYSAES and reared on broccoli initially before being moved to a non-tetracycline based, artificial larval diet in September 2014. Adults were supplemented with non-tetracycline sugar water after eclosion. Both strains were maintained on a 16 h:8 h light to dark cycle at 23 °C and 30 % humidity.

### *5.3.2 Field site and cage design*

All field cage experiments were performed at Research Farm North, NYSAES, New York in August and September 2015. Three field cages were set up, each 24 x 12 x 6 ft (L x W x H), each consisting of a fine mesh (50 squares/cm) covering a metal frame. Each cage was split into two halves using a wooden framed divider covered in mothproof material, resulting in 6 experimental cage areas measuring 12 x 12 x 6 ft, of which three were selected based on them having approximately equal cabbage coverage. The

edges of the divider were sealed with fiberglass insulation to ensure the two halves were completely isolated. One corner of the fabric was attached to the frame using heavy duty Velcro to allow passage from one half of the cage to the other.

Around the entrance to each cage, a wooden framed vestibule was constructed to create double containment within the cages, allowing the cages to be entered without allowing moths to exit. This frame, again, was covered with mothproof material and the edges sealed with fiberglass. Within the vestibule were housed disposable lab coats, a fly swat, and stick traps to ensure containment of the moths within the cages. Each cage contained approximately 24 mature, planted cabbages in four rows of six plants (Fig. 5.1)

Figure 5.1 – Photo showing the set-up inside the field cages, each containing four rows of approximately six cabbage plants.

*5.3.3 Reproductive competitiveness of OX4319L male DBM against GA males*

Males from both OX4319L and GA strains were grouped into cohorts of 500 and housed in release cylinders (20 Ø x 30 cm) containing a sugar water source to standardise pre-release conditions between strains. 500 males from each strain were co-released into field cages (n=3) in the early afternoon and given four hours to acclimatise. Once this acclimatisation period had finished, 500 GA females were added

to each cage using the same release cage setup. The resulting moth populations were allowed to mate freely for 3 days.

On day three post-release, all living males and females from each cage were removed from the cage by aspiration, and isolated into individual Eppendorf tubes. Plants were agitated and removed from the ground if necessary to ensure high recapture of living moths. Once isolated, moths were taken back to the laboratory and the number of each sex recorded.

In order to test mating competitiveness between the two male strains, females from individual cages were housed in cohorts of 20, where possible, in a 16 oz deli pot with a sugar water source and a cabbage painted Parafilm strip for egg laying. Any spare females unable to be grouped into full groups of 20, were housed together in smaller numbers within their respective cages. On day four post-release, the cabbage Parafilms were removed and replaced. Eggs were placed into fresh deli pots and surrounded with sticky traps. These eggs were allowed to hatch, and all resulting L1 larvae were captured on the sticky traps. These L1 larvae were then subjected to PCR analysis to assign paternity to either an OX4319L male or a GA male, based on the detection of expression of the tTAV gene carried on the transgenic construct within the OX4319L descendants.

On day six post-release, the cabbage Parafilms were removed and again replaced. However, this time, each egg sheet was placed into an individual petri dish containing approx. 1 $cm^3$ of TET diet, and sealed with a sheet of absorbent paper. Eggs were allowed to hatch, and larvae allowed to feed and develop until late larval instar or pupation. Upon pupation, individuals underwent PCR analysis to assign paternity. This method of egg collection and rearing was then repeated with the final egg collection made on day eight post-release.

Progeny counts were adjusted for OX4319L crosses to allow a comparison to wildtype matings. It is known that OX4319L heterozygotes have a lowered survivorship to pupation than wildtype counterparts, with 88.3% of RIDL heterozygotes surviving to pupation in relation to wildtype individuals (Morrison, personal communication).

Therefore raw counts of the number of OX4319L progeny were taken, and corrected using this value before reproductive competitive was assessed.

*5.3.4 Male longevity*

In order to assess male longevity between the two strains, the recapture rate of males from each strain on day three post-release was used as a proxy. Recaptured males were subjected to PCR analysis to determine their strain of origin. The cages were reentered on day 6 post-release, and rechecked for any living moths. Any male moths collected at this time were also analyzed using PCR, to assign strain of origin.

*5.3.5 Molecular analysis of recaptured moths and progeny*

Genotyping by PCR was conducted to identify samples carrying the OX4319L transgene insertion versus those that did not. This genotyping was not only able to identify the strain of the released and recaptured male moths, but also identify whether progeny collected from recaptured females were fathered by an OX4319L male or a Georgia (GA) strain male.

The genotyping PCRs were conducted as follows:

Primer Set A - Detects OX4319L transgene insertion, expected 637 bp; primers 'OX4319LF2' (5'-CACAGATATCACCAGAGCATTGGA-3') and 'PB5out' (5'-CTCTGGACGTCATCTTCACTTACGTG-3').
Primer Set B - Detects ribosomal RNA locus (control for gDNA quality), expected 458 bp; primers 'dbm17StaqF' (5'- CCAAGCCTC TTCGTAACAAGATCG -3') and 'dbm17StaqR' (5'- GATGGAGATACCACGCACCTGG -3').

Genotyping PCR was conducted on purified genomic DNA and subjected to the following PCR program using Biotaq polymerase: [2 min at 95°C, 10× (10 s at 95°C, 30 s at 60°C minus 0.5°C per cycle, 1 min at 72°C), 25× (10 s at 95°C, 30 s at 55°C, 1 min at 72°C), 7 min at 72°C. To detect the presence of PCR product, all reactions were run on a 1% TAE agarose gel for 20 min and photographed on a UV gel documentation system. The presence of a PCR product from Primer Set B was used as a positive control assay,

and presence of PCR product from Primer Set A indicated the presence of the transgene insertion.

*5.3.6 Data analysis*

For the analysis of reproductive competitiveness data, a Chi-squared test of independence was used to determine the strength of the association between the numbers of each strain (OX4319L or GA) among the progeny of recaptured females. For male longevity, a Chi-squared test of independence was also used to determine the strength of association between the identity of the male strain and the proportions of each strain recaptured. All data analysis was carried out using R (R Core Team, 2016).

**5.4 Results**

*5.4.1 Male longevity*

After 3 days in the field cages, a total of 360 male moths were collected from the three test cages. PCR genotyping of these males showed that, of all male moths recaptured, 42 % were OX4319L males and 58 % were non-transgenic males (Table 5.1; Figure 5.2). There was no significant difference in recapture rates of the two strains, and by inference longevity, after 3 days ($\chi^2 = 2.5995$, df = 2, p = 0.2726).

Table 5.1 - Numbers of OX4319L and non-transgenic male moths recaptured from each field cage 3 days post-release.

| Cage | OX4319L | Non-transgenic |
|---|---|---|
| 7A | 54 | 87 |
| 7B | 56 | 91 |
| 8B | 35 | 37 |
| Total | 145 | 215 |

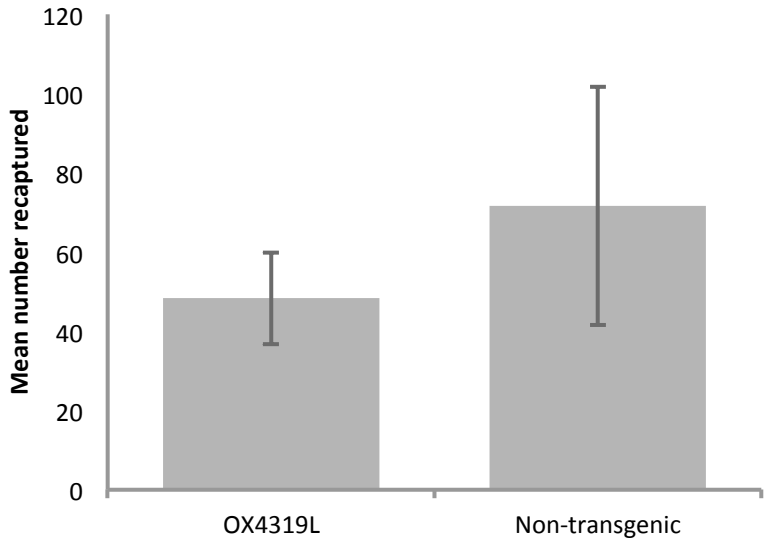


Figure 5.2 - Mean number of OX4319L and non-transgenic male moths recaptured from field cages (n=3) 3 days post-release. Mean ± 1 standard deviation.

These data allowed us to estimate daily probability of survival (DPS) of each male moth type as: 45.9% for OX4319L males and 52.3% for non-transgenic males (DPS = ((final number of males)/(initial number of males))$^{1/d}$; where d = interval between release and recapture, or number of days; Clements and Paterson, 1981). Average Life Expectancy (ALE) was calculated as 1.29 days and 1.53 days for OX4319L and non-transgenic males, respectively (Clements and Paterson, 1981). These are most likely underestimates of survival, because the presence of some moths in the field cages after day 3 showed that not all surviving released moths had been recaptured.

*5.4.2 Reproductive competitiveness of OX4319L male DBM against GA males*

The results from genotyping the progeny of recaptured female moths indicated that non-transgenic males were more competitive in mating than were OX4319L males (Table 5.2; Figure 5.3; $\chi^2 = 30.3047$, df = 2, $p < 0.001$). OX4319L males acquired an estimated paternity of 23 % of the progeny in the cages, equivalent to a mating fraction (proportion of progeny sired) of 0.23. These data translate into OX4319L male moths achieving an estimated mating competitiveness of 0.29 against their GA counterparts in cages, assuming equal numbers of each male type (Mayer *et al.*, 1998).

Table 5.2 - Genotype and frequency of progeny from the three field cages, from recaptured females grouped into cohorts of ≤20 individuals (designated as 'family'). Progeny were genotyped as pupae or late larval instar. The 'Corrected totals' column shows OX4319L numbers of recaptured OX4319L progeny corrected to account for the lower rate of survival (88.3 %) to pupation of this strain in comparison to controls.

| | | Family | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Cage | Strain | 1 | 2 | 3 | 4 | 5 | 6 | 7 | Total | Corrected totals |
| 7A | OX4319L | 5 | 24 | 0 | 0 | 0 | 0 | 4 | 33 | 37 |
| | Non-transgenic | 35 | 15 | 12 | 20 | 1 | 26 | 4 | 113 | 113 |
| 7B | OX4319L | 1 | 0 | 1 | 4 | 1 | 27 | 14 | 48 | 54 |
| | Non-transgenic | 25 | 0 | 24 | 19 | 11 | 26 | 16 | 121 | 121 |
| 8B | OX4319L | 0 | 0 | 1 | | | | | 1 | 1 |
| | Non-transgenic | 39 | 24 | 23 | | | | | 86 | 86 |

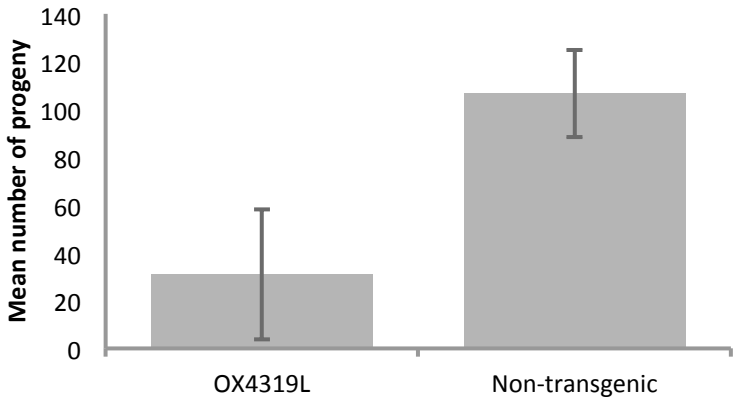


Figure 5.3 - Mean number of OX4319L and non-transgenic progeny produced by females collected from field cages 3 days post-release. Error bars indicate standard deviation. OX4319L data are corrected to account for lower rate of survival (88.3 %) to pupation of OX4319L heterozygotes relative to non-transgenic counterparts.

