Genetic Control of the Diamondback moth (*Plutella xylostella* L.)

Tim Harvey-Samuel
St Hilda's College

Department of Zoology
University of Oxford

A Thesis Submitted in Fulfilment
of the Degree of
Doctor of Philosophy

Trinity Term 2014

Acknowledgments

First and foremost, I would like to thank Neil and the rest of the moth team. Adam, Mat, Thea, Nerys and Debs you guys have not only been there to help me every step of the way, but have made the last 4 years a truly enjoyable experience. I'll never forget my time in the moth team and will miss you guys loads. Neil, in particular I should thank for, in addition to dealing with my often intractable opinions and generally recalcitrant nature, was perhaps the best supervisor I could have asked for. Thanks must also go to the molecular team at Oxitec, in particular Tarig and Caroline. Tarig for having the patience of a saint in teaching me everything I know about molecular biology, and Caroline for all her help with the infamous Q-system, and for putting up with my frequent laboratory indiscretions. You will never have to tell me to put on a labcoat again Caroline, I promise. Thanks also to Luke, Seb and Tony for their advice, guidance and encouragement over the last 4 years. Finally, a massive thank you to my parents, whose unwavering support and constant interest in my work has picked me up whenever I needed picking up.

Table of Contents

Abstract 6

Declaration of Authenticity 7

Chapter 1: Introduction 9
1.1 Modern agricultural systems 9
1.1.1 Pest management in agricultural systems 9
1.1.2 Ecological and economic consequences of pesticide use 11
1.2 Integrated Pest Management and the Sterile Insect Technique 13
1.2.1 SIT and the Lepidoptera 16
1.2.2 Lepidopteran radioresistance 16
1.2.3 Lepidopteran sexing systems 20
1.3 Transgenic control of pest insects 22
1.3.1 Expanding SIT with RIDL: the diamondback moth 28
1.4 Investigating alternative gene expression systems 32
1.5 Goals 35
1.5.1 Project aim 35
1.5.2 Project objectives 35

Chapter 2: Developing an fsRIDL system in the diamondback moth 37
2.1 Introduction 37
2.2 Results and Discussion 41
2.2.1 Transformation of diamondback moth with OX4319 41
2.2.2 Characterisation of the female-lethal phenotype 42
2.2.2.1 Test cross 1 42
2.2.2.2 Test cross 2 44
2.2.3 Investigating the evolutionary conservation of *dsx* in Lepidoptera 46
2.2.4 Characterisation of the transgene insertion locus 49
2.2.5 Comparing fsRIDL phenotypes as homozygotes 53
2.2.5.1 Survival to adulthood and rearing characteristics 54
2.2.5.2 Longevity 60
2.2.5.2.1 On tetracycline 60
2.2.5.2.2 Off tetracycline 62
2.2.6 Analysis of heterozygous fsRIDL phenotype 64
2.2.6.1 Temporal investigation of fsRIDL penetrance on host plant material ... 64
2.2.6.2 Tetracycline dose-response in heterozygotes 67
2.2.7 Transgene-associated fitness costs 69
2.2.7.1 Circadian rhythm of mating 71
2.2.7.2 Relative sterility index 75
2.2.7.3 Lifetime mating performance 76
2.2.7.4 Population genetics of transgene-associated fitness costs 79
2.2.7.4.1 Selection on transgene under permissive conditions: 81
2.2.7.4.2 Selection on transgene under restrictive conditions: 83
2.2.7.4.3 Discussion of population-level fitness costs 85
2.3 Conclusion and Summary 89
2.4 Materials and Methods 91

2.4.1 General experimental details 91
2.4.1.1 Laboratory conditions and insects used 91
2.4.1.2 Statistics 91
2.4.1.3 Micro-injection 92
2.4.2 Characterisation of the female-lethal phenotype 92
2.4.2.1 Test cross 1 92
2.4.2.2 Test cross 2 92
2.4.3 Investigating the evolutionary conservation of *dsx* in Lepidoptera 93
2.4.4 Characterisation of transgene insertion locus 93
2.4.5 Comparing fsRIDL phenotypes as homozygotes 95
2.4.5.1 Survival to adulthood and rearing characteristics 95
2.4.5.2 Longevity 96
2.4.5.2.1 On Tetracycline 96
2.4.5.2.2 Off Tetracycline 96
2.4.6 Analysis of heterozygous fsRIDL phenotype 97
2.4.6.1 Temporal investigation of fsRIDL penetrance on host plant 97
2.4.6.2 Tetracycline dose-response in heterozygotes 97
2.4.7 Transgene associated fitness costs 98
2.4.7.1 Circadian rhythm of mating 98
2.4.7.2 Relative sterility index 99
2.4.7.3 Lifetime mating performance 100
2.4.7.4 Population genetics of transgene-associated fitness costs 101
2.4.7.4.1 Selection on transgene under permissive conditions 101
2.4.7.4.2 Selection on transgene under restrictive conditions 103
Chapter 3: Proof-of-principle cage studies 107
3.1 Introduction 107
3.2 Population suppression 108
3.2.1 Results and Discussion 110
3.3 Insecticide resistance management 121
3.3.1 Results and Discussion 125
3.5 Conclusion and Summary 137
3.4 Materials and Methods 138
3.4.1 Population suppression 138
3.4.1.1 General conditions and summary 138
3.4.1.2 Establishment phase 139
3.4.1.3 Suppression Phase 140
3.4.2 Insecticide resistance management 141
3.4.2.1 General conditions and summary 141
3.4.2.2 Experimental diamondback moth strains 142
3.4.2.3 Experimental broccoli cultivars 142
3.4.2.3 Restocking of plant material 143
3.4.2.4 Establishment of cage populations 143
3.4.2.5 fsRIDL treatments and modelling 143
3.4.2.4 Data collection 147
3.4.2.4 Statistics 149
Chapter 4: Investigation of the Q inducible expression system for transgene control in the diamondback moth 152
4.1 Introduction 152

4.2 Results and Discussion 156
4.2.1 Quinic acid toxicity 156
4.2.2 Design and transformation of modular constructs........ 158
4.2.2.1 Repressor construct........ 158
4.2.2.2 Activator/effector construct 160
4.2.2.3 Transient expression assays........ 171
4.3 Conclusion and Summary........ 178
4.4 Materials and Methods 182
4.4.1 Micro-injection 182
4.4.1 Quinic acid toxicity 182
4.4.2 Construction of OX4673 (repressor construct) 183
4.4.2.1 OX4673A expression profiling using RT-PCR........ 184
4.4.3 Construction of OX4651 (activator construct) 185
4.4.4 Construction of basal expression control construct OX4658 187
4.4.5 Construction of modified activator construct OX4765 187
4.4.6 Construction of modified activator construct OX5046 188
4.4.7 Transient expression assays........ 189

Chapter 5: Conclusions........ 191

Appendix 203
Summary of injection success model 213
Papers........ 214
Chapter cover page photos 214

References 215

Abstract

Insect pests represent major threats to food production, biodiversity conservation, and human and animal health. Currently, the most widespread strategy to control their populations is through the spraying of synthetic chemical insecticides. However, the overuse of these compounds has had significant negative environmental consequences. Additionally, our reliance on insecticides has resulted in major reductions in their efficacy through pest-evolved resistance. To successfully manage insect pests, while avoiding environmental degradation, thus requires the development of novel, more sustainable, pest management strategies. Recent advances in our understanding of recombinant DNA methods and molecular biology have allowed the application of transgenic tools to pest management. Here, synthetic genes can be engineered, transformed into the genomes of pest species, and transported into wild target populations through the natural mating behaviour of the insect. A strategy in which these transgenes are lethal to those insects inheriting them in the field is known as RIDL – Release of Insects carrying a Dominant Lethal. A variant of RIDL limits this lethality to females – female specific RIDL (fsRIDL) – which explicitly targets the reproductive capacity of a target population. The aim of this thesis is to investigate the application of such an fsRIDL strategy to the diamondback moth (*Plutella xylostella* L.). This economically important pest of brassica crops is highly adept at developing resistance to insecticides and is considered extremely difficult to manage effectively. I present findings which demonstrate the power of diamondback moth lines transformed with fsRIDL transgenes to eliminate target pest populations, and combine synergistically with other transgenic control strategies such as *Bt* crops in counteracting the evolution of pesticide resistance. Additionally, an exploration into an alternative gene expression system to that used in current RIDL strategies – the Q system – suggests that not all expression systems will be suitable for transgene control within this highly specific framework. It is hoped that this work will contribute towards the effective control of the diamondback moth, and form a model for the sustainable control of other lepidopteran species through genetic pest management

Declaration of Authenticity

This work was made possible through the financial support of the Biotechnology and Biological Sciences Research Council (BBSRC) CASE studentship scheme and Oxitec Ltd. All work presented in this thesis was conducted by the author, except where explicitly noted in the text. None of the following has been submitted previously for a qualification at the University of Oxford, or any other institute of higher education.