## 5.5 Discussion

I found no significant difference in recapture rates of OX4319L and GA males after 3 days, which suggests comparable field longevity after three days for males from these two strains (Fig. 5.2). Mating competitiveness of OX4319L in comparison to GA males was assessed by collection of progeny from mated females housed together with males from both strains. OX4319L males gained significantly lower paternity in comparison to GA males (Fig. 5.3; proportion of progeny sired by OX4319L males = 0.23). Although the results showed that the OX4329L males were less competitive at gaining fertilisations than the control, their relative competitiveness was within the range of successfully deployed SIT and RIDL release strains in other insects (FAO/IAEA/USDA, 2003). The reduced competitiveness was not unexpected given the documented fitness cost of transgene insertions (e.g. Harvey-Samuel *et al.*, 2014). It is also possible that the fitness deficiencies of strains carrying transgene insertions might be more apparent under field conditions, which are likely to be more hazardous than the laboratory environment. As shown in **Chapter 4**, OX4319L males have significantly lower pupal masses than wildtype counterparts, which will likely result in smaller body size in the adults. This size deficit could also account for OX4319L males being less competitive to some degree, in addition to any possible insertion effects of the transgene.

Counts of the collection of moths from field cages on day three post-release, showed a recapture rate of approximately 10 %. This is consistent with the recapture rate of GA males in mark-release-recapture trials achieved previously (see **Chapter 4**). This recapture rate suggests that in future suppression programs of RIDL DBM strains, three releases of OX4319L males a week would be required to ensure an acceptable and stable standing population of OX4319L males within the pest population. This release rate of three releases per week has previously been shown to effective in releases of transgenic *Aedes aegypti* in field suppression trials (Carvalho *et al.*, 2015; Harris *et al.*, 2012; Gorman *et al.*, 2015).

Although significantly lower than that of their GA counterparts, the mating fraction (relative competitiveness) of 0.23 gained by OX4319L males was still above the standard for sterile males in widely practiced sterile insect technique (SIT) releases. Currently, the International Atomic Energy Agency (IAEA; 2003) states that irradiated

males for release should gain a mating fraction of 0.2 as a minimum requirement for suppression. The results of this chapter are promising because OX4319L males gained this mating fraction in competition against a recently colonized GA wildtype strain, and under testing field conditions.

When this mating fraction was transformed into a mating competitiveness, the value was 0.29 for the OX4319L males in competition with GA males (Mayer *et al.*, 1998). This value was considerably higher than the mating competitiveness calculated for non-transgenic, irradiated DBM males, which showed a mating competitiveness of 0.07 during SIT releases (Apu, 2002). The OX4319L mating competitiveness value was also higher than has been shown in successful suppression releases of transgenic mosquitos, where a mating competitiveness of 0.031 was observed in Brazil (Carvalho *et al.*, 2015), 0.14 in Panama (Gorman *et al.*, 2015) and 0.059 in the Caribbean (Harris *et al.*, 2012). Large scale, irradiation-based SIT release programs for New World screwworm and the Mediterranean fruit fly have also shown mating competitiveness values of 0.1 (Mayer *et al.*, 1998) and <0.01 (Rendon *et al.*, 2004) respectively. The data clearly suggest that OX4319L has promise for use in in population suppression of DBM. The mating competitiveness here is drawn from field cage trials and the estimates of mating competitiveness should be taken conservatively, and may be lower in larger scale releases under open field conditions.

The method for collecting progeny used in this chapter, of collecting mated females from field cages after 3 days and isolating them for egg laying, does not give complete resolution of the paternity gained by each cohort of males. After mating, females begin laying eggs within a few hours (Talekar and Shelton, 1993), with mating stimulating egg laying (Wang *et al.*, 2005) and continue laying for approximately 5-8 days. This means that in ensuring that males were given maximum time to mate with females, early egg laying will have been missed, and therefore the estimate of paternity gained may not be the whole story. It is possible that a cohort of males may have had a higher reproductive competitiveness during the first 3 days of egg laying. This matter should be further investigated to ensure the data presented here are not skewed towards later laid eggs.

The diminished reproductive competitiveness shown by the OX4319L strain compared to that of their wild-caught counterparts could be caused by a wild variety of factors. Laboratory adaptation is a key factor that must be considered. Laboratory-reared strains, such as the OX4319L strain, as well as its progenitor strain Vero Beach, have been maintained in laboratory settings for a number of years, facing very different selection pressures from wild individuals of their species. Conditions faced in the laboratory are stringently maintained, with little to no environmental fluctuation or stressors. The loss of resistance to such stressors is well documented in other insect species (e.g. Hoffman *et al.*, 2001; and Matos *et al.*, 2000), although not well known in the DBM. It could therefore be feasible that selection towards reproductive traits could also be affected in the same way. Husbandry involving small cages may limit the opportunity for sustained flight, but maximise encounters with the opposite sex, meaning increased mating occurrences but decreased courtship. Similar trends have been described in Medfly populations where high densities within cages are common (Briceño and Eberhard, 1998).

Both factors could play a key role in the reduced competitiveness evident in transgenic lines, however the reduction in competitiveness has not been a limiting factor is transgenic approaches to pest control thus far. Although methods to improve competitiveness of individuals would be very beneficial, it is often easier and cheaper to simply adjust the overflooding ratio of any suppression based trials in order to ensure a high level of mating success from the release population.

The data presented in this chapter provide information on the use of the OX4319L transgenic strain of DBM as a method of suppressive pest control against wild DBM populations. Although no suppression trials were carried out in field cages, previous work in glasshouses has already shown that OX4319L is capable of suppressing wildtype DBM populations (Harvey-Samuel et al., 2015). The data also shed light on other important questions when releasing transgenic insects in this way. For example, the male longevity data suggest the frequency of releases that would need to be implemented in a suppression trial in order to keep a stable level of OX4319L males within a pest population. The data collected on mating competition between OX4319L and GA males offer an insight into the overflooding ratios that might be needed in order to achieve the desired suppression of a pest DBM population.

The next steps for the development of this strain could involve open field releases, both to assess the dispersal characteristics of the OX4319L strain and to determine whether the mating competitiveness reported here is maintained when OX4319L males are released into wild populations and are forced to seek out females and compete for them in open competition.

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# 6 General Discussion

## 6.1 Introduction

This thesis has focussed on the testing of an important, and potentially environmentally benign, method for insect pest control - the Release of Insects carrying a Dominant Lethal (RIDL) - in two key agricultural pests: the Mediterranean fruit fly (medfly) and the Diamondback moth (DBM). In this chapter I summarise the key findings from the data presented in the thesis and use these results to suggest potentially fruitful lines of future research. I discuss the prospects for RIDL technology as a whole, particularly focussing on RIDL strains of medfly and DBM. I also discuss the broader implications of the successful use of genetic engineering in insect pest control in terms of both technology and for public opinion.

## 6.2 Key findings

### *6.2.1 The effect of larval dietary components on the penetrance of a female-specific RIDL construct in the Mediterranean fruit fly*

In **Chapter 2**, I tested the effect on lethal penetrance of varying carbohydrate source and protein levels in the larval diet of developing RIDL medfly heterozygotes to simulate a population control, release style scenario. Male-only populations of the OX3864A strain of medfly, homozygous for a female-specific RIDL (fsRIDL) construct, were obtained by rearing in the absence of a dietary tetracycline supplement. These males were then allowed to mate with wildtype females, producing $F_1$ offspring heterozygous for the fsRIDL construct. These progeny were seeded and allowed to develop on larval diets with varying carbohydrate sources and protein content. Changes in the carbohydrate source of the larval diet had no effect on the penetrance of the RIDL construct, i.e. female lethality, however changes in protein level did lead to the emergence of some females. Hence a low level of female 'escape' was evident in heterozygous offspring seeded onto a low protein, sucrose-based diet.

The level of breakdown of RIDL lethality I observed does not pose immediate concern for the control potential of this lead RIDL strain. It falls below the level of female

escape for RIDL strains of other species and below the fertility threshold for irradiation treatment in SIT programmes. However, even though this laboratory experiment was conducted at a large scale, it should be noted that this experiment involved relatively low numbers of medfly in comparison to the numbers of individuals that would be released in an open-release suppression trial. In agricultural pests, it is generally the females, which cause damage to the crops. Therefore, any increase, however small, in the number of females surviving could represent a potential concern. However, in reality, a threshold-type system, as characteristic of integrated pest management (IPM) systems (e.g. Peterson and Hunt, 2003) could be implemented to determine an acceptability level for female survival, on a case-by-case basis. In some scenarios, a low level of female survival may not be acceptable. However, in other situations it might not represent any elevated risk.

Female escape could be the subject of extensive modelling to assess any potential problems that arise from females surviving to adulthood, taking into account all the relevant factors (i.e. number of individuals released, background population level, and level of escape). However, currently, this has not been explored in regards to RIDL systems in the medfly or the Diamondback moth, with current modelling efforts focussed on how RIDL systems may be able to reduce the spread of resistance to conventional control techniques like insecticides. Perhaps the easiest way to assess an acceptable level of female escape is to focus on the results of suppression trials, both in terms of pest population numbers, but also by tracking other measurable variables such as crop damage or disease prevalence, depending on the pest insect in question. If an increase in these is witnessed, and this increase gets to an unacceptable level using IPM-based thresholds, then the escape of females could provide possible causes for such effects.

An issue of perhaps greater concern is the potential for small numbers of females to survive in an fsRIDL system to lead to the development of resistance. Any females that survive represent a target for selectable resistance to the RIDL construct. Agricultural pests are frequently successful invasive species because of life history traits that make them highly adaptable to new environments (Carey, 1984). For example, high population numbers, overlapping generations, and multiple generations per year may all increase the likelihood of these types of pests to develop resistance, as seen in

response to the application of many different pesticides and over very short timescales (ARPD, 2012; Magaña *et al.*, 2007; Couso-Ferrer *et al.*, 2011). The potential for resistance against genetic control systems is as yet relatively untested.

*6.2.2 Experimental evolution of responses to tetracycline hydrochloride*

In **Chapter 3** I determined tetracycline dose response curves to assess the minimum concentration of tetracycline at which female survival is possible. I then used these data to inform the initiation of selection lines to select for increased female survival at low concentrations of tetracycline. After a number of generations of selection, dose response curves were again performed to investigate whether dose responses to tetracycline had changed over the course of selection.

Following 8 generations of selection for female survival at low tetracycline concentration, dose response curves indeed showed a change in tetracycline responses, with females surviving at much lower concentrations (approximately 3 times lower) of tetracycline than at the start of the experiment. In the selection lines themselves, rearing at a 50x-reduced level of tetracycline initially resulted in a significantly male biased sex ratio in surviving offspring, as expected. However, over subsequent generations of selection under low tetracycline, the sex ratio rebalanced and became more female as greater numbers of females escaped the RIDL construct lethality. A sex ratio equivalent to the control lines was achieved by generation 9. The results show the development of potential resistance to the female killing construct, if low levels of the dietary antidote, tetracycline, are available and hence if some females escape and selection is sufficiently strong. Molecular analysis of expression of the lethal transgene did not give any evidence that the underlying mechanism by which females survived was a change in gene expression. Therefore, the mechanism of female escape is as yet unknown. It would be interesting to discover the contribution to the resistance phenotype of modifiers of gene expression. Overall, the data presented in this chapter highlight the importance of good stock husbandry practice to ensure that selection pressures for female escape are non-existent, which is how mass-rearing facilities currently operate.