Chapter 1

Chapter 1: Introduction

1.1 Modern agricultural systems

The mass-conversion of arable land to agriculture has dramatically altered the landscape of earth (Matson et al., 1997; Tilman, 1998). Since the 1700's, the global area employed in the production of food has increased by 555% (Goldewijk, 2001) with over 40% of total land surface area currently under crop production or pasture (Foley et al., 2005). The most significant increases in crop yield, however, have been achieved only within the last 60 years. This period witnessed unprecedented agricultural intensification, known as the "green revolution", through the development of synthetic pesticides, artificial fertilisers, irrigation systems and high-yielding crop varieties (Evenson and Gollin, 2003). While successful in improving yields, the green revolution exacted a significant environmental toll that now threatens the sustainability of agro-ecosystems and the areas of biodiversity with which they are associated (Pimentel and Pimentel, 1990). Research into ecologically sustainable agricultural systems is thus imperative, both to safeguard food production for an expanding human population and to prevent the degradation of the environments on which these populations depend.

1.1.1 Pest management in agricultural systems

Prior to the green revolution, commercial agricultural systems consisted of polycultures with multiple species being cropped together (Figure 1.1). While lacking the productivity of modern monocultures, these systems enjoyed a stability imparted on them by their complexity. The management of insect pests in these systems was to a

great extent reliant on the spatial and temporal resistance to attack provided by crop-species genetic diversity, as well as within-farm biodiversity (Liebman, 1995). This diversity worked to minimise the proportion of the crop susceptible to attack by any one pest species or genotype as well as providing habitat for a wide variety of beneficial species such as predators and parasitoids (Andow, 1991). These natural enemy complexes are estimated to contribute between 50-90% of insect pest control in contemporary agro-ecosystems and on traditional farms would have been the primary means of pest population control (Pimentel, 2005). As agriculture has intensified, the majority of this complexity has been lost. Polycultures have been replaced with uniform monocultures, growing a select few specialised crop varieties, each displaying very low genetic diversity. These monocultures primarily exist within areas of low habitat diversity where little area is given over to the non-crop areas vital for maintaining and promoting natural enemy communities (Landis et al., 2000). Modern agro-ecosystems therefore remain highly vulnerable to pest damage, with average global losses due to insects alone estimated at 15% (Oerke, 2006; Maxmen, 2013). These highly modified systems are now heavily reliant on synthetic chemical insecticides to control pest populations, a situation which has led to a number of serious social and environmental consequences.

Figure 1.1: Examples of polyculture (left) and monoculture (right) cropping. The primary crop in each system is maize; however, six other crop varieties are also planted in the mixed polyculture system. Polyculture photo taken from JPKC (http://jpkc.jluhp.edu.cn), monoculture photo taken from Nicholas Tonelli (www.flickr.com)

1.1.2 Ecological and economic consequences of pesticide use

The use of synthetic chemical pesticides has increased by c. 854% since the 1960s (FAO, 2014). Along with this increase has come a growing awareness of the large-scale and long-term damage the indiscriminate use of these compounds has caused (Carson, 1962; Krebs et al., 1999). Pesticides have contributed to significant biodiversity loss (McLaughlin and Mineau, 1995), including regional and national extinctions (Geiger et al., 2011), in aquatic (Thompson et al., 2006), terrestrial, and marine ecosystems (Shahidul Islam, Tanaka 2004). They have been shown to affect multiple non-target groups including invertebrates (Beketov et al., 2013), birds (Mineau and Whiteside, 2013; Hallmann et al., 2014), mammals (including humans (Rauh et al., 2011)), reptiles (Lambert, 1997), amphibians (Christin et al., 2013), plants (White and Boutin, 2007) and fish (Dunier and Siwicki, 1993). These reductions in biodiversity have subsequently had negative effects on community structure, ecosystem function and resilience to further disturbance (Relyea et al., 2005; Schäfer et al., 2007; Schäfer et al., 2012; Sabatier et al., 2013).

The overuse of these compounds has furthermore resulted in widespread resistance to their active ingredients. As of 2008, 7,747 reports of insecticide resistance had been recorded in 553 arthropod species (Whalon, 2008). The economic cost of this resistance, primarily in crop production, is estimated to be between $4-10 billion globally (Pimentel, 2009). However, as the largest number of cases of resistance is reported in dipterans of medical importance, the social costs in terms of vector-borne disease transmission may be much greater.

The non-selective employment of these compounds (both in terms of mode of action and usage) has caused reductions to the predator and parasitoid communities which function as natural enemies within agricultural landscapes (Pimentel, 2009; Geiger et al., 2011). In addition to the direct lethal effects of pesticides on these beneficial groups, their complex prey/host finding behaviours and social structures may be negatively affected by sub-lethal exposure. Moreover, they often lack the range of detoxifying mechanisms that have evolved in herbivorous pests in response to plant defence mechanisms, but which may also be effective in reducing pesticide toxicity (Tao et al., 2012; You et al., 2013). These factors combine to make these groups relatively susceptible to insecticides and less likely to become resistant to them. The loss of natural pest population regulators through exposure to insecticides has resulted in the resurgence of previously well-controlled secondary pest species and their elevation to major economic concerns (Huberson et al., 1998; Hajek, 2004). It is no coincidence that the three most insecticide-resistant arthropod species were originally secondary pests in their respective agro-ecosystems (Whalon, 2008). In these cases the consequences of natural enemy loss are exacerbated as the primary remaining means of controlling these populations (insecticides) has been rendered ineffective.

1.2 Integrated Pest Management and the Sterile Insect Technique

Synthetic chemical insecticides are cheap, easy to use and effective (when used properly, or in the short-term). However, despite their widespread use over the last 60 years, insects continue to represent serious threats to human health, biodiversity and food production (Ruttan, 2002; Oerke, 2006; Kenis and Branco, 2010). This is primarily due to our over-reliance on these compounds as the most widely used means of pest control. Under this scenario, evolutionary pressure against insecticides is expected to be very high, leading to strong selection for resistant genotypes or behaviours. Integrated Pest Management (IPM) seeks to avoid this by utilising all effective and environmentally benign methods of pest control in ways directed by the target ecosystem's ecology and economics. Reducing the intensity of management in this way leads to increased stability in agro-ecosystems and the long-term sustainability of the management tools used to protect them.

An existing alternative to synthetic insecticides that has been effective in an IPM context is the Sterile Insect Technique (SIT). SIT involves the mass-sterilisation of pest insects, which are then released inundatively within a target area. Mating between (sterile) released and wild insects results in the production of non-viable offspring and a reduction in the reproductive potential of the population (Klassen and Curtis, 2005). As a mating-based means of population control, successful use of SIT is dependent on detailed knowledge of the sexual competitiveness of released insects and the population biology of the target population. In particular, the reproductive rate (R_0) of the target pest will determine the proportion of total matings which must be 'won' by released insects in order to prevent or reverse population growth (Table 1.1). The feasibility of

achieving this proportion will be affected both by the competitiveness and overall number of released insects relative to their wild counterparts.

Table 1.1: Percentage of total matings required to be 'won' each generation by released sterilised insects in order to prevent population growth (maintain a stable population) for pests with varying rates of reproductive increase (R_0). Pests with high R_0 require a high level of control from released insects to prevent population increase. In a suppression programme, the aim is not to maintain but to reduce a target population and thus the percentage matings achieved by released insects desired will be higher than those listed here. This simple model assumes an equal competitiveness of released and wild insects as well as a lack of density dependent population dynamics. If the sexual competitiveness of sterilised insects is significantly impaired, an increased number of insects will need to be released to achieve the required mating percentage, at greater cost. As such, the competitiveness of released insects is vital to the success of an autocidal release programme. Adapted from (Klassen, 2005).

R_0 of pest	Number progeny per female	Number of these required to live	Number of these required to die	Percentage matings required by released sterile insects
2	4	2 (1/2)	2 (1/2)	50%
3	6	2 (1/3)	4 (2/3)	67%
4	8	2 (1/4)	6 (3/4)	75%
5	10	2 (1/5)	8 (4/5)	80%
10	20	2 (1/10)	18 (9/10)	90%
20	40	2 (1/20)	38 (19/20)	95%

In conventional SIT, sterilisation is achieved by exposing a suitable life-stage of the pest to ionising radiation from a suitable gamma ray source, e.g. Cobalt-60. This induces dominant lethal mutations (chromosomal breaks) in the gametes and somatic tissue of exposed individuals. Ideally, these lesions have no phenotypic effect on the gamete itself but will, however, result in lethal imbalances of genetic material when it is used to form a zygote and mitotic cleavage divisions occur (Figure 1.2A) (Robinson, 2005). As SIT relies on the natural mating behaviour of the pest insect both to locate targets and suppress populations, it is much more efficient and species-specific than the spraying of insecticides, the vast majority of which do not reach the pest. Moreover,

SIT shows no long-term persistence in the environment beyond the life-span of released insects and minimal off-target effects, which are major advantages over many synthetic insecticides.

The SIT has been shown to be an extremely effective form of area-wide pest control (Nagel and Peveling, 2005). First trialled in the 1950's (Bushland et al., 1955; Knipling, 1955) it has been involved in numerous successful eradication/suppression programmes since, in a variety of insect orders. Most notable amongst these was the eradication of the New World screwworm (*Cochliomyia hominivorax* Cocquerel) from the southern United States, Mexico and Central America (ceasing at Colombia) between 1958 and 2001. Female adults of the screwworm lay eggs in open wounds of warm-blooded animals and upon hatching, larvae feed on exposed tissue. Prior to the SIT eradication programme, larval feeding caused significant economic damage to the livestock industry and was treated by direct application of insecticides to wounds (Knipling, 1985). Releases of sterilised screwworm adults began in Florida in 1958 and were rolled southwards as wild populations were successively removed. By 1966, the screwworm had been eradicated from the USA and by 2001 had been driven from most of Central America. Currently small-scale releases are continued indefinitely at the Colombia-Panama border to prevent reinvasion. This programme, costing US$1.3 billion over its 45-year period, is currently estimated to save producers at least US$1.3 billion per annum in livestock losses and treatments (Klassen and Curtis, 2005).