It is currently thought that levels of tetracycline present in the natural environment are far less than those needed to prevent female lethality in this kind of system. This is likely to be the case in agricultural pests, where the insect is feeding on fruit crops, but may be more of an issue in other insect pests. For example, mosquitoes may develop in pools of water, which in theory could be contaminated with tetracycline through run-off, due to the wide scale use of tetracycline in livestock agriculture. The experiment described in this chapter is a proof-of-principle to investigate the theoretical possibility of resistance development, with the aim of studying the process and mechanism by which resistance could be achieved. However, the conditions under which such resistance could occur should be far different from those that any released insect would encounter.

Further work in this area could focus on the possible mechanisms behind female escape, as the present study did not show any indication of possible mechanisms. Work from **Chapters 2** and **3** could be combined, to launch an investigation of the possible mechanisms underlying female survival in adverse dietary conditions and / or low levels of tetracycline availability. Artificial selection could be performed on populations of individuals reared on larval diets with low protein, taking the small numbers of surviving RIDL heterozygous females and allowing them to mate to surviving wildtype. Arguably, this is more representative of the possible route for selection that might occur during a suppression release.

### *6.2.3 Response to artificial pheromone sources by OX4319L transgenic Diamondback moth*

In this chapter I investigated to ability of males from the OX3419L strain of DBM containing an fsRIDL construct, to respond to an artificial source in wind tunnel experiments. Despite having significantly smaller pupal masses (and hence body sizes) than all control groups, OX4319L males showed no diminished ability to respond to an artificial pheromone source. This is an important test for the genetically engineered strain or two reasons.

First, the concept of RIDL as a control strategy depends on released RIDL males being able to seek out and effectively mate with wild individuals within the pest population.

Long distance mate recognition is largely down to sex pheromones released by the females, which males can sense and allow them to seek out female mates (Chow *et al.*, 1974; Talekar and Shelton, 1993). Therefore, it is important for any released males to be able to sense and react to female sex pheromones in a comparative manner to that of wild-caught males.

Second, during open field trials and possible suppression trials using such strains, tracking of released individuals is needed to assess the effectiveness of the release treatment. Monitoring of field populations can be done using pheromone-baited traps (e.g. Baker *et al.*, 1982), which allow the easy capture of released males. Having comparable attractiveness of artificial pheromone lures to both OX4319L males and males from other strains allows for better comparisons in recapture and dispersal rates. The data presented here suggest pheromone baited traps would be suitable for use with the OX4319L strain of DBM in the field.

*6.2.4 Dispersal characteristics of a laboratory-reared, wildtype DBM strain*

**Chapter 4** also investigated the dispersal characteristics of a long-term, laboratory-reared wildtype strain, Vero Beach, of DBM. The Vero Beach strain is the progenitor strain from which the OX4319L strain was developed. Selection pressures on laboratory-reared strains are very different to field conditions (e.g. Shelly *et al.*, 1994; Lance *et al.*, 2000). With the compact cage conditions and no need for extended flight, this key ability may well be diminished in laboratory-reared strains. This represents a potential problem for the ability of such strains to achieve effective control.

Dispersal of released individuals from the Vero Beach strain was measured within a circular cabbage field of approximately 10 acres using both passive and pheromone-baited sticky traps. Dispersal to the outer perimeter of the host field was observed, showing evidence for moths with a long history of laboratory rearing being able to disperse up to, and potentially over, 100 m. The average dispersal distance was also consistent with findings in the literature (e.g. Mo *et al.*, 2001). This data is promising for RIDL OX4319L moths, as, assuming no transgene insertion effects on flying, it is likely that a RIDL strain developed from the Vero Beach strain would show similar dispersal capabilities.

Experiments demonstrating the dispersal of OX4319L males have been planned, where RIDL males will be released alongside Vero Beach and wild-caught males in mark, release, recapture studies in order to examine the dispersal capabilities compared to males from other strains. These dispersal data are essential for planning and implementing an effective suppression programme where release distances must be incorporated to gain a sufficiently high presence of RIDL males throughout a pest population.

*6.2.5 Mating competitiveness and longevity of OX4319L males in field cage trials*

In **Chapter 5**, I investigated field longevity and mating competitiveness of the OX4319L strain of DBM in field cage trials. Males from a wild-caught strain were used as a control throughout. No significant difference was found between recapture rates of males from the two strains after 3 days in the field, suggesting similar field longevity after three days. Again these data are vital for planning suppression trials. For example, the correct rate of release is essential to ensure effective coverage from released males. In releases of transgenic *Aedes aegypti*, release rates of 3 times per week have been used successfully in field suppression trials (Carvalho *et al.*, 2015; Harris *et al.*, 2012; Gorman *et al.*, 2015).

Mating competitiveness of OX4319L males was significantly lower than males from the wild-caught strain for matings with wild-caught females. This drop in competitiveness is perhaps unsurprising, given the known fitness cost of transgene insertions of this nature (Harvey-Samuel *et al.*, 2014). However, this competitiveness was well within the range of previously successful SIT and RIDL release programmes in other insect species (FAO/IAEA/USDA, 2003). This mating competitiveness is also well above the mating competitiveness of non-transgenic, irradiated DBM males (Apu, 2002), irradiated medfly (Mayer *et al.*, 1998) and screwworm (Rendon *et al.*, 2004) males, and transgenic *A. aegypti* males (Carvalho *et al.*, 2015; Harris *et al.*, 2012; Gorman *et al.*, 2015).

Field cage suppression trials are being planned for the OX4319L RIDL strain of DBM. In these trials, the ability of OX4319L males to suppress an established wild population of

DBM in an outdoor cabbage crop will be tested. Previous trials in a glasshouse setting have proven successful (Harvey-Samuel *et al.*, 2015). Therefore, field cage trials are the next step before an open-release field suppression test is performed.

### *6.2.6 Overview*

The data presented in this thesis provide support for the viability of RIDL technology as a functional method of pest control in agricultural insect species. Thorough testing of product strains is vital to ensure such strains are functional in the adverse conditions that may be encountered during field releases. It is also vital to understand and anticipate the possibility of these new technologies going wrong in the field and what the adverse effects might be. Questions such as whether any negative implications are reversible or whether they have knock-on effects for the wider ecosystem must be assessed before wild scale release of these kinds of insects. Currently, it is believed that the use of RIDL technology poses no more of a threat to the ecosystem than other commonly used technologies, such as SIT.

The data presented in this thesis suggest that the OX3864A strain of medfly and the OX4319L strain of DBM are suitable for further study in order to fully test the effectiveness of RIDL systems in the field. Future work will have to consider the position of RIDL within current pest management regimes. Many studies show the usefulness of new technologies in slowing the spread of resistance to currently used methods of control, and this may in itself be the most useful aspect of a technology such as RIDL. RIDL technologies should not be considered as the be all and end all of insect control; it merely is another tool in the arsenal of farmers looking to protect their crops from often devastating pests.

## 6.3 Genetic engineering, public understanding and policy

As discussed by Leftwich *et al.* (2015), and in purely scientific terms, the success of the RIDL technology depends on the ability of a construct to induce lethality, the life stage at which lethality is induced (Phuc *et al.*, 2007), the sex specificity of the lethality (Fu *et al.*, 2007), the stability of the transgene, the stability of the insertion, and any fitness costs of the insertion or expression of the construct (Harvey-Samuel *et al.*, 2014). In

reality, the success of a new technology also depends on a multitude of other inputs. Effective testing is vital, as is public understanding and support for newly implemented technologies. Regulatory policy allowing the effective implementation of the technology must also be satisfied. I was involved in the process of obtaining regulatory permission to conduct trials of genetically engineered DBM in the US during my PhD and I outline some of this process and its hazards, below.

Open field trials using RIDL *A. aegypti* have been carried out in multiple locations across Central and South America (Carvalho *et al.*, 2015; Gorman *et al.*, 2015; Harris *et al.*, 2012). Field cage trials using the OX3864A strain of medfly have occurred in Crete (Leftwich *et al.*, 2014). Field cage experiments presented in this thesis have also been conducted, using the OX4319L RIDL strain of DBM. Regulations surrounding the production and subsequent release of these insects are strict and licences must be obtained. During the application process, environmental assessments are carried out and production facilities are checked to ensure adequacy. During release programmes, all releases must be recorded, with limits on the maximum numbers of individuals released each week.

Despite these stringent regulations, there is still heavy opposition from the public about the use of genetically engineered insects as a means of pest control. Misinformation on the topic on the Internet is rife, with slogans such as “Hell no, GMO!” being prominent and scarce account being taken of the specific, evidence-based science. This is a significant problem for scientists to overcome if the use of new technologies is to be successful. They must address the public distrust in scientists and allay fears generated from the consumption of diverse on- and off-line, non peer-reviewed sources.

### *6.3.1 Public engagement*

During trials of RIDL strains, public communication sessions are organised in order to attempt to explain the technology fully and describe both its advantages and disadvantages. These sessions can have varying degrees of success, but they are important for scientists to have the opportunity to try to engage with the public about their concerns with the use of relatively unknown technologies that could have

beneficial effects. During my PhD I experienced a public session of this type and witnessed it to be hijacked by organised groups who came not to discuss, or to be educated, but instead to try to stop the programme. This kind of situation is unproductive and potentially damaging for all involved. Scientists have a responsibility to be open and engaged with the public about their science, but it is also essential that the public have the capacity to show reciprocity and be open to that engagement.

*6.3.2 Regulatory permissions*

The regulatory systems for the use of genetically engineered organisms in open field trials and in control procedures vary between countries. However, approval is always sought from the corresponding national governing body. Although some of these governing bodies may have protocols in place for genetically engineered crops, many are not equipped with a relevant protocol for legislating on genetically engineered insects. For this reason, in the USA, the application process can vary substantially between two insect species, even if both contain a similar type of RIDL construct.

A RIDL DBM strain, for example, requires an application via the US Department of Agriculture (USDA), as it is classed as a method of agricultural pest control. However, a RIDL *Aedes aegypti* mosquito requires an application via the Food and Drug Administration (FDA) as it is classed as a method for disease control. Although many of the steps in regulatory processes taken by both regulating bodies are similar, or even the same, this highlights the issue that there are few truly fit-for-purpose regulatory frameworks in place to deal with genetically engineered insects.

When applying for a Federal licence to release RIDL DBM, an application is made to the Biotechnology Regulatory Services (BRS) Department of the USDA Animal and Plant Health Inspection Service (APHIS). BRS-APHIS consider the application and its risks and carry out an Environmental Assessment (EA) on the technology. This determines any potential impacts on human health or the environment. An EA alone is enough if the risks are determined to be low. However, if risks are perceived to be high, and there are potential hazards to the environment or people, then an Environmental Impact Statement (EIS) is produced. An EIS goes into more depth than an EA. EAs and EISs take into account any previous work on the technology, the nature of the proposed

licence, and combine all of this information to fully assess whether a licence should be permitted.

If a technology is approved then the EA or EIS will return a provisional finding of no significant impact (FONSI) and the application will go out for public comment. In the case of the USDA application, this public comment period is a minimum of 30 days but can be extended in blocks of additional 30 days. In this time the application is available to view online and any comments or queries can be made on the website. The BRS consider all comments and any substantive issues are addressed. The applicant may be asked to provide further information to answer these queries. Public comments show notable breadth and depth. Some comments prove to be useful points for consideration and many may also show support. However, it is often the case that the majority of comments are not based on fact or any scientific knowledge and have the aim of stopping the scientific testing of these types of technologies.