1.2.1 SIT and the Lepidoptera

Despite its proven record as an economically and ecologically robust method of insect population control, SIT remains underexploited (Whitten and Mahon, 2005). Prominent amongst those insect orders in which SIT has been underutilised are the Lepidoptera (Bloem et al., 2005). This is surprising as lepidopteran species rank amongst the most destructive pests of agriculture, stored products and natural forest ecosystems, worldwide. Owing to this status, the non-phytophagous nature of their adult stages, and an almost unrivalled ability to evolve insecticide resistance, the potential to control pest Lepidoptera with SIT is considered very high. However, operational SIT programmes have only been implemented against three pest moths: pink bollworm (*Pectinophora gossypiella* Saunders) in USA, a major cotton pest; codling moth (*Cydia pomonella* L.) in Canada, a pest of apples and pears; and painted apple moth (*Orgyia anartoides* Walker) in New Zealand, a polyphagous forestry and horticulture pest (Bloem et al., 2005). Two primary constraints on the further application of SIT to Lepidoptera include their relative resistance to radiation-induced sterility (Bloem and Carpenter, 2001) and the lack of mass-rearing-compatible sexing systems (Bloem et al., 2005; Knipple, 2013).

1.2.2 Lepidopteran radioresistance

SIT relies upon ionising radiation to induce chromosomal breaks in adult insects, prior to their release into the field. While not lethal to these adult stages, these breaks will constitute dominant lethal mutations in offspring inheriting them in the field (Figure 1.2). Cells that are mitotically active (i.e. dividing at a rapid rate) are much

more sensitive to this chromosomal damage than mitotically inactive counterparts. Developing embryos, with their rapid rounds of cell replication, are therefore extremely sensitive while adult somatic tissue is less so. The challenge for SIT is to expose adult insects to a dose of radiation high enough to sufficiently damage their gonadal cells (such that the majority of offspring inheriting this genetic material die), but low enough to prevent somatic abnormalities that might lead to their reduced mating success (Bakri, Mehta et al. 2005). In most insect species, female germ-line cells are more sensitive to irradiation than males, as mature oocytes are pre-meiotic. As the release of fertile females is extremely detrimental to a SIT programme, irradiation rates are thus usually designed around doses required to sterilise males. The situation in Lepidoptera, however, is complicated by their unusual genetics. Lepidopteran chromosomes are partially holokinetic; that is, the properties of their centromeres are spread out along the length of their chromosomes. Thus, whereas in a dipteran, inheritance of a radiation-induced single stranded break can result in two acentric fragments and a dicentric bridge (leading to further breaks and the loss of genetic material), it is probable that the same break in lepidopterans would effectively form two smaller chromosomes. These smaller chromosomes would retain the ability to accurately pair at mitosis, be inherited correctly and result in fewer imbalanced gametes (Figure 1.2B). This feature, in combination with a unique, inducible cell recovery system and DNA repair process, confers a high degree of radioresistance on lepidopterans. In particular, male germ-line cells appear to be extremely tolerant of radiation damage and doses required to induce sterility in males are very high (Lachance and Graham, 1984; Koval, 1996). On average, the dose of radiation required to induce complete sterility in lepidopterans is nearly 300% higher than in dipterans (Bakri, Mehta et al. 2005). As a result of the unacceptable reductions in insect competitiveness which result from such high doses, a

compromise in the form of inherited sterility (IS) has been advocated in lepidopteran SIT.

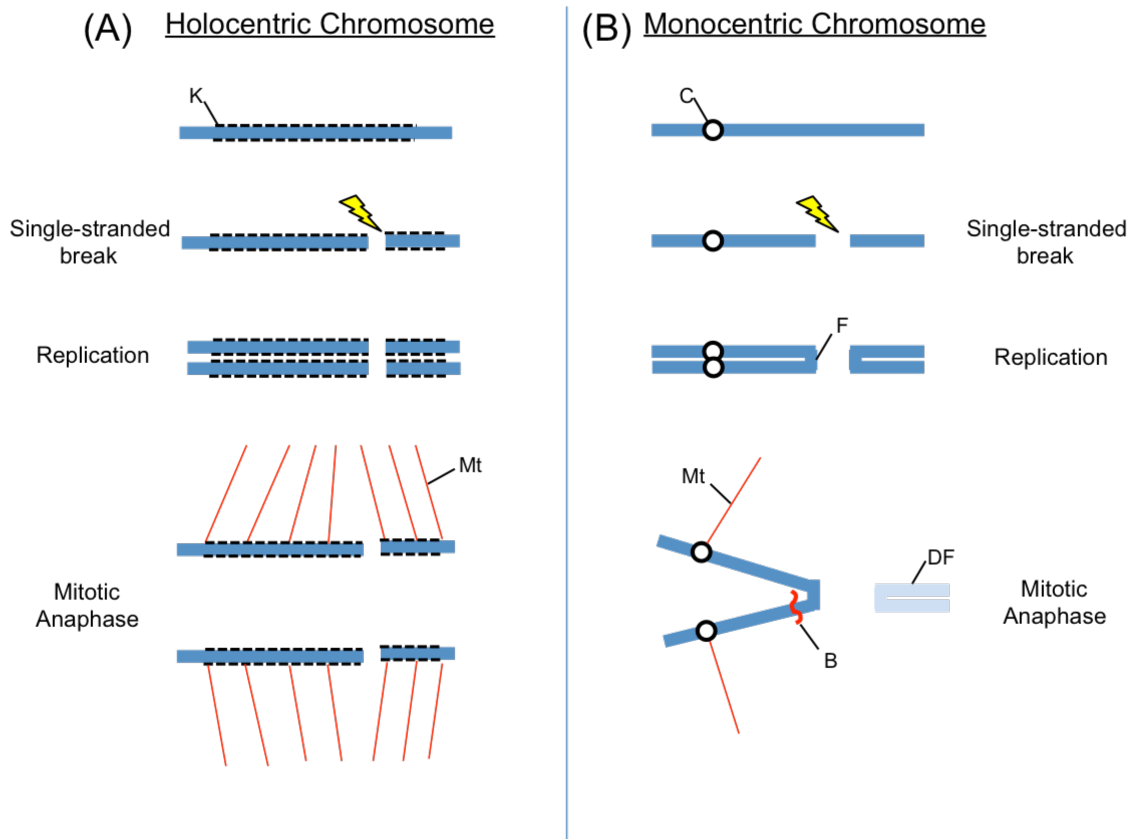


Figure 1.2: The mitotic fate of radiation induced single-strand breakages in A) a lepidopteran holokinetic chromosome and B) a monocentric chromosome, typical of the Diptera. In A) the kinetochore plate (K) extends across more than 50% of the chromosome. If a breakage occurs within K, the resulting fragments each contain points for spindle microtubules (MT) to attach (are kinetic) and will be inherited correctly. In B), strand breakage results in an acentric fragment (without a centromere) and a dicentric bridge. The ends of these fragments fuse (F) and during anaphase, the acentric fragment will be lost (DF) while the dicentric fragment will form a bridge, causing further chromosome breaks (B). Modified from (Carpenter, Bloem et al. 2005).

Inherited or F1 sterility (IS) describes a phenomena unique to the Lepidoptera, Hemiptera and Acari whereby a large proportion of the progeny of adults treated with sub-sterilising doses of radiation survive, but show complete sterility. This is a result of the dispersed centromeres in these orders. Here, irradiation damage results in a higher percentage of chromosomal translocations than in orders with monocentric

chromosomes. As these translocations occur within gametes that possess full haploid genomes (at least in males), a complete complement of genetic material will be passed to the F1 generation. It is in the meiotic divisions of the F1 germ-line where aberrations such as translocation multivalents are likely to occur, leading to the production of chromosomally unbalanced gametes (Tothova and Marec, 2001). As the parental, released, generation receives only a partially sterilising radiation dose, their competitiveness is often significantly higher than that of fully sterilised moths (Carpenter et al., 2005; Hight et al., 2005). Although potentially extending the list of lepidopteran species to which SIT may be applied, IS has rarely been used at the field scale. Of the three, operational, lepidopteran SIT programmes only that targeting the painted apple moth in New Zealand has utilised sub-sterilising radiation doses. The primary concern with using IS has been that survival of the F1 generation on the crop would cause unacceptable damage, particularly to those of high value such as fruits. However, field studies suggest that, when used over the course of the growing season, damage to crops is not significantly different to when fully sterile insects are released, at least in apples (Bloem and Carpenter, 2001). Where IS does pose a particular challenge is to the monitoring of SIT programmes. The markers currently used on released insects such as fluorescent or ingested dyes are not inherited. Discerning wild and F1 sterile moths may therefore be very difficult and cause significant operational challenges, particularly where both sexes are released in the parental generation.

1.2.3 Lepidopteran sexing systems

A long-standing question in SIT is whether it is preferable to release males alone or both sexes (male-only or bi-sex release). As the reproductive biology and life history dynamics of individual pest species may differ, the optimum approach may differ between species. However, some general principles hold when addressing this question.

Male-only release can improve the per-unit efficiency of sterilised insects by preventing them from simply mating with each other at release sites (Hendrichs et al., 1995; Rendon et al., 2004). As males can usually mate multiple times, and females are less prone to do so, release of males only may further increase the genetic load introduced per insect released. Additionally, the release of females can be detrimental as they may cause crop damage through their ovipositional behaviour and their progeny may temporarily increase the target pest population within the crop (Hoa and Tien, 2001). Taking the optimum approach is a financial incentive, as suppression/eradication will be achieved faster and with reduced rearing, release and monitoring costs. Conversely, field cage studies investigating the efficacy of male-only and bi-sex releases in SIT for the cactus moth (*Cactoblastis cactorum* Berg.) suggest that released females may play a significant positive role in lepidopteran SIT programmes by acting as “sperm sinks” for wild males (Hight et al., 2005). This is surprising as it is generally assumed that in polygynous species, male reproductive effort is in great excess relative to the number of female mating opportunities in a population, i.e. that male sperm production is not normally a limiting factor (Bateman, 1948; Koyama et al., 1984). In any case, the net benefit that released females would have at the field scale has been disputed (Marec et al., 2007). Without a direct comparison of bi-sex and male-only release for Lepidoptera at the field level it is difficult to predict which of these strategies

will be most effective. However, at the very least it is expected that a male-only release programme would reduce assortative mating and rearing costs, both of which can be major constraints in large-scale SIT programmes.

The most serious impediment to the use of male-only releases in Lepidoptera is that there are no efficient means to sort males from females. In SIT programmes targeting the Mediterranean fruit fly (medfly, *Ceratitis capitata* Wiedemann) Genetic Sexing Strains (GSS) containing a Y chromosome-autosome translocation of a *temperature-sensitive lethal* (*tsl*) mutation have been developed using x-ray mutagenesis (Franz, 2005). The use of these strains has significantly improved the efficiency and cost-effectiveness of medfly SIT (increasing per male efficiency by 3-5 × (Rendon et al., 2004)). In this system, mass-reared males are heterozygous for the tightly linked *tsl* and *white pupae* (*wp*) alleles, whereas females are homozygous-recessive for both of these mutations. This allows females to be selected out of the pre-release population both visually and through exposure as eggs to restrictive temperatures. In Lepidoptera, however, the conventional route of generating GSS as described above is inappropriate due to the homogametic nature of males (ZZ). Lepidopteran sexing systems are therefore limited to dominant W-linked visible markers (Nagaraju and Goldsmith, 2002) and balanced-lethal GSS (Strunnikov, 1975; Marec et al., 2005a), neither of which is compatible with mass-rearing or available in a species appropriate for SIT (Morrison et al., 2010). SIT programmes against lepidopterans have thus been limited to bi-sex release (pink bollworm and codling moth) or male-only release via manual sex-sorting (painted apple moth). Development of an effective GSS in pest Lepidoptera would greatly enhance the prospects of SIT against this economically important Order (Bloem et al., 2005).

1.3 Transgenic control of pest insects

Advances in molecular biology and germ-line transformation have permitted the tight control of synthetic gene expression in insects. This capability has been harnessed to circumvent many of the issues that limit the efficiency of SIT and its uptake in other pest species. The most developed of these technologies is termed RIDL (Release of insects carrying a Dominant Lethal), in which the radiation-induced lethality of SIT is substituted with a conditional-lethal transgene cassette. RIDL typically employs the *piggyBac* transposon to transform pest insects with a tetracycline-repressible lethal genetic system (Thomas et al., 2000). At its most basic, this system comprises two tightly linked loci: the hybrid transactivator tTAV, a fusion of the tetR tetracycline-responsive DNA binding domain from *Escherichia coli,* and the C-terminal region of VP16, an early-acting transcriptional activator from the herpes simplex virus; and *tetO*, binding site specific to tetR. Under permissive conditions, tetR is bound by tetracycline (or its analogues), resulting in a conformational change that prevents the tTAV protein from binding *tetO* (Stebbins et al., 2001). The co-presence of tTAV and *tetO* under restrictive conditions (in the absence of tetracycline) results in high levels of tTAV expression and the expression of sequences downstream of *tetO*. If *tetO* is placed upstream of the tTAV sequence, this results in a positive feedback loop leading to cell death through transcriptional squelching and the eventual death of the insect (Phuc et al., 2007a) (Figure 1.3).

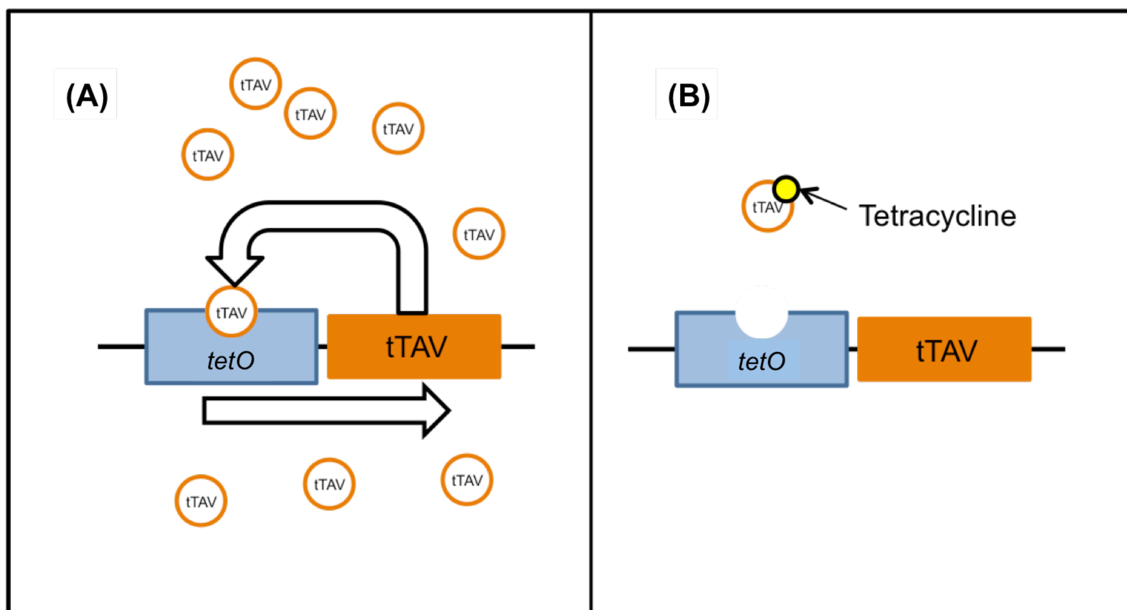


Figure 1.3: Schematic showing the mode of action in RIDL based lethality. In the absence of tetracycline (A), basal expression of the transactivator tTAV binds its upstream binding site *tetO*, forming a positive feedback loop and thus enhancing its own further expression. This produces large amounts of tTAV protein which cause cell and insect death through transcriptional squelching as the resources of the cell are monopolised. In the presence of tetracycline (B), tTAV is bound by this molecule and prevented from binding to *tetO*. Thus while there is some minimal basal expression of tTAV, a positive feedback loop is not formed and lethality is repressed. Adapted from (Gong et al., 2005).

In a RIDL release programme, transformed pest insects are mass-reared under permissive conditions, with tetracycline added to the larval diet/habitat, under which lethality is repressed. Upon release into the field, the progeny of transgenic and wild insects develop under restrictive conditions as tetracycline is not available in sufficient quantitates in the field to suppress lethality, and the progeny die before reaching adulthood. This form of RIDL, known as bi-sex RIDL, is analogous to conventional SIT as both sexes exhibit the lethal trait. Bi-sex RIDL, with manual sex sorting prior to release, has been shown to be effective at suppressing wild mosquito populations in the field (Harris et al., 2011a; Harris et al., 2012).

A female-specific form of RIDL, fsRIDL, has been achieved by exploiting the sex-alternate splicing of the medfly sex-determination gene *transformer* (*tra*), in olive

fly *Bactrocera oleae* (Rossi) and medfly (Fu et al., 2007; Ant et al., 2012), and by using a female-specific indirect flight muscle promoter in the tiger mosquito *Aedes albopictus* (Skuse) (Fu, Lees et al. 2010). In fsRIDL fruitflies, the tTAV locus is inserted downstream of an alternatively spliced *tra* sequence. When pre-mRNA from this minigene is spliced in males, translational machinery encounters stop codons found within male-specific exons and tTAV, which is located downstream of these sequences, is not translated. A female-specific splice variant lacks these male-specific stop codons and thus functional tTAV protein is produced in females only (Figure 1.4).