After the consultation period, if comments have not raised any serious concerns, then the regulator issues a final FONSI and EA and the licence is granted. The licence is usually very specific about details of any releases or experiments, dictating the number, location and frequency of any releases and requires adequate recording of all experiments. In the USA, this is not the final level of regulation. The licence is granted at a Federal level. However, individual states may also require further approval for releases that take place within their borders. This can vary hugely between states and lengthen the application process further.

Whilst having these protocols in place is vital for safeguarding safety of both human populations and the environment, the current protocols are not entirely suitable and need to be put into a more relevant context for the use of genetically engineered insects. The bureaucracy involved slows down the progress of scientific testing of potentially life-saving technologies. The technologies do need to be scrutinised and questioned, and their overall safety assessed from a variety of standpoints. However, an improved, fit-for-purpose system would make the process far more efficient.

The next step for testing of the OX3864A and OX4319L RIDL strains tested in this thesis is open field releases. Only this can rigorously demonstrate their potential for effective

pest control. Unfortunately, even with public and political backing, the required permits for such trials have become increasingly hard to obtain and the progress of this kind of science slows down as a result. Until this is improved, the true potential of these RIDL strains remains to be fully tested.

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## Appendices

**Appendix 1 – Evolutionary biology and genetic techniques for insect control. Evolutionary Applications. 2015.**

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REVIEW AND SYNTHESES

# Evolutionary biology and genetic techniques for insect control

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## Abstract

The requirement to develop new techniques for insect control that minimize negative environmental impacts has never been more pressing. Here we discuss population suppression and population replacement technologies. These include sterile insect technique, genetic elimination methods such as the release of insects carrying a dominant lethal (RIDL), and gene driving mechanisms offered by intracellular bacteria and homing endonucleases. We also review the potential of newer or underutilized methods such as reproductive interference, CRISPR technology, RNA interference (RNAi), and genetic underdominance. We focus on understanding principles and potential effectiveness from the perspective of evolutionary biology. This offers useful insights into mechanisms through which potential problems may be minimized, in much the same way that an understanding of how resistance evolves is key to slowing the spread of antibiotic and insecticide resistance. We conclude that there is much to gain from applying principles from the study of resistance in these other scenarios – specifically, the adoption of combinatorial approaches to minimize the spread of resistance evolution. We conclude by discussing the focused use of GM for insect pest control in the context of modern conservation planning under land-sparing scenarios.

## Introduction

Insects spread disease and destroy millions of tons of crops each year. With global climate change and an ever-increasing population size, there are significant challenges associated with safeguarding people from disease and maintaining food supplies. This provides an urgent stimulus to develop new methods for insect control. Traditional approaches include pesticides, integrated pest management, and biological control. However, each has serious drawbacks because of environmental and social costs and/or lack of (cost-) effectiveness. For example, synthetic insecticides have been widely applied against a wide variety of pests and disease vectors – but their continual application selects strongly for resistance and is also nonselective, destroying natural enemies of the pests as well as perturbing the ecosystem as a whole. In addition, with increasing concerns of off-target effects of pesticides, the range of chemicals available for control is diminishing.

In light of these concerns, and due to potential problems with existing methods, there has been increasing interest in applying genetic modification (GM) techniques for insect control (Thomas et al. 2000; Deredec et al. 2008). In these, the aim is to harness the natural mating system of the pest in order to introduce into the pest population traits that will ultimately lead to its demise. Genetic methods that are transmitted or inherited through one sex, and which sterilize, kill, or cause sex change in the other, offer the greatest control potential (Bax and Thresher 2009). A goal in developing and assessing new methods, and in refining existing ones, is to understand whether it is ultimately better to optimize control strategies over a range of different species, environmental, and biotic conditions, or instead to employ highly species- and/or environment-specific targeting (see section Control strategies made evolution-proof or evolution-resistant, below; Leftwich et al. 2014).

Insect pests can be particularly difficult to control effectively using traditional nonselective methods such as biocides or insecticides if they are hard to target, or occur in close proximity with humans. For example, agricultural pests that exhibit flexible host use may have refugia across multiple host species, making them difficult to locate and eradicate. For pests in which the larvae reside within host fruits, the delivery of exogenously applied control agents

may also be relatively ineffective at targeting the relevant life history stages. Insecticides used to treat disease vectors such as mosquitoes need to be accurately targeted because of the co-occurence of the insect vector with human residences. However, mosquitoes such as *Aedes aegypti* are opportunistic in their breeding and resting sites; hence, finding all potential habitable spots for these insects may be difficult and labor intensive. Selective and species-specific mechanisms, that is, those in which control is achieved when released males seek out wild females for matings, transmitting to them sterility or genes that kill offspring, therefore offer many advantages. A major one is that they rely upon the natural mating system of the pest, honed by natural selection over many millennia.

Broadly speaking, the control technologies that employ genetic mechanisms fall into two types: (i) those that act to suppress local populations and are themselves self-limiting, eventually becoming extinct, and (ii) those in which the pest population is replaced by a more benign form or in which a trait is self-sustaining and driven through the pest population to reduce the harm caused by the pest (Alphey 2014). These different strategies differ in the extent to which the introduced genes persist in the population. From an environmental perspective, the longer-term impacts of these contrasting strategies upon the population structure and population genetics of the pest species involved may be very different, as discussed further below.

## Self-limiting population suppression mechanisms

### Sterile insect technique

A key breakthrough in achieving effective and environmentally benign insect control was the introduction of the sterile insect technique (SIT) over half a century ago (Knipling 1955). This is a species-specific method of insect suppression (Hendrichs et al. 1995; Krafsur 1998) in which insects are mass-reared under factory conditions, sterilized by irradiation, and then released. The released males mate with wild females and these sterile matings lead to a reduction in the size of the pest population. SIT is generally more effective if only males are released (Rendon et al. 2004). This prevents the introduction into the population of females that can damage fruit crops or transmit disease and minimizes any reduction in the efficiency of suppression arising due to assortative mating among released individuals (rather than between released males and wild females). However, male-only releases require that there is an efficient mechanism for sex sorting. SIT males must be able to seek out females and mate. However, SIT males are typically less competitive than wild males because of their history of adaptation to factory, rather than natural, conditions and because the irradiation used for sterilization significantly reduces fitness (Briceño and Eberhard 1998; Briceño et al. 2002; Lux et al. 2002; Parker and Mehta 2007). This reduced competitiveness can be mitigated to some extent by releasing more insects and increasing the overflooding ratio. Therefore, SIT males are often released periodically in large numbers to flood the resident population in order to achieve control. SIT has had great successes – but also some failures and trials in which only limited success was reported (e.g., in some mosquito trails conducted in the 1970s, reviewed by Benedict and Robinson 2003).

SIT has been used with success against the New World screwworm fly (Krafsur 1998), melon fly (Iwahashi et al. 1983; Kuba et al. 1996; Koyama et al. 2004), medfly, and tsetse fly (Hendrichs et al. 1995). There are, however, acknowledged problems, which may be responsible for examples in which SIT has not been successful or has had limited impact (e.g., Benedict and Robinson 2003). Sterilization by irradiation is perhaps the most significant problem because of the deleterious impact it has on the fitness of the released insects. A lower irradiation dose can be used to reduce harmful effects on fitness, but will allow some fertile individuals to be released. The sterilization dose used therefore needs to balance the degree of sterilization achieved versus its fitness impact. Additional problems are the deleterious side effects caused by 'domestication' of wild strains during mass rearing, leading to poor field performance of released males (Cayol 2000).

As noted above, 'male-only' releases are advantageous for control (Rendon et al. 2000; Rendon et al. 2004), provided that efficient sex-sorting mechanisms can be achieved, because they reduce the collateral damage caused by the release of sterile females (e.g., fruit 'stings' or biting) and prevent matings among released insects that have zero control value. Efficient sex-sorting mechanisms can reduce the impact of some potential drawbacks of SIT, such as the evolution of 'behavioral resistance', that is, discrimination by wild females against mating with SIT males (e.g., McInnis et al. 1996) and the evolution of changes in the timing of mating that lessen the probability of matings between released and wild flies (e.g., Economopolous et al. 1971; Economopolous 1972; Miyatake and Shimizu 1999). However, poor reliability of sex-sorting mechanisms for such male-only releases can result in additional problems for maintaining productivity and release strain stability (Seawright et al. 1978; Papadopoulos et al. 1998; Hendrichs et al. 2002; Lux et al. 2002; Robinson et al. 2002; Barry et al. 2003; Mossinson and Yuval 2003; Robinson et al. 2004; Windbichler et al. 2008). An increased frequency of remating by wild females mated to sterile SIT males, which can significantly reduce the effectiveness of SIT, is also possible (Kraaijeveld and Chapman 2004). Together these factors can explain examples of poor field performance and/or mating discrimination against SIT males (McInnis et al. 1996; Cayol 2000).

### Release of insects carrying a dominant lethal

To circumvent problems identified with the application of SIT, there has been intense interest in GM technologies (Handler and James 2000; Heinrich and Scott 2000; Thomas et al. 2000; Horn and Wimmer 2003; Robinson et al. 2004; Fu et al. 2007). One such method that has been developed for a range of different pests and tested in laboratory through to open field conditions is the 'release of insects carrying a dominant lethal' (RIDL; Thomas et al. 2000). RIDL offers potentially significant improvements over SIT (Schliekelman and Gould 2000; Thomas et al. 2000) perhaps most importantly because it circumvents the need for sterilization using irradiation. All fitness costs of irradiation are therefore eliminated. RIDL technology can target both sexes, but as noted above, the delivery of sex-specific action offers significant benefits for control. As an example of the application of RIDL technology, the female-specific (fs) 'fsRIDL' system (Fu et al. 2007) induces female-specific lethality through alternative splicing of sex-specific introns, leading to the production of a tetracycline-repressible transactivator fusion protein (tTA) in females, resulting in a lethal tTA positive feedback loop. Adding tetracycline to the diet suppresses lethality – but in the wild, the lethality is expressed and kills females at the pre-adult stage.

Transgenic RIDL insects have now been produced for pests of economic (Gong et al. 2005; Ant et al. 2012; Jin et al. 2013) and medical (e.g., Phuc et al. 2007; Fu et al. 2010; Harris et al. 2011) importance. Using the female-sterile system, male-only releases are easily achieved by removing tetracycline from the diet in the release generation. fsRIDL therefore offers a simple way to reduce or eliminate females in the pest population. In addition, the use of autofluorescent markers for transformation (Horn et al. 2002; Fu et al. 2007) facilitates the detection of released individuals in the field. Caged and field-caged trials of RIDL medfly (*Ceratitis capitata*; Leftwich et al. 2014), olive fly (*Bactrocera oleae* Gmelin; Ant et al. 2012; Harvey-Samuel et al. 2014), diamondback moth (*Plutella xylostella* L.; Jin et al. 2013), mosquitoes (*Aedes aegypti*; Phuc et al. 2007; Wise de Valdez et al. 2011), and pink bollworm (*Pectinophora gossypiella*; Jin et al. 2013) show the potential for RIDL strains to eliminate or control the spread of wild-type populations. More recent tests of RIDL strains are now employing more complex setups and following fitness outcomes in multigenerational designs (e.g., Harvey-Samuel et al. 2014). These studies have the potential to highlight sensitivities of strains that are not apparent under simpler glass house or laboratory tests. This approach could be further expanded in the future to capture likely performance under an ever-broader range of ecological and environmental conditions. Despite the findings that GM strains may sometimes show evidence of reduced competitiveness in direct comparisons with wild types, there are nevertheless many examples of their potential effectiveness to control pest populations (Thomas et al. 2000; Fu et al. 2007; Harris et al. 2011; Wise de Valdez et al. 2011; Ant et al. 2012; Jin et al. 2013; Harvey-Samuel et al. 2014; Leftwich et al. 2014). Any fitness loss of the GM strains can normally be countered by procedures such as increasing the frequency or number of released individuals.