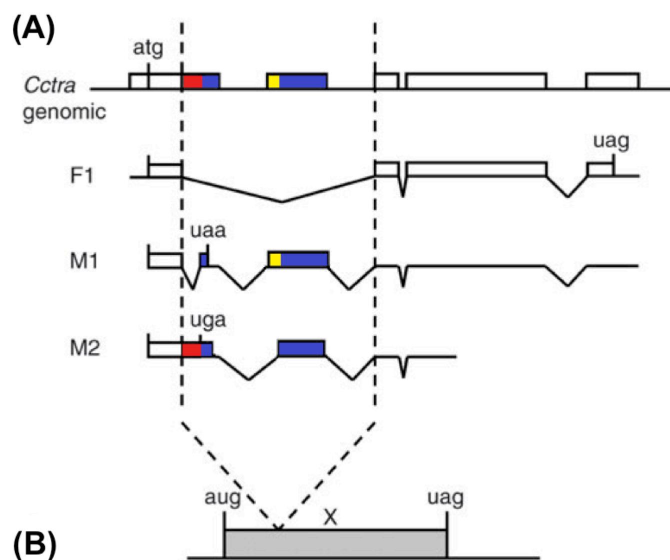


Figure 1.4: Sex-alternate splicing of the *Ceratitis capitata transformer* (*Cctra*) gene (A), and its use within a hypothetical fsRIDL construct (B). The top schematic of (A) represents the genomic form of *tra* with the areas involved in alternative splicing bounded by the vertical dashed lines. Endogenous splicing of *Cctra* results in one female-specific transcript (F1) and two male specific isoforms (M1,2). Blue boxes represent male-specific exons while yellow and red boxes represent the exonic regions specific to M1 and M2, respectively. In male transcripts, stop codons (uaa, uga) are present within the reading frame while in female-specific transcript, the exonic area downstream of the alternatively spliced section is also translated (terminating at the final stop codon, uag). By placing tTAV downstream of the alternatively spliced region (marked with an x in B) it will only be translated in females. Modified from (Pane et al., 2002; Fu et al., 2007).

In *Ae. albopictus*, tTAV expression was made female-specific at the transcriptional level. Engineered lethality is repressed by tetracycline in mass-rearing colonies, as in bi-sex-RIDL. In fsRIDL, however, tetracycline is withdrawn from the generation of release, allowing only males to develop to adulthood. Thus, fsRIDL acts both as a GSS and an autocidal method of population suppression. Females inheriting a copy of the fsRIDL transgene in the field will not develop to adulthood while male progeny will be heterozygous carriers (Figure 1.5), leading to death of 25% of their offspring in the next generation.

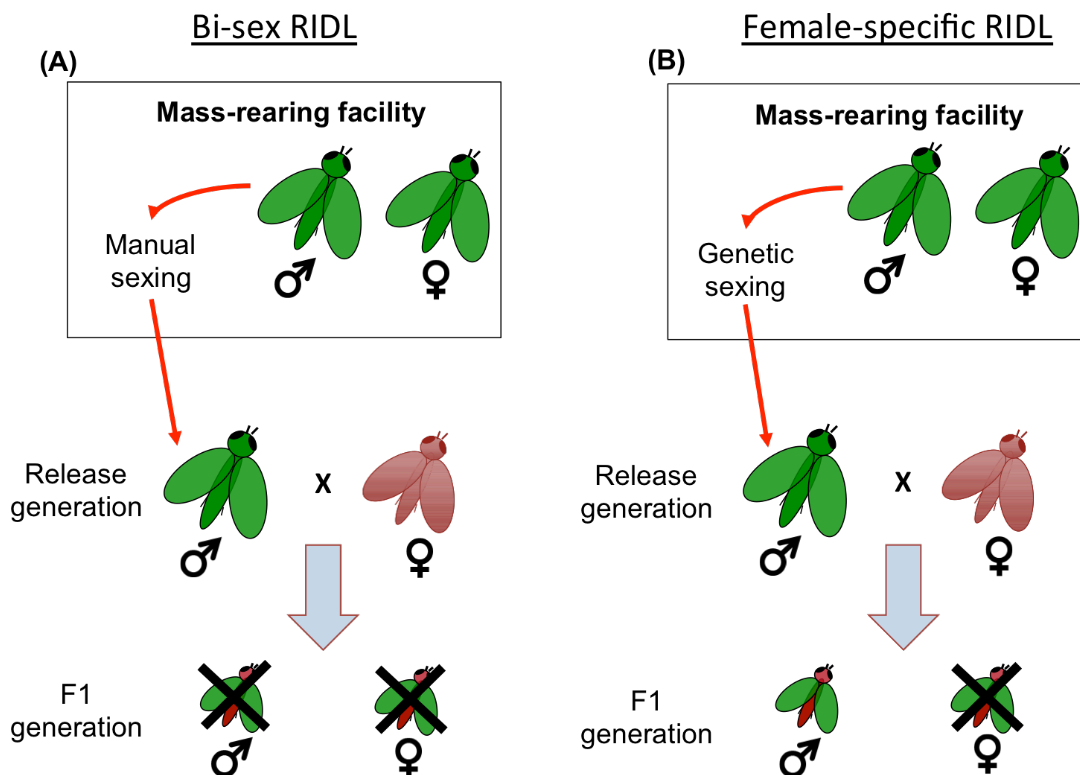


Figure 1.5: Schematic showing the use of Bi-sex and female-specific RIDL in a release programme. In Bi-sex RIDL (A), all generations of RIDL insects (green) in the mass-rearing facility are reared on tetracycline diet to suppress lethality. In release generation, males are manually sexed and released. When these males mate with wild females in the field (red) all F1 progeny die. In female-specific RIDL (B), Insects are reared on tetracycline, but in the release generation tetracycline is removed, leading to genetic sexing as only males survive to be released. The F1 female progeny of these released males die, but male heterozygotes survive to pass on fsRIDL genes to the following (F2) generation (not shown).

Modelling studies have shown this form of control, where females are specifically targeted and male progeny survive to cause further suppression, to be amongst the most effective and robust methods of genetic pest management (Schliekelman and Gould, 2000; Bax and Thresher, 2009). fsRIDL strains have successfully eradicated experimental caged populations of olive fly, medfly and the dengue vector *Ae. aegypti* (de Valdez et al., 2011; Ant et al., 2012; Leftwich et al., 2014). Additionally, modelling suggests that their use can act as a potent insecticide resistance management strategy through the introgression of susceptibility alleles (through surviving heterozygous males) into wild populations (Alphey et al., 2007; Alphey et al., 2009).

The most significant advantage of RIDL over traditional SIT is that it avoids the deleterious effects of irradiation on released insects and the reductions in sexual competitiveness this can cause. The relative performance of released insects in any autocidal control strategy is of great importance, as it will significantly affect the release rates required to achieve population suppression and therefore the cost of the programme. This is of particular relevance to species that show high radioresistance, for instance lepidopterans, or to species that are extremely radiosensitive, such as mosquitoes. Although some transgene-associated fitness costs are likely in any transformed organism, the use of molecular tools such as tissue/sex-specific promoters and splicing introns can be employed to minimise their impact on insect competitiveness. This is a great advance over conventional SIT, where chromosomal damage (and the behaviours this may affect) is essentially random. Additionally, the inclusion of a tightly linked fluorescent protein marker in the transgene allows for easy and accurate monitoring of the transgene both in release and subsequent field generations (Walters et al., 2012). This is a vital tool for SIT programmes as it provides

information both on the competitiveness of released insects - and consequently, the appropriateness of a particular release rate - and because released insects should be distinguishable from wild ones to prevent false positives and unnecessary further releases (Simmons et al., 2011). In conventional SIT this information is collected by two separate methods, the marking of released insects with visible dyes and the monitoring of sterility in field-collected egg masses, both of which have potential issues with reliability. Finally, due to the universality of DNA and, in fsRIDL, the use of conserved sex-regulatory regions, RIDL constructs and components are highly transferrable between species. For example, fsRIDL strains have been created in four dipteran and three lepidopteran species using a single transgene construct for each order (Ant et al., 2012; Jin et al., 2013; Tan et al., 2013; Leftwich et al., 2014). In the case of the Lepidoptera, the three families involved - Yponomeutidae; Gelechiidae and Bombycidae - represent a large phylogenetic range (Regier et al., 2013). This is a significant improvement over current GSS methods used in SIT where each chromosomal translocation must be generated independently with no way to transfer successful systems between species.

1.3.1 Expanding SIT with RIDL: the diamondback moth

The diamondback moth (*Plutella xylostella* L.) is a lepidopteran within the family Yponeumetidae, also known as the ermine moths (Figure 1.6). The diamondback moth is a specialist of brassica plants and is the most significant economic pest of this plant group, worldwide, costing growers in excess of US$5 billion annually in losses and control measures (Zalucki et al., 2012). The brassicas include high-value crops such as cabbage, broccoli and oilseed rape, as well as varieties that are nutritionally important for subsistence farming.

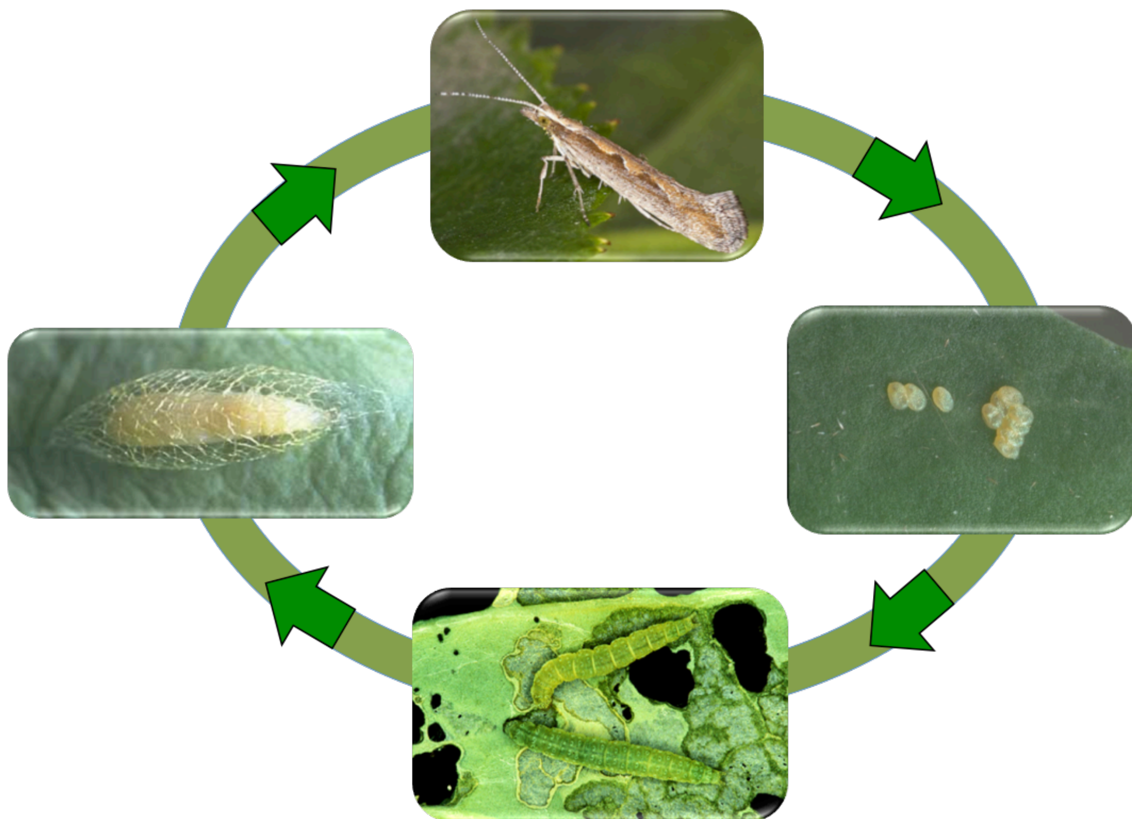


Figure 1.6: Life-cycle of the diamondback moth, *Plutella xylostella* L. Duration spent in each life stage (at 25°C) is as follows: egg, 3 days; larva, 7 days; pupa, 3 days. Adults become sexually mature within 24 h of eclosion. Photos (clockwise from adult) taken from www.wikipedia.com, www.inra.fr, www.inhs.illinois.edu, http://www.sardi.sa.gov.au.

The diamondback moth is believed to have originated in the Mediterranean (Harcourt, 1954), has spread to wherever crucifers are grown, and is considered amongst the most difficult insect pests to control (IRAC, 2013). Their ability to migrate long distances (up to 1000km per day) and the massive expansion of crucifer crops over the last 60 years - global brassica production area increased by 76% since the 1960's - has made this pest the most widespread of all lepidopteran species(Talekar and Shelton, 1993; Furlong et al., 2013; FAO, 2014). The globally projected distribution of this species is almost ubiquitous (Figure 1.7).

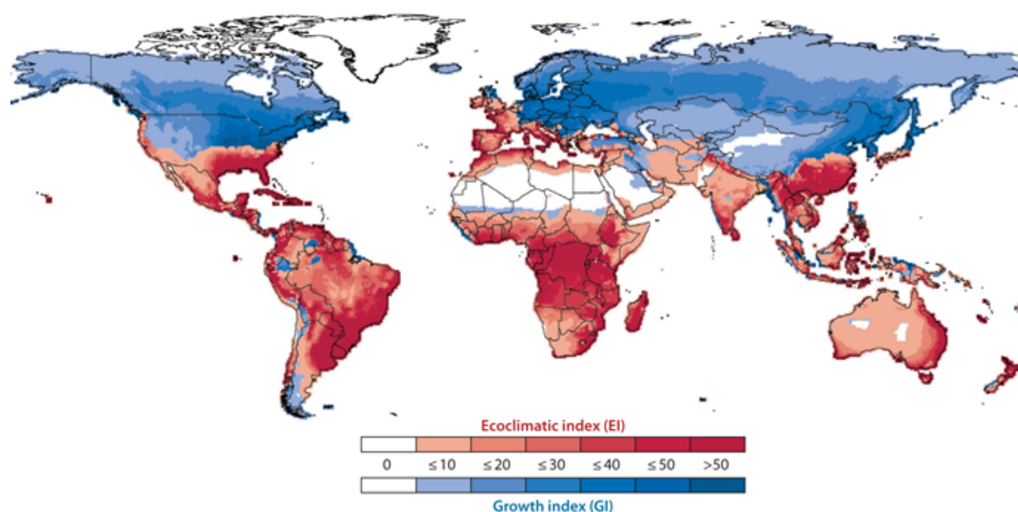


Figure 1.7: Projected distribution of diamondback moth based on a validated bioclimatic model. Red shading represents areas of the world where this species can persist year round. Blue shading represents areas where populations of diamondback moth can become seasonally established but will be unable to persist. White areas are where populations are absent. Taken from (Furlong et al., 2013).

The primary means of controlling diamondback moth is through the intensive use of synthetic chemical insecticides. The high value of brassica crops and the relative low monetary cost of insecticides has often led farmers to employ prophylactic or calendar spraying with cocktails of different insecticides. This in turn has led to the

suppression of natural enemy complexes, the widespread development of insecticide resistance, and the elevation of this secondary pest to its status as a major economic concern (Furlong et al., 2013). A classic example of this process is in the Cameron Highlands of Malaysia, where it is estimated that over 90% of growers spray insecticides targeted at diamondback moth at least once a week, using between three and four active ingredients in each 2-month growing cycle. This, combined with a year-round growing season, and this pest's short life-cycle (approximately 14 days in tropical climates) provides constant, strong, selection for resistant traits. Newer, more selective compounds such as spinosad, avermectins and *Bt* (*Bacillus thuringiensis* toxins) have become common (in response to mass-resistance formation in older compounds), however these are often delivered in combination with older non-selective pesticides and used sequentially. Thus, resistance to new compounds can develop in the field in as little as 2-3 years, while recovery of natural enemy complexes is often minimal. As new insecticides lose their effectiveness, growers resort to increased spraying regimes and older compounds as the cycle repeats itself. Tellingly, after more than 40 years since research began into developing an IPM system in the Cameron Highlands, pesticides are still the dominant control method and the diamondback moth remains a major economic pest (Mazlan and Mumford, 2005).

This situation, however, is not limited to developing countries and tropical regions. Globally, the diamondback moth is one of a select few species of insect which has developed field resistance to all major insecticide classes (Whalon, 2008; IRAC, 2013). It ranks second on the list of most resistant pest insects, both in terms of total number of compounds and number of compounds shown by at least one population, and was the first insect to develop field resistance to DDT (Ankersmit, 1953) and *Bt* Cry toxins (Kirsch and Schmutterer, 1988). This is in part due to a unique genetic ability to

detoxify insecticides (You et al., 2013), but is also a result of the lack of alternative control methods available for protecting high-value crucifer crops.

Previous research into the use of IS/SIT for the control of diamondback moth has ranged from determining effective radiation dosages (Omar and Mansor, 1993; Sutrisno and Hoedaya, 1993; Binbahari, 1994), to field cage (Maung, 1998; Hoa and Tien, 2001) and open field release trials (Maung, 1998; Sutrisno, 1998; Yang et al., 1998). However, no area-wide SIT programme for this pest has been implemented (Furlong et al., 2013). This is primarily due to concerns over the effect of irradiation on the biology of released males, the lack of a GSS to enable large-scale male-only releases, and the additional costs these issues would impose on a SIT programme (Hoa and Tien, 2001; Jin et al., 2013). Estimates of effective radiation doses for diamondback moth vary between 150-250 Gy. Sutrisno (1998) found that radiation doses of 200 Gy were high enough to induce F1 sterility. However, Hoa and Thien (2001) found that doses of this level cause an approximately 20% increase in the level of deformities formed in parental generation male adults, while Maimun and Mahani (1996) report a 79% reduction in pupal eclosion in the parental generation when dosed with 175 Gy. The variation in these results illustrates the difficulty in calibrating effective radiation doses in SIT and the degree to which differences in strain genetic background, irradiation technique and other factors can complicate this. Regardless, both of these results imply a potentially significant detrimental effect of radiation on SIT programme cost-effectiveness. Indeed, the only field study to examine the economic viability of a diamondback moth IS programme found that it was less cost-effective than insecticide-based control, primarily due to the high costs of insect production (Yang et al., 1998). Furthermore, radiation doses of 200-250 Gy have been shown to reduce male re-mating ability by >50%, longevity by around 33%, and increase development time in the F1

generation by 3-4 days (Binbahari, 1994; Yang et al., 1998; Hoa and Tien, 2001). The latter of these points may be important if the emergence times of F1 adults (as the primary providers of sterility in IS programmes) become de-synchronised with those of the wild population. If an fsRIDL system analogous to that achieved in dipterans could be engineered in the diamondback moth, this could potentially circumvent many of these issues and allow for sustainable, SIT-like control of this important pest species.