RIDL technology is advanced in terms of its application under open field conditions in comparison with other GM control strategies. For example, strains of RIDL mosquitoes (Phuc et al. 2007) have already been subject to open field testing in the Cayman Islands (Harris et al. 2011, 2012), Malaysia (Lacroix et al. 2012), and Brazil (Alphey 2014). In these field trials, the released male insects were found to persist in the environment, to locate wild females and successfully mate with them and to achieve pest population suppression (Harris et al. 2011, 2012; Alphey 2014). Genetically sterile RIDL *A. aegypti* strains have also been tested under field release conditions. They show similar field longevity and maximum dispersal distances to a progenitor strain, but exhibit reduced mean dispersal distances (Lacroix et al. 2012). The potentially reduced flight potential of RIDL insects such as mosquitoes should be considered when developing facets of the release programs such as release sites and release densities (Bargielowski et al. 2011).

The success of GM technology itself depends on the effectiveness of the construct in killing, whether its effects are sex-specific (e.g., Fu et al. 2007), the life history stage at which it kills (e.g., Phuc et al. 2007) the stability of the transgene construct, the stability of the insertion, any fitness costs arising from insertion of the construct, and any fitness costs of the expression of the construct. The killing potential of the strains for potential release can be isolated in initial laboratory testing, as can the exact life history stages affected (Thomas et al. 2000; Fu et al. 2007). The life cycle stage that is targeted depends upon the specific pest and the reagents available. For example, for RIDL programs against agricultural pests in which larvae live within commercially important crops, early-acting lethality might be advantageous to limit larval penetrance into fruits and hence reduce spoilage. However, under female-sterile programs (e.g., Fu et al. 2007), any such benefit is negated as male RIDL larvae survive and continue to damage fruit (Leftwich et al. 2014). In contrast, for non-RIDL programs that target vectors of disease such as mosquitoes, transgenes that act to reduce the probability of disease transmission should ideally be much later acting in order to enhance additional control arising from increased density-dependent mortality among larvae (which do not themselves cause disease, e.g., Wise de Valdez et al. 2011).

  

Stability of GM construct locations in the genome can be achieved by removing the mechanisms or sequences needed for the gene carriers (e.g., transposable elements) to remobilize (Dafa'alla et al. 2006). Internal stability of the GM constructs themselves is also important to avoid breakdown of the mechanisms they deploy. The potential for such breakdown can be assessed using stress tests of GM strains, subjecting them to heat and food stresses and testing whether killing ability is compromised. Fitness costs arising from the insertion site of constructs causing mutagenic effects in the host genome are normally circumvented by producing and comparing multiple lines with different insertion sites and then selecting those with the least impact on performance. Docking mechanisms to introduce constructs (e.g., Nimmo et al. 2006) into the same, low fitness impact, genomic location each time (similar to the 'Gateway' technology) would be a useful development for the future.

Fitness effects associated with the expression of the transgenes (e.g., of markers) that are separate from the killing effects are also possible. These can be measured under controlled conditions by comparing the fitness of individuals bearing the transgenes in the activated or nonactivated form. Although it is possible to do this in practice, it has proved more fruitful to compare the overall performance and fitness of the GM in comparison with progenitor (e.g., Massonnet-Bruneel et al. 2013) and/or wild-type strains (e.g., Leftwich et al. 2014). These tests combine the sum of the effects noted above as well as any deleterious effects arising from the process of domestication (Table 1).

### Probiotics to enhance SIT and RIDL performance

It is now widely recognized that a significant contribution to host fitness comes from associations with commensal gut bacteria (the gut microbiome; Dillon and Dillon 2004). As in vertebrates (Turnbaugh et al. 2006; Vijay-Kumar et al. 2010), the gut microbiome in invertebrates can have widespread and significant effects on fitness (Dillon and Dillon 2004; Ben-Yosef et al. 2008a). In pest and nonpest fruit flies, changes in the gut microbiome can alter life span, mate choice, reproductive physiology, development, and metabolism (Behar et al. 2008a,b; Ben-Yosef et al. 2008a,b; Sharon et al. 2010; Shin et al. 2011). The number and diversity of gut bacteria of laboratory- and mass-reared pest and nonpest fruit flies is diminished in comparison with wild flies (Ben Ami et al. 2010; Chandler et al. 2011). Hence, there is evidence that the gut microbiome changes significantly during domestication. While the diet can alter the composition of the gut microbiome to some extent, there is an emerging picture that there are core members of this community irrespective of diet. Almost nothing is currently known, however, about how these core components colonize the gut, the role of the host in that process and transmission routes. With these factors in mind, attention has turned to the potential for probiotic treatments to improve sterile SIT male reproductive performance (Gavriel et al. 2011).

Changes to the gut microbiome are of particular interest for GM strains such as those using the RIDL technology, which all utilize tetracycline-repressible promoters (e.g., Thomas et al. 2000; Alphey 2002; Alphey and Andreasen 2002). As noted above, in these strains, the lethality or manipulated gene expression is under the control of a tetracycline-repressible promoter. The effects of the construct are suppressed during normal culture in the laboratory or factory using dietary tetracycline (e.g., Fu et al. 2007; Phuc et al. 2007). The continual exposure of RIDL strains to antibiotics is likely to (i) alter the composition of gut bacterial communities through a reduction in gut bacterial diversity and (ii) select for tetracycline-resistant gut bacteria. The effect on host fitness of gut bacterial communities that are altered in these ways is not yet known.

It is therefore important to understand whether any loss of gut bacteria in domesticated laboratory strains and in those maintained on antibiotic diets can be slowed or reversed by variation in dietary regimes or supplementation with bacteria in the diet. That such 'probiotic' treatments have significant promise is shown by experiments in which the reproductive performance of sterilized male medflies was improved by diet supplementation with *Klebsiella oxytoca* (Gavriel et al. 2011).

### Incompatible sterile matings

The sterile-male incompatible insect technique (IIT) can lead to a type of population suppression that is similar, in principle, to SIT and RIDL (Brelsfoard and Dobson, 2009; Laven 1967). It can be conferred by incompatible matings between individuals infected/not infected by strains of maternally inherited intracellular bacterial parasites such as *Wolbachia* (Brelsfoard and Dobson, 2009). Control is achieved through the cytoplasmic incompatibility phenotype (CI) that occurs when *Wolbachia*-infected males mate with uninfected females resulting in female sterility. The exact mechanism is still not fully known, although it results in early development arrest in the embryos produced from incompatible matings.

Control could therefore be achieved if *Wolbachia*-infected males were released into a non-*Wolbachia*-infected population to mate with noninfected females. This strategy has been considered for several insects, including mosquitoes and medflies (Brelsfoard and Dobson, 2009; Zabalou et al. 2009). It has been realized that, in mosquitoes, males can be released without increasing the number of biting insects (only females bite and transmit disease), and because *Wolbachia* is inherited solely through the maternal

**Table 1.** Selection on focal traits arising from rearing under laboratory or factory conditions and potential strategies to minimize deleterious impacts for insect control.

| Focal trait(s) | Direction and nature of selection applied in the laboratory or factory | Strategies to minimize deleterious impact on control potential |
|---|---|---|
| *Traits under selection in laboratory or factory* | | |
| Diet utilization | Strong selection for high productivity on novel artificial diets, likely to select for adaptation to utilize new food components efficiently and to affect many different life history traits (e.g., Hood-Nowotny et al. 2012; Yahouedo et al. 2014). Adaptation to artificial, standardized, and simpler diets is expected to reduce gut microbial diversity (Behar et al. 2008a; Chandler et al. 2011), reducing host fitness. Dietary changes could also alter pheromone components, thus affecting reproductive success (e.g., Sharon et al. 2010). Diet can also deleteriously impact on gut microbial diversity if antibiotics are added (Behar et al. 2008a,b; Ben Ami et al. 2010), for example, to diets in laboratory or factory settings to suppress dominant lethality (e.g., Thomas et al. 2000) | Use of more complex natural and varied diets or diet supplements (e.g., Kaspi and Yuval 2000). Use of probiotics to restore gut microbial diversity (e.g., Niyazi et al. 2004; Gavriel et al., 2011) |
| Inbreeding | Genetic bottlenecks that occur upon adaptation of the pest species to the mass-rearing conditions may reduce genetic diversity (e.g., Cayol 2000; Ciosi et al. 2014; Parreno et al. 2014) | Can be countered by periodic introduction of 'fresh blood' into mass-rearing strains and therefore releasing individuals with greater genetic diversity (e.g., Cayol 2000; Gilchrist and Meats 2012) |
| Development time | Likely to be selection for rapid development, but depends upon timing of pupal collections for seeding the next parental generations. A genetic correlation between development time and mating traits is reported. Hence, selection on development time can lead to correlated selection for altered timing of mating, with the potential to result in reproductive isolation between wild and factory strains (e.g., Miyatake and Shimizu 1999) | Avoid collection and use of only the first pupae to emerge to propagate the next generation |
| Larval density | Selection for success under elevated larval density. Variation in larval density has the potential to affect body size (e.g., Medici et al. 2011) and survival (e.g., Marti and Carpenter 2008; Medici et al. 2011) and hence has multiple effects on fitness | Could reduce larval densities during culturing to a level with minimal impact on body size. However, given that reduced density may also increase costs and reduce overall efficiency, one could instead optimize density and size across potential trade-offs between overall effectiveness/efficiency/cost (although this optimum is harder to measure) |
| Time to sexual maturity | Selection for rapid sexual maturity and first egg laying (e.g., Miyatake 1998; Hernandez et al. 2014), because those individuals that mature quickly contribute more to the next generation | Avoid taking the very first fertilized eggs that are laid. Use of other measures such as avoidance of selection for rapid development and small body size |
| Body size | Selection on body size is possible depending upon diet and development time regimes chosen (e.g., Cayol 2000; Cendra et al. 2014). Avoidance of inadvertent selection for small body size likely to be important and large male body size generally associated with increased mating success (e.g., Rodriguero et al. 2002). Larger females may also be more fecund. Changes in body size may also alter blood-feeding rates in disease vectors such as mosquitoes, with smaller females feeding more often (e.g., Nasci 1986; De Xue et al. 1995; Farjana and Tuno 2013) . Variation in body size could therefore potentially alter the probability of disease transmission | Monitor body size, adjust diet, and development time regimes if practical (e.g., Cayol 2000) |

(continued)

 