1.4 Investigating alternative gene expression systems

At present, RIDL technology is based on the 'tet-off' gene expression system (Gossen and Bujard, 1992). While this system has proven to be highly compatible with autocidal control, the development of additional, orthogonal, gene expression systems would be valuable for a number of reasons.

The primary advantage of having a system which could act in conjunction with, but independently of, the tet-off system is that it would make possible a higher level of phenotypic control in RIDL strains. Currently, the RIDL phenotype is limited to either bi-sex or female-specific lethality. While both of these strategies are capable of managing pest populations, their efficacy would be increased if their two phenotypes were combined in a single strain. This would allow females to be selected out of the pre-release generation whilst all the progeny of any released males would die. However, achieving this phenotype is complicated if both systems are under the control of the same 'antidote' (tetracycline). If either of the systems can be controlled by an alternative, orthogonal expression system, this combined phenotype could be achieved relatively easily.

Additionally, a potential disadvantage of using the tet-off system in an area-wide control strategy is its reliance on the antibiotic tetracycline. Tetracycline and its analogues are amongst the most widely used antibiotics (de la Torre et al., 2012). In agriculture, they are commonly fed to livestock for therapeutic purposes, but also to promote growth (Lipsitcht et al., 2002). Although RIDL is not currently employed against livestock pests such as the screwworm, it is possible that in the future such insects could be targeted. If this were the case, and were it possible that the levels of tetracycline found within host animal tissue were sufficiently high, this could lead to a breakdown in RIDL control. Tetracycline is also known to exist at sufficient quantities in agricultural waste/manure that resistance levels are elevated in field soil microbes (Schmitt et al., 2006) and there is experimental evidence that tetracycline is taken up by agricultural crops from manure-treated soil (Kumar et al., 2005). Therefore, ingestion of sufficient quantities of tetracycline via plant tissue by the progeny of released RIDL insects could lead to a breakdown in control. While these examples remain highly hypothetical, they nonetheless represent situations where a tet-off based control system could potentially fail. This uncertainty may prove a potential hurdle to regulatory approval of this technology.

An inducible expression system (in contrast to the repressible tet-off system) would, furthermore, open up opportunities for RIDL to be employed in more species. Due to the suppressive nature of the tet-off system, when lethal constructs are being tested, the G_0 generation (those insects which have been injected) must be reared on a tetracycline-supplemented artificial diet. If not, any transformed progeny would die before being identified. Artificial diets exist for a number of agriculturally important insects. However, as new pest threats emerge around the world, a reliance on a suppressive system will limit those species in which preliminary RIDL research could

be performed. This situation is far from hypothetical and recently precluded the development of RIDL lines in the tomato leaf mining moth, *Tuta absoluta* (Meyrick). An inducible system would not be subject to these limitations as progeny could be reared on host plant material without the system being induced, and transformants identified using their fluorescent central marker.

These issues highlight the need for further research into alternative expression systems to work in synchrony with those already employed successfully in RIDL. This would both counteract some of the limitations associated with the current tet-off RIDL system and facilitate the extension of this technology to more sophisticated means of insect pest control.

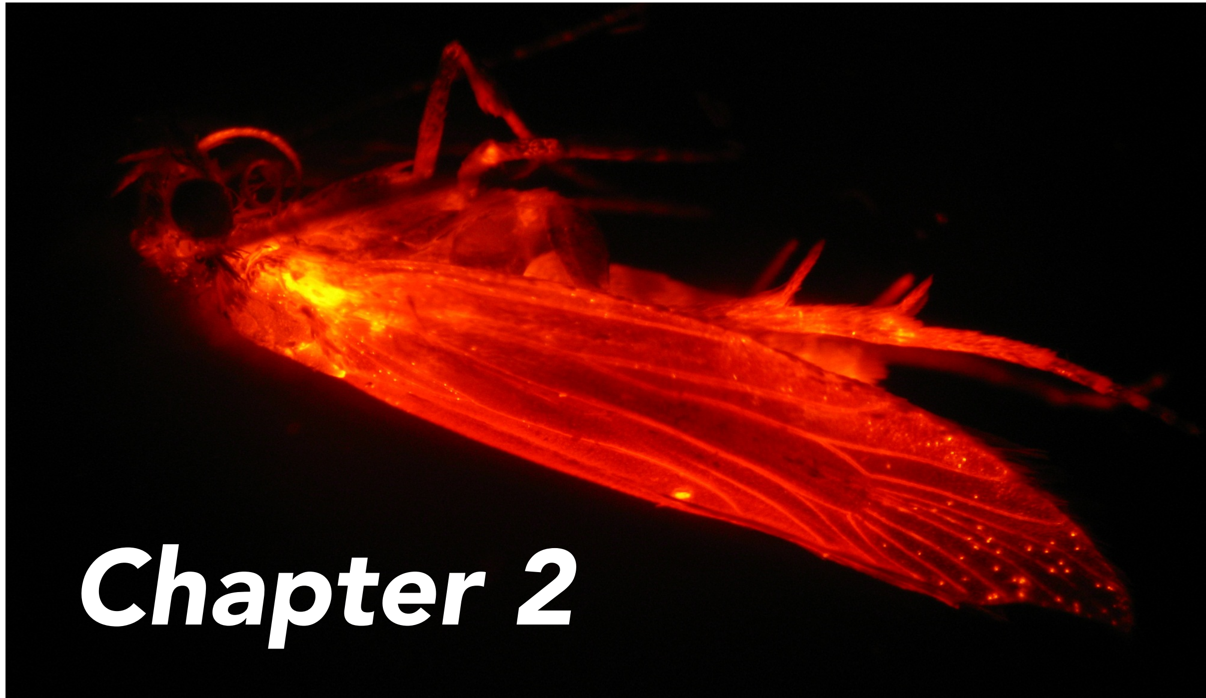
1.5 Goals

1.5.1 Project aim

The aim of this project was to develop a novel tool for the sustainable control of the diamondback moth through the application of fsRIDL technology to this highly damaging pest. In addition, research was undertaken into an orthogonal, inducible gene expression system (Q System) to further increase the efficiency of genetic control strategies.

1.5.2 Project objectives

1. Using sex-specific alternative splicing introns from the pink bollworm *doublesex* gene, generate fsRIDL lines in the diamondback moth. Analyse lines at the molecular and genetic level to confirm the locus of transgene insertion, and fidelity of the lethal phenotype.
2. Analyse fsRIDL lines biologically, assessing transgene-associated fitness costs to behaviours and phenotypes including mating competitiveness, longevity and rearing efficiency which are important in autocidal control strategies. Choose best-performing line for future studies.
3. Trial the population suppression and insecticide resistance management capabilities of the selected line in greenhouse cage trials.
4. Investigate the quinic acid inducible "Q-system" as an orthogonal alternative to the tet-off system for gene expression control in RIDL.


Chapter 2

Chapter 2: Developing an fsRIDL system in the diamondback moth

2.1 Introduction

fsRIDL requires a means of limiting engineered lethality to females. Previously, this has been achieved in Tephritids through the use of alternatively-spliced intronic sequences from the sex-determination gene, *transformer* (*tra*). Amongst the wide array of genetic mechanisms used to determine sex in insects, the roles of *tra*, and its downstream target *doublesex* (*dsx*), are very highly conserved. In general, upstream signals induce the expression of *tra,* which is functionally spliced in females only. Tra protein subsequently induces female-specific splicing of *dsx*, leading to a female sex-determination cascade. In the Lepidoptera, however, no *tra* homologue has yet been found (Nagaraju et al., 2014). A *transformer2* (*tra2*) homologue has been identified in the domesticated silkworm *Bombyx mori* L. but unlike in *Drosophila*, it appears to play no role in the sex-determination pathway. Instead, in male lepidopterans, masculinizer (*masc*) protein from the Z sex chromosome induces the male-specific splicing of *dsx,* which then initiates the male sex-determination cascade. Females prevent this from occurring through the *feminizer* (*fem*) gene found on the female-specific W sex chromosome. *fem* mRNA cleaves *masc* pre-mRNA and thus dsx protein is expressed in its female-specific isoform (Ohbayashi et al., 2001; Suzuki et al., 2001; Suzuki et al., 2008; Shukla and Nagaraju, 2010; Kiuchi et al., 2014).

With the goal of creating an fsRIDL system in lepidopterans, the pink bollworm *Pectinophora gossypiella* (Saunders) homologue of *dsx* (*Pgdsx*) was

identified, cloned and its sex-alternate splicing characterised (Jin et al., 2013). As lepidopteran *dsx* mRNA is constitutively female, this allowed the design of an fsRIDL system where tTAV was excised in male transcripts, rather than simply untranslated as in the analogous system used in Tephritids (Fu et al., 2007). Previous work has identified the importance of the second female-specific exon in correct splicing of lepidopteran *dsx* mRNA (Suzuki et al., 2008). A highly conserved sequence within this exon (TTAATAATATAAGTGGTGTA), known as 'CE1', has been suggested as the binding site for the splicing repressor *Bmpsi*, a homologue of the *Drosophila P*-element somatic inhibitor, *PSI*. In males, *Bmpsi* binds to CE1, which prevents splicing machinery from encountering female-specific splicing sites and causing them to "skip over" the two female exons, excising them in the process. As in other lepidopteran species, work on *Pgdsx* identified two female-specific exons. Designing fsRIDL constructs using these exons confirmed the importance of the second exon in regulating sex-alternate splicing, as any modification to it (such as the insertion of the tTAV coding sequence) caused a breakdown in correct splicing (Jin et al., 2013). Additionally, this work underlined the importance of highly conserved sequence blocks (AGTGAC/T) within the 5' intron of female exon 2 which, when removed, also caused abnormal splicing. For these reasons, the upstream female exon (exon 1) was chosen as the site for tTAV sequence insertion and exon 2 was retained within the construct (Figure 2.1).