**Table 1.** (continued)

| Focal trait(s) | Direction and nature of selection applied in the laboratory or factory | Strategies to minimize deleterious impact on control potential |
|---|---|---|
| Longevity/life expectancy | Longevity *per se* is not expected to be a target of direct selection under mass rearing, but is likely to change as a side effect of changes to development time, body size, oviposition behavior and timing (e.g., Cayol 2000; Hernandez et al. 2014) | Changes to longevity and life expectancy will be minimized by measures to reduce selection for divergent traits under mass rearing (Cayol 2000) |
| Oviposition | Strong selection for a different type of oviposition behavior in comparison with the field, into artificial diets or through artificial egg laying devices. Likely to alter oviposition behavior substantially and select for traits such as an earlier, shorter, and more productive oviposition period (e.g., Suenaga et al. 2000; Hernandez et al. 2014) | Use of natural host-mimicking devices for egg laying in addition to artificial ones, although operational constraints may render such enrichment impractical |
| Productivity | Selection for high fecundity (e.g., Hernandez et al. 2014) and productivity arising from requirement for sufficient numbers of pupae to release | May be difficult to address by itself, although implementation of all the other measures could help minimize this problem |
| Courtship behavior | Crowded conditions and adaptation to mass rearing are likely to select for truncated courtships (e.g., Briceño and Eberhard 1998; Briceño et al. 2002), alterations to courtship songs (e.g., Briceño et al. 2009), increased courtship interruptions (e.g., Briceño et al. 2002; Briceño and Eberhard 2002), more male–male mounting (e.g., Gaskin et al. 2002; Weldon 2005), and potentially altered courtship thresholds in females (e.g., Briceño et al. 2002) | Reduce density of adult cages and increase complexity of the environment, to the extent practical. Could consider reducing the number of adult males recruited to the cages (Leftwich et al. 2012) to reduce intensity of male–male competition |
| Pheromones | The use of artificial diets and mass-rearing conditions may be associated with alterations to pheromones (e.g., Sharon et al. 2010; Benelli et al. 2014). The close proximity of females may also select for differences in male pheromone strategies. Large numbers of pheromone-fanning males within large cages are likely to result in a pheromone 'fog'. This may lead to selection for decreased pheromone emission. Individuals may also become desensitized to pheromones (Briceño et al. 2002; but see Kuriwada et al. 2014) | Reducing densities within adult cages to the extent that is practical. Consider periodic selection for ability to produce (males) and track (females) pheromones (e.g., wind tunnels). Diet enrichment to promote production of diverse pheromone blends (e.g., Kaspi and Yuval 2000; Niyazi et al. 2004; Gavriel et al., 2011) |
| Male–male competition and female mate choice | Crowded conditions and adaptation to mass rearing are likely to select for intense male–male competition leading to divergent mating strategies in comparison with the wild type. Frequent disturbance and potentially truncated or reduced thresholds for female choice decisions are also expected (see courtship behavior, above) | Reduce densities within adult cages and increase complexity of the environment (e.g., Liedo et al. 2007) to the extent practical |
| Mating frequency | Crowded conditions likely to select for more frequent matings and rematings (e.g., Vera et al. 2003; Kraaijeveld et al. 2005). This may select for changes in male ejaculate allocation and competition strategies (e.g., Linklater et al. 2007) and for other mating behaviors that are distinct from those that occur in the field | Reduce densities within adult cages and increase complexity of the environment (e.g., Liedo et al. 2007) to the extent practical |
| Assortative mating | Not evident under domestication, there is the potential for assortative mating to occur if there are changes to the sexually selected traits listed above. This could result in resistance of released males to mate with wild females (e.g., McInnis et al. 1996). Assortative mating due to evolved differences in the time of mating is also possible (Economopolous et al. 1971; Economopolous 1972; Miyatake and Shimizu 1999). A different kind of problem may be 'mating failure' between released males and wild females (e.g., Perez-Staples et al. 2013) | Assortative mating (and damage arising from female release) can be eliminated through the use of single-sex release programs (e.g., Hendrichs et al. 1995), if practical and cost-effective. Avoid selection for traits that increase reproductive success under laboratory-/factory-specific conditions. Increase overflooding ratios of released males into the wild. Increase diversity of age classes of male introduced into the wild (e.g., Gilchrist and Meats 2012) |

(continued)

**Table 1.** (continued)

| Focal trait(s) | Direction and nature of selection applied in the laboratory or factory | Strategies to minimize deleterious impact on control potential |
|---|---|---|
| Living in a simpler environment | Laboratory and factory conditions are simple environments that lack many of the important complexities of field environments (even 'simpler' ones such as agricultural environments) | Behavioral enrichment, for example, artificial lekking/perching sites, more horizontal surface area (e.g., Liedo et al. 2007). Artificial trees/host plants |
| *Traits not under selection in laboratory or factory conditions* | | |
| | Selection for flight ability is minimized | Use of large, lower density cages, consider periodic selection for flight ability (e.g., use of flight tunnels) |
| | Selection for long-range mate finding is minimized | Use of large, lower density cages, consider periodic selection for mate finding ability (e.g., use of flight tunnels with pheromone release). Use of parapheromones and other chemical agents (e.g., Shelly 1995; McInnis et al. 2011; Benelli et al. 2014) to enhance male mating success |
| | Selection for predator evasion | Hard to achieve, but general increases to competitiveness of released individuals might increase agility and hence predator evasion |
| | Selection for disease resistance and avoidance of trade-offs diverting resources from mate finding to combating infection if disease is encountered by individuals released into the field | Hard to achieve other than by periodic reintroduction of wild-type genetic variation |

line, the released insects do not spread *Wolbachia* through the population, and hence, males represent an evolutionary 'dead end' (O'Connor et al. 2012). It is important under this control scenario that no infected females are released, as all matings with infected females are compatible (indeed, this is the mechanism for driving *Wolbachia* through populations by gene driving, see below). The risk of simply contributing to the expansion of the pest population can be reduced if the target population is also infected but with a different strain of *Wolbachia*, giving bidirectional CI and sterility in eggs resulting from both types of matings that could occur.

The potential success of this strategy was first demonstrated in *Culex quinquefasciatus* mosquitoes many decades ago (Laven 1967). However, it was thought not to be generally applicable because there were perceived to be limited numbers of examples of bidirectional CI. However, with increased ability to artificially transfect species (e.g., with *Wolbachia* strains), this technique may now offer new opportunities for control. For example, this type of self-limiting control using *Wolbachia* has been observed in *Aedes polynesiensis* mosquitoes. This species carries a natural, single-strain *Wolbachia* infection. Release of males artificially transfected with a different *Wolbachia* strain derived from another *Aedes* species resulted in successful bidirectional incompatibility with the wild-type *Aedes polynesiensis* population, including in open field tests (Brelsfoard et al. 2008; O'Connor et al. 2012).

## Population replacement or introduction of traits that reduce the deleterious impact of the pest

For population replacement to confer insect control, mechanisms to drive genes through populations to effect control are needed. Driving mechanisms are required in which genes exhibit non-Mendelian transmission, to enhance their own representation above that of other genes in the genome. Several such driving genes or systems are known, including *Wolbachia*-based (Hoffman et al. 2011), homing endonuclease genes (HEGs; Burt 2003), and transposable element-based systems (e.g., *Medea*). We focus in this section primarily on the *Wolbachia* and HEG systems. The recently described 'mutagenic chain reaction' (MCR) system conferred by CRISPR gene editing is discussed in the following section on newer technologies.

Key to successful invasion of traits that will lead to control of the target pest is an understanding of the ease of driving genes conferring the control trait through the population. The initial establishment and spread of drive is the crucial step and depends on many factors which sum to a property known as the 'invasion threshold'. However, some drive systems can theoretically spread from any initial frequency (Deredec et al. 2008; Alphey and Bonsall 2014) although


 

stochastic effects are expected to be significant at low initial frequencies (which will be true for any type of release program). Whether the invasion threshold, if it exists, is high or low determines the size and frequency of the initial inoculum into the pest population required to achieve control (Alphey 2014). These issues are not unique to drive-based systems, and overflooding thresholds for achieving suppression are also critical for success in the SIT and RIDL methods described above. These ratios determine whether the released flies reduce damage to below the relevant economic threshold or disrupt disease transmission efficiently.

### Driving refractoriness to pathogen transmission using *Wolbachia*

One of the best-known gene drive systems is that, associated with *Wolbachia*, a maternally inherited intracellular parasite. *Wolbachia* infection can result in a number of different driving phenotypes such as male killing and cytoplasmic incompatibility (CI), depending on the species infected and *Wolbachia* strains involved. It is the CI phenotype that offers the potential for control because, through females, it can drive *Wolbachia* infection (and any control potential offered by the parasite) through populations. *Wolbachia*-infected females have a substantial fitness advantage over uninfected females (which become sterile following matings with infected males) and given that the *Wolbachia* parasite is maternally inherited, and this will result in an increase in *Wolbachia* in the population as a whole (Turelli and Hoffmann 1995).

To date, *Wolbachia* infection has been used to control disease (e.g., dengue virus) transmission in mosquitoes. *Wolbachia* infection is known in mosquitoes to interfere (by as yet unknown mechanisms) with the efficiency with which hosts can transmit pathogens such as dengue virus. Hence, the driving of *Wolbachia* through such species using CI can potentially reduce the spread of disease (Hoffman et al. 2011; Yeap et al. 2011). A proof of principle for insect control by this method comes from the spread of a strain of *Wolbachia* derived from *Drosophila* through natural populations of *Aedes aegypti* in Australia (Hoffman et al. 2011). Similarly, *Wolbachia*-induced refractoriness to the spread of *Plasmodium* by the mosquito *Anopheles stephensi* has also reported (Bian et al. 2013). More recently, improvements to the potential spread and penetration of *Wobachia* into natural populations are proposed by linking the introduction of *Wolbachia* to insecticide resistance (e.g., Hoffmann and Turelli 2013).

### Homing endonucleases

Homing endonuclease genes (HEGs) are found naturally among fungal genomes and represent a potentially powerful mechanism for driving genes through populations to achieve insect control (Burt 2003; Deredec et al. 2008). Although the primary focus is on HEG as gene drivers, it should be noted that self-limiting forms of HEG control are also possible (Burt 2003). In the heterozygous state, the protein encoded by HEG genes causes a double-stranded break to occur in the homologous chromosome at the same position. If the break is repaired using the HEG-bearing chromosome as template, the HEG becomes homozygous as a result of gene conversion or homologous recombination. This mechanism therefore represents a powerful means for driving genes through populations, using HEGs as vehicles. In agricultural pests, potential control agents that could be loaded into HEGs are genes that decrease viability or that decrease female fecundity or distort the sex ratio. The latter could be especially effective, for example, if HEG activity could be restricted to the male germ line but act on female-specific traits or inactivate or degrade the X chromosome.

Proof of principle experiments for insect control via engineered HEGs has been conducted in the fruit fly *Drosophila melanogaster*, in which sperm development and the female germ line were targeted by the HEG I-*SceI* (Chan et al. 2011). HEG-derived drive has also been shown *in vivo* using the same drive gene in *Anopheles gambiae* mosquitoes, where it appears to occur at much higher efficiency (Windbichler et al. 2011). This is partly because in *D. melanogaster*, the homologous recombination needed for the drive to occur appears to be restricted to specific sperm cell stages within the testis. However, the efficiency of HEG drive can, in principle, be improved by trialling different genetic constructs. The overall efficiency of HEG drive is also significantly affected by temperature (Chan et al. 2013), which will be an important consideration if this technology moves into field trials.

The effects of population genetics upon the spread of HEG-based systems have also been investigated using theoretical approaches (Alphey and Bonsall 2014). The results show that the success of HEG-based drive depends critically upon the interaction of population genetic and ecological factors such as density-dependent effects during larval competition, the timing of the impact upon fitness of HEG drive, and the relative fitness of the different wild-type and HEG genotypes present in the population.

## Control potential of new, or underutilized, techniques

### CRISPR and the mutagenic chain reaction

A new, and potentially revolutionary, gene drive system recently gained attention in the context of insect control (Esvelt et al. 2014), with a recent study in *D. melanogaster* reporting 97% transmission (i.e., well over the expected

25% Mendelian outcome) of a normally recessive, loss of function *yellow* pigmentation gene (Gantz and Bier 2015). This was achieved using the increasingly popular CRISPR gene-editing tool (Jinek et al. 2012) to create a 'mutagenic chain reaction' (MCR). The transmission efficiency reported by this new method far exceeds that which can currently be achieved with the HEG strategies described above and this technology therefore offers a highly potent prospect for gene drive control. The MCR technique used was, however, criticized on the basis of its lack of safeguards (Bohannon 2015). The editing and targeting sequences were contained within the same gene cassette, meaning that there was no way to stop or 'recall' the gene drive once initiated. However, such safeguards can be built in and, with such efficient driving, the possibility to drive through subsequent neutralizing genes should also be considered.

### Control through gene manipulation via RNA interference

Concerns about the use of GM technologies, and variation in the length of time needed to address regulatory concerns in different countries, have prompted interest in the use of RNA silencing to produce sterile males for control releases (e.g., Thailavil et al. 2011; Whyard et al. 2015). Such methods are currently considered non-GM technologies. The RNA silencing method relies on the introduction into the target insects of double-stranded RNA that is complementary to the endogenous gene to be silenced. The double-stranded RNA (dsRNA) then catalyzes the degradation of the target RNA via the RNA interference (RNAi) mechanism (reviewed by Bartel 2004). dsRNA can be introduced into invertebrates via feeding or injection and exert a significant silencing phenotype. There are several possibilities for control, including the silencing of testis-expressed genes in order to sterilize males or to manipulate genes in the sex determination pathway in females, for example, to change females into sterile pseudomales (e.g., Thailavil et al. 2011; Whyard et al. 2015). A recent study fed dsRNA to larvae of *Aedes aegypti* mosquitoes and showed reduced fertility in groups in which male testis genes were silenced and an increase in the number of males: females in groups in which female-specific *doublesex* RNA was targeted (Whyard et al. 2015). Key to success of RNAi for control will again be the relative competitiveness of the released insects, the efficiency of sterilization (to minimize the release of fertile males), cost, and the likelihood of resistance evolution.

### Insect control through reproductive interference and the actions of seminal fluid proteins

Incomplete mate recognition, leading to reproductive interference in matings between closely related species, is of core interest in evolution and ecology because of its role in maintaining species barriers. It may often also be asymmetric (when reciprocal interspecific matings incur different fitness costs). This 'satyrization' has long been considered of potential interest in insect control because of its potential to result in competitive displacement of species (DeBach 1966). For example, there is the potential for control if an insect vector exhibiting low disease transmission characteristics could be introduced to replace a resident species with high disease causing potential.

Such a phenomenon is thought to have occurred in the USA in mosquitoes of the genus *Aedes*. *Aedes aegypti*, a major vector of dengue virus, suffered competitive exclusion following the spread over the last 3 decades of the Asian tiger mosquito *A. albopictus* (Bargielowski and Lounibos 2014). *Aedes albopictus* itself can carry and transmit dengue and chikungunya viruses, although it is generally thought to represent a lower risk to human health. Hybrid matings are costlier to *A. aegypti* than to *A. albopictus* females, as seminal fluid proteins (Sfps) from *A. albopictus* males transferred into *A. aegypti* females render the latter refractory to conspecific matings (Tripet et al. 2011). There is no such effect in the reciprocal mating, conferring the observed asymmetry in fitness costs. This asymmetry and the associated costs of hybrid matings predict selection for rapid evolution of reproductive character displacement in areas where the two species occur in sympatry, to prevent such matings. Interestingly, evidence for just this phenomenon has recently been described (Bargielowski et al. 2013). Asymmetry in fitness following hybrid matings across many species of *Drosophila* is well known (Coyne and Orr 1989). However, the contribution of Sfps in this context has not been studied, even though it was first described decades ago (Fuyama 1983). Further research into the potential for control via reproductive interference could therefore be useful. A potential problem for insect control under satyrization, however, is that successful competitive exclusion could select for resistance, leading to the potential reinvasion of the pest.

The biodiversity and potential control toolkit represented by Sfps is extensive. These molecules vary hugely in structure (Mueller et al. 2005) and function (Ram and Wolfner 2007) and cause a profound remodeling of female behavior and physiology following their transfer during mating (e.g., Chapman 2001; Sirot et al. 2014). They can alter female sexual receptivity, ovulation and egg laying, feeding and sleeping, sperm storage, retention and usage, and immunity gene expression (Sirot et al. 2014). These phenotypes have significant effects on fitness (Chapman et al. 2003; Chapman 2006) and some genes that encode Sfps evolve extremely rapidly (Swanson et al. 2001; Clark and Swanson 2005).

  

In pest species such as medflies Sfp transfer can alter female behavior from that associated with seeking mates to that associated with searching for oviposition sites (Jang et al. 1998). This offers the potential for self-limiting control strategies in which females might be prevented from switching on behaviors associated with crop damage (egg laying). There has also been much research on Sfps in *Anopheles* mosquitoes (e.g., Baldini et al. 2013; Gabrieli et al. 2014; Shaw et al. 2014). These studies offer much in the way of raw material for exploring new control strategies (Davies and Chapman 2006). The potential for Sfp engineering, perhaps combined with asymmetric reproductive interference, is so far relatively untapped and could offer useful complementary additions to the control strategies described above. It is worth anticipating that, as with other methods, those based upon Sfp engineering have the potential to become compromised by the evolution of resistance (e.g., behavioral resistance against mating with Sfp-manipulated males). Strategies to mitigate such effects should therefore be simultaneously considered.

#### Underdominance for driving control mechanisms

Underdominance occurs when the fitness of a heterozygote is lower than for both corresponding homozygotes. In theory, this can be used to drive an underdominant transgenic construct into a population to replace wild-type alleles (Davis et al. 2001; Altrock et al. 2010; Reeves et al. 2014). The likelihood of population allele replacement depends upon the initial frequency of introduction and does not require that both wild-type and introduced homozygotes have equal fitness, just that both their fitnesses are greater than the heterozygote. Such a system would be geographically limited and reversible (by reintroduction of the wild-type allele), hence represent a self-limiting form of control.

The principle of insect control through drive resulting from underdominance has been around for decades. However, a recent study successfully developed proof of principle in *D. melanogaster* (Reeves et al. 2014). The expression of a *Minute* locus was knocked down in heterozygotes. In this, RNAi was used to knock down the expression of one of the many *Minute* loci. *Minutes* are haplo-insufficient; therefore, the knockdown resulted in a dominant, deleterious fitness effect (significantly delayed development, small size). The transgenic homozygote was rescued from this effect by the inclusion of a rescue gene to elevate the level of the Minute transcript to a functionally wild-type level. The introduction of the underdominant transgene caused successful replacement of the wild-type allele in as little as 5 generations in laboratory population tests. The introduction of transgenes that render hybrid matings costly could though select for the rapid evolution of mating barriers between the wild type and transgenics, which might reduce its efficiency.

With the ever-increasing opportunity to design constructs for greater stability and efficiency, further work into these new or under-employed genetic mechanisms might be very useful in light of the findings that they have at least the potential for efficient gene drive.

## Risks of existing and new technologies

The risks of the various control methods and mitigation strategies are discussed elsewhere (e.g., Alphey 2014; see also Bohannon 2015) and summarized only briefly here. The relative risks are generally held to be lower for suppression in comparison with replacement or driving mechanisms. This is because suppression mechanisms are inherently self-limiting and drive themselves extinct, whereas driving mechanisms have greater persistence and longer-term consequences should the technology fail. RIDL technology is further advanced than any of the other current GM control methods and has been successfully subjected to laboratory greenhouse, field cage, and open field trials (Wise de Valdez et al. 2011; Ant et al. 2012; Harris et al. 2012; Jin et al. 2013; Harvey-Samuel et al. 2014; Leftwich et al. 2014). The open field release of GM insects is not without controversy, and any such release obviously requires extensive licencing, technical, regulatory, and public engagement activity to investigate the safety of the technology in terms of to the environment and human health. Public engagement activities are also essential to inform and address potential concerns. Upholding the ideal of maximum transparency at all stages is of prime importance.

In terms of GM, concerns are often raised about the stability of the GM constructs and the possibility of escape. Both are possibilities, however remote, whose risks need to be calculated and assessed. In principle, single- and tightly linked genetic units should be less resistant to recombination and hence breakdown than larger or multicomponent systems. It is also important to understand whether the ultimate consequences of such a breakdown are likely to be the inadvertent spread of introduced genes or gradual loss of the introduced genetic material. In general, risk mitigation and recall strategies for all GM methods are essential to consider from the initial proof of principle stage.

## A perspective from evolutionary biology

The general importance of bridging the gap between evolutionary biology and genetic pest management to develop effective and long-lasting control strategies has been well recognized (Gould 2008). This dialogue can usefully inform

the most effective way in which to target pests and to prevent the control strategies employed being degraded by the evolution of various forms of resistance.

The basics are straightforward and well understood; if we apply a selective pressure for any trait, then, given sufficient genetic variation, the population will respond. The response of the population to that selection pressure will be determined by the size of the selection differential (the difference in the mean value of the trait under selection in the original versus selected parent populations). The heritability of the trait under selection can be calculated by the ratio of the response over the selection differential (Falconer and Mackay 1996). The selection differentials that exist when wild strains are brought into the laboratory or factory can be huge – covering all aspects of life histories (Table 1). In effect, when pest strains are domesticated, a large-scale artificial selection experiment is conducted upon the ability of individuals to survive and prosper in the novel environment. We should therefore expect released insects to have compromised performance when placed into field environments to which they are no longer adapted.

There are many ways in which the process of generating insects for control programs has the potential to result in selection for traits that are likely to lessen the effectiveness of released insects in the field. The life history consequences for laboratory selection in this context have been considered in some detail (e.g., Cayol 2000). What has been less well implemented are strategies to tackle them, even though with adjustments to husbandry practices such effects could, in principle, be circumvented or minimized (Table 1).

The general consequences of domestication are a significant reduction in genetic diversity. The initial stages of domestication often involve a fairly savage bottleneck, which can significantly reduce genetic diversity in comparison with wild progenitor populations. This increases the net effect of genetic drift and the likelihood that rare gene variants important for success in the field may be lost. However, unless the bottleneck is particularly drastic, of greater importance are changes in allele frequencies that subsequently occur due to strong selection for domestication. A proposed solution to this problem is to conduct periodic introduction of wild individuals into the domesticated strains (the 'fresh blood' technique). This would help to reduce the impact of many of the concerns listed in Table 1, although increases in the genetic diversity, and any associated benefits, may be temporary as wild alleles are likely to be selected against in the laboratory or factory environment. An associated problem is the loss of genetic diversity in the accompanying microbiome of the domesticated individuals. There are obvious cost implications of the potential solutions above as they decrease productivity. However, it is also important to consider that little work has yet been performed on the relationship between the improvements suggested above and the gain in control effectiveness.

Comprehensive stress testing of GM strains is crucial to prevent unwanted surprises down the line. For example, the effect on strain stability of temperature, food availability, humidity, and pathogens should all be examined. For control mechanisms in which the sex-specific lethality relies upon the absence of dietary additives, it must be clear that such additives cannot be encountered at anything close to an effective dose by the released insects in the field or urban settings. Further insight into predicting the likely effectiveness of released insects may also come from a better integration of population genetics. For example, understanding population genetics, gene flow, and the effects of partial reproductive isolation are important for understanding the impact and efficiency of release programs (e.g., Endersby et al. 2011).

### Understanding sexual selection and the mating biology of pests is crucial to improving control via GM and non-GM technologies

As emphasized above, the production of safe and fit insects for release is key to success of all SIT and GM technologies (Scolari et al. 2011). One important lesson relevant to all control strategies discussed above, both GM and non-GM, is that knowledge of the life history and reproductive biology of the pests involved is as important now as it has ever been (e.g., Briceño and Eberhard 1998; Briceño et al. 2002; Leftwich et al. 2012; Oliva et al. 2013; Perez-Staples et al. 2013). Important insights into the success of SIT and genetic control programs have come from knowledge of compounds that affect male mating behavior (e.g., Kouloussis et al. 2013), the best attractants for traps, and the effect of sterilization on mating and remating behavior (Kraaijeveld and Chapman 2004). Direct comparisons between the fitness and competitiveness of strains carrying GM technologies versus controls and the wild-type populations remain an essential part of the toolkit for validation of these technologies (e.g., Morrison et al. 2009; Massonnet-Bruneel et al. 2013; Leftwich et al. 2014). Also of great importance is knowledge of the effects of domestication on the control potential of released insects. This knowledge can be used to minimize the effects of selection for traits that compromise control efficiency.

Trapping and detection methods are also key to successful insect control and just as important to the successful implementation of genetic control methods as the basic genetic technology itself. Therefore, continuing research into attractants is important to gain knowledge into the incidence and distribution of pest populations, and to

  

easily and reliably detect released versus wild insects in the field (e.g., Juan-Blasco et al. 2013). This includes theoretical and empirical investigations of the effects of environmental factors (Dufourd and Dumont 2013). Understanding of the dispersal of released insects is also important to predict the likely effectiveness of insect control from released programs (e.g., Gavriel et al. 2012). To date, there has been little consideration of the age structure of the population into which insects will be released. This is of particular importance if there is any assortative mating by age and can affect the numbers of released insects likely to give effective control (Huang et al. 2009). Future empirical research into these factors may be useful.

We conclude that ultimately it should be possible to minimize the impact of natural selection on the effectiveness of insect control by SIT and GM methods by understanding what are the principal and key elements of reproductive success of the pests in the natural environment and building the understanding of that knowledge into rearing practices.

## Control strategies made evolution-proof or evolution-resistant

Evolution is inevitable given the existence of genetic variation, and, given this, evolution by natural selection is also assured whenever a selective force is applied. The evolution of resistance to control strategies of all kinds is, therefore, inevitable. Control programs cannot be made evolution-proof, but the deleterious impact of natural selection on control efficiency can be substantially mitigated. There is recognition that combinations of simultaneous and diverse approaches are needed to prevent degradation in the effectiveness of individual approaches over time (Deredec et al. 2008; Alphey 2014). However, there has been very little exploration to date of the most efficient combinations of genetic techniques for insect control. A combination of approaches is needed not just to spread risk in a general sense, but to diffuse the strength of natural selection focused on specific traits likely to diminish the effectiveness of control.

We suggest that there is much to learn from the study of insecticide, chemotherapy, and antimicrobial resistance (AMR). AMR in particular is a grand challenge, representing a major global threat to human health in terms of our ability to combat infectious disease as well as to treat cancer via chemotherapy. Although the contexts are different, the underlying principles of how to slow the spread of resistance are conceptually similar as they all rely upon the same evolutionary principles. It has also been recognized that facets of resistance are predictable according to mechanisms of resistance and the environment in which resistance evolves. Therefore, an approach that integrates across these levels is needed (Maclean et al. 2010). Thinking across insecticide resistance and AMR has led to the proposal of four major strategies to slow and manage the evolution of resistance (REX consortium, 2013) as outlined below.

1 *Responsive alternation* refers to the strategy of sequential use, applying different control methods in series (but not cycling them). For example, one method might be applied continually until resistance is observed and then the next method applied.
2 *Periodic application* is when control methods are cycled or rotated; hence, a pesticide might be used for 6 weeks then a second used then back to the first.
   Note that in methods (1) and (2), the application of control varies over time but not space (i.e., is uniformly applied everywhere).
3 *Mosaic* is an approach that varies space but not time. For example, at least two different control methods are applied simultaneously but in different places and the places in which they are used do not overlap. An example might be the use of different antibiotics in different hospitals or different pesticides in different fields.
4 *Combination* is when 2 or more approaches are applied concomitantly over time and space. An example is the use of combinatorial therapy for HIV infection, with multiple drugs being applied simultaneously.

Variation of all of these approaches using full- and half-strength control strategies is also possible. Allied to the thinking that less than total eradication might be useful is recent research into the need to prevent chemotherapy resistance, which suggests that managing cancer, rather than eradicating it, may sometimes be a more successful strategy overall (Greaves 2007; Read et al. 2011).

A recent review of the efficacy of these methods applied across very different contexts in medical and agricultural settings (REX Consortium 2013) suggests that, in terms of their ability to slow the evolution of resistance, *combination* methods were best, outperforming *periodic application* and *mosaic* approaches (which were equivalent) and all were better than *responsive alternation.* The *combination* approach works well because it ensures that individuals are killed even if they are resistant to one of the approaches applied.

The basic underlying principle is to create scenarios in the target population (be it microbes, insects, or crops) in which there is greater variation in selection pressure on the pest to evolve resistance. This strategy will ultimately give rise to more sustainable pest control over the long term. Imposing variation in selection pressure for resistance is important because it presents a less strong but, more importantly, a moving target. Encouragingly, initial suggestions for combinatorial approaches are being made. For

example, Deredec et al. (2008) suggest that the evolution of resistance to HEGs could be slowed by simultaneously targeting multiple genes using multiple HEGs, or by targeting multiple sites within the same gene. HEG constructs should also be rigorously designed to reduce the probability that the expression of the gene product becomes separated from the recognition site. Other possibilities are to combine RIDL systems that employ female-specific lethality with releases of engineered males susceptible to other control methods (e.g., to insecticides or to *Bacillus thuringiensis* (*Bt*)-engineered toxins expressed by GM crops). Such methods could provide dilution of resistance across the different mechanisms (Alphey et al. 2007; Alphey et al. 2009; Alphey et al. 2011). Genes introduced into wild populations by released males will be inherited by males in systems that employ female-specific lethality and by both sexes if resistance permits some progeny to survive the effects of the engineered 'lethal' genes. Theory suggests that inheritance of susceptibility genes through this mechanism can slow or potentially reverse the spread of resistance mutations to RIDL, prolonging the effectiveness of this technology (Alphey et al. 2011). This resistance dilution would potentially work for release programs, such as RIDL or SIT, in which releases are sustained over time, but is not expected to occur in drive-based systems that employ limited, inoculative releases.

An important consideration for combination approaches, should they be adopted for insect control, is that SIT and GM approaches have well-documented fitness costs, as outlined above (e.g., costs of bearing GM constructs, loss of fitness upon irradiation, costs of bearing *Wolbachia* infection). Such fitness costs incurred simultaneously under a combination approach have the potential to impose a greater fitness 'load' upon the release population and potentially reduce its effectiveness. These costs would therefore have to be weighed up against the advantages. Fitness costs to released insects of SIT and GM technologies have been mentioned in several different contexts, and their magnitude is a key determinant for successful control. Under a traditional model in which there is a fixed resource pool that can be allocated to different life history traits but which cannot maximize them all simultaneously. The costs of bearing a GM construct or driving strain of intracellular microorganism are therefore likely to lead to trade-off with other life history traits with effects on fitness.

The need to recognize and minimize resistance has not yet permeated deeply into discussions of SIT and GM insect control. An approach similar to responsive alternation is sometimes used in SIT programs – for example, pesticides may be used to reduce initial population sizes before SIT intervention. Combination control has, though, been used in other agricultural contexts. GM crops engineered using *Bt* technology have been developed that produce several different toxins against their target pests (Cui et al. 2011). A *combination approach* involving the use of *Bt* crops and sterile insect releases to target pink bollworm (*Pectinophora gossypiella*) removed the need for insecticide sprays and was effective at reducing pest abundance while maintaining current resistance levels to *Bt* cotton (Tabashnik et al. 2010).

**Box 1:** General principles for maintaining fitness and competitiveness of control strains and increasing effectiveness in control programs

- Keeping the domesticated progenitor and GM strains in an outbred genetic background with frequent outcrossing to promote the maintenance of a wild-type ancillary genome.
- Keeping the domesticated environment as complex and varied as is feasible.
- Diet variation and supplementation may be useful to maintain variation in traits related to nutrient acquisition and to maintain diverse gut microbiomes.
- Knowledge of the ecology, life history, and reproductive success of wild-type strains is essential to inform best practice in husbandry and in trapping technology.
- Simple GM constructs and vehicles seem more likely to be stable and hence less likely to break down than more complex ones.
- Drive systems should have built in safeguards.
- Theory, parameterized by real world data, is essential to predict and test program-specific optimal invasion thresholds, release ratios, release frequencies, release timing (with respect to season and resident population size), release population composition (e.g., age structure).
- Strategies from the study of insecticide resistance and antimicrobial resistance (AMR) could lead to improved strategic and combined deployment of GM and non-GM strategies.

### Improved targeting of insect control

Consideration of the problems created by the blanket use of broad spectrum antibiotics that has hastened in the current potentially catastrophic problem of AMR has led to increased interest in improved diagnostics coupled with the use of newer narrow spectrum (highly selective) antibiotics. Such a strategy facilitates the use of combination therapies discussed above.

  

**Box 2:** Perspectives – a focus on the contribution of women

The very fact that we are contributing articles to this special issue tells us something about the increased focus on gender equality. We welcome the way in which our behavior is being nudged ever more frequently – to encourage us to think about equality in new ways and to challenge the unconscious biases and schema that we all carry about what makes a successful scientist. These biases matter because they impact on issues such as visibility and we need to guard against the selective citing of studies on that basis. With this in mind, in writing this article, we relied heavily upon search engines rather than on our collective memories or our knowledge of people in the field, to try to make sure that we were comprehensive and had scrutinized all the relevant sources, not just those that had higher visibility. We have not tested systematically whether this approach altered the gender profile of the work we have cited in our review, although we note that female researcher (first author) papers certainly feature prominently (e.g., by Anne Deredec, Nina Alphey, Irka Bargielowski). Future comparisons of these different approaches to citing work (and their knock-on effects for visibility) would be useful.

Translating this into the control strategies considered here, diagnostics would equate to developing a better understanding of the pest problem (its ecology, population dynamics, fluctuation, location, intensity), and narrow spectrum antibiotics to an understanding (based upon the diagnostics) of which selection of diverse GM and non-GM specific control strategies available could be targeted most effectively. There is no reason why this new thinking on rapid, point-of-need strategies combined with better stewardship could not, in principle, be applied in terms of GM technologies for control. We offer some general thoughts on principles to maintain fitness and competitiveness of control strains and hence increase effectiveness of control programs (Box 1).

## Insect control and conservation

In this final section, we conclude by discussing briefly an emerging idea that GM technologies for insect control are not necessarily in conflict with modern conservation planning. These research areas have typically proceeded along very separate lines, but dialogues led by new thinking in conservation practice may offer opportunities for synergy. For example, recent research in conservation has advanced the controversial idea that 'land sparing' has the potential for greater conservation value than does 'land sharing' (Phalan et al. 2011, 2014). Under this scenario, there is greater preservation of biodiversity through the intensification of farming on existing land. This is because it allows for less land to be used for the same yield and therefore more land to be freed up to return to its natural state, or be preserved, and support a greater number and diversity of natural species than is true under other conservation scenarios. Increases in productivity in the order of a few % per annum could support this scenario and are predicted to be possible. Control of agricultural pests using GM technologies could play a role under this scenario. They allow relatively cost-effective and targeted control of insect pests with less environmental impact than is true for pesticides. This sets up the interesting situation that rather than being in opposition to the preservation of biodiversity, the development of advanced GM technology could actually be part of the solution to preserve it. Future work on integrating the likely efficiency savings for yield of the application of GM control programs would be especially useful to ground truth these interesting ideas.

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## Author contributions

TC wrote the first draft of the manuscript, and all authors contributed to editing and revisions.

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