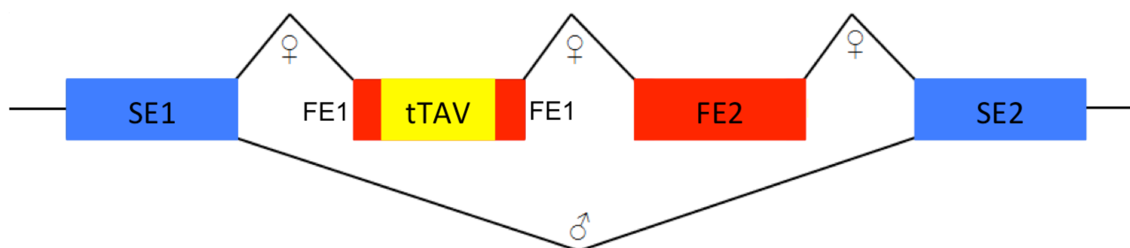


Figure 2.1: Simplified representation of pink bollworm *doublesex* (*Pgdsx*) within the OX4319 minigene construct. Exons appearing in both male and female mRNA transcripts are blue (shared exon, SE); female-specific exons (FE) are red (excised in males). Introns are indicated by peaked lines with the sex-specific nature of each intron displayed. Numbers represent the 5'-3' direction in the endogenous *dsx* gene. Sex-alternative splicing of *Pgdsx* pre-mRNA results in tTAV being translated in females only.

Various permutations of this modified *Pgdsx* gene and the *tetO* binding site were constructed and transformed into both pink bollworm and diamondback moth. Lethality in these early lines was not well-controlled (mortality on and off tetracycline) and showed a lack of sex-specificity. A later construct in which the copy number of *tetO* repeats was reduced from 21 to seven and an extra VP16 cassette was removed, displayed promising results in terms of tetracycline repression and female-specific mortality off tetracycline. This construct was named OX4319.

Building on the success of these preliminary experiments conducted by researchers at Oxitec Ltd, the aim of the work described in this chapter was to generate an OX4319-transformed strain of diamondback moth which was suitable for the exploration of fsRIDL as a population management tool for lepidopterans. Such a strain must not only display a highly penetrant female-lethal phenotype, but this should be tightly controlled, both under permissive conditions (on tetracycline) and in males. Moreover, fitness costs associated with the transgene should be minimal. The deleterious effects of transgenesis and, specifically, of highly lethal constructs (as OX4319 is designed to be) can be substantial (Mackay et al., 1992), but may be variable

between species and transgene insertion sites, so each line must be analysed independently. The research in this chapter primarily consisted of fsRIDL line generation, molecular characterisation, and the assessment of transgene-associated fitness costs, particularly those affecting male mating competitiveness. As RIDL is a mating-based strategy, released insects must show sexual competitiveness relative to the wild males against which they will be competing. This measure of performance, along with the over-flooding ratio chosen, is the prime determinant of whether a release programme will succeed, and whether it will do so economically. Other biological characteristics useful as general proxies for insect performance and pertinent to mass-rearing suitability, such as longevity and survival to adulthood, were also assessed.

These data, along with the background biology of the strain and other information relevant to gaining regulatory approval, were used to determine the appropriateness of further testing the OX4319 female-lethal system as a population control and insecticide resistance management strategy.

2.2 Results and Discussion

2.2.1 Transformation of diamondback moth with OX4319

A total of 1480 wild-type diamondback moth embryos were micro-injected with the OX4319 construct using a micro-manipulator 5171 (Eppendorf – Hamburg, Germany). From these, 610 surviving G_0 pupae were collected. Subsequent crosses of these G_0 individuals yielded one male transformant, which (after out-crossing to wild-type females) established the line OX4319L (Figure 2.2).

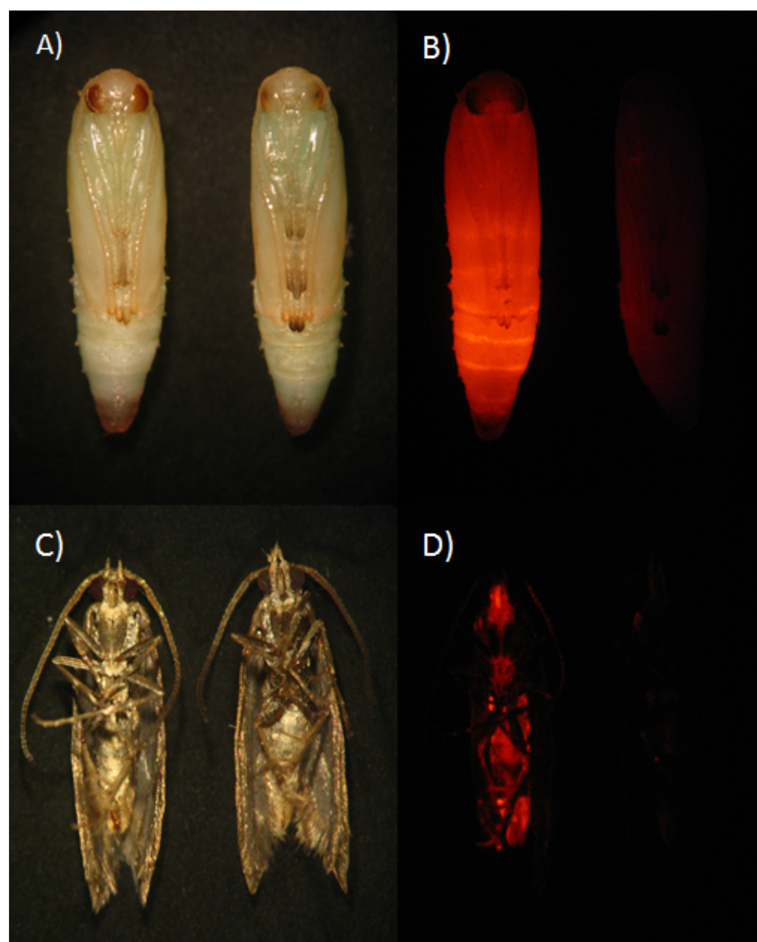


Figure 2.2: Photographs of diamondback moth pupae (A and B) and adults (C and D) taken under bright field (A and C) and red fluorescent protein excitation filters (B and D). In each panel, the left-hand individual is transgenic (OX4319L) and the individual on the right is wild-type. Under bright field (normal light), OX4319L is indistinguishable from wild-type. However, when exposed to the correct wavelength of light, DsRed2 protein in the transgenic individuals fluoresces red, allowing accurate identification of transgenic individuals.

2.2.2 Characterisation of the female-lethal phenotype

The fsRIDL phenotype in OX4319L was analysed in two test crosses. For fsRIDL lines to be appropriate for population control, female-specific lethality should be highly penetrant off tetracycline and highly suppressed on tetracycline, thus these crosses were designed to identify large deviations from the expected results. The fsRIDL phenotype was analysed in more detail in later experiments.

2.2.2.1 Test cross 1

In this cross OX4319L-heterozygous males and wild-type homozygous females were crossed. Resulting larvae were reared on either tetracycline or non-tetracycline diet. Progeny were scored for deviations from Mendelian inheritance ratios.

Results:

For an autosomal, dominant, non-deleterious gene showing Mendelian inheritance, we would expect this test cross to give a 1:1 ratio of transgenic:wild-type progeny in both sexes. For females on tetracycline, the number of individuals surviving to adulthood observed did not differ significantly from this expected ratio (Figure 2.3) ($G = 0.068$, $n = 526$, $p = 0.794$). For males, however, the observed number was significantly lower than expected on tetracycline ($G = 6.637$, $n = 508$, $p = 0.009$) but not significantly different off tetracycline ($G = 0.742$, $n = 652$ $p = 0.389$). These results are interesting as we might expect that if there were substantial fitness costs associated with a tetracycline-repressible female-lethal system, they would be greatest in females and in males off tetracycline (where the system is unrepressed). It is possible that the deviation from expected results in the male cohort (on tetracycline) was therefore an artefact of

rearing or starting conditions rather than a deleterious consequence of transgenesis. From this preliminary assessment it appeared that the transgene (in its heterozygous state) is relatively well-repressed by tetracycline in the larval diet. Also, as the transgenic:wild-type survival ratios were approximately 1:1 it is likely that only a single insertion event had occurred (multiple insertions would bias the segregation ratio in favour of the transgene). Off tetracycline, female survival was close to 0%. This implies that, in this line, the sex-specific nature of tTAV expression conferred by the *Pgdsx* minigene in OX4319 is functioning as designed.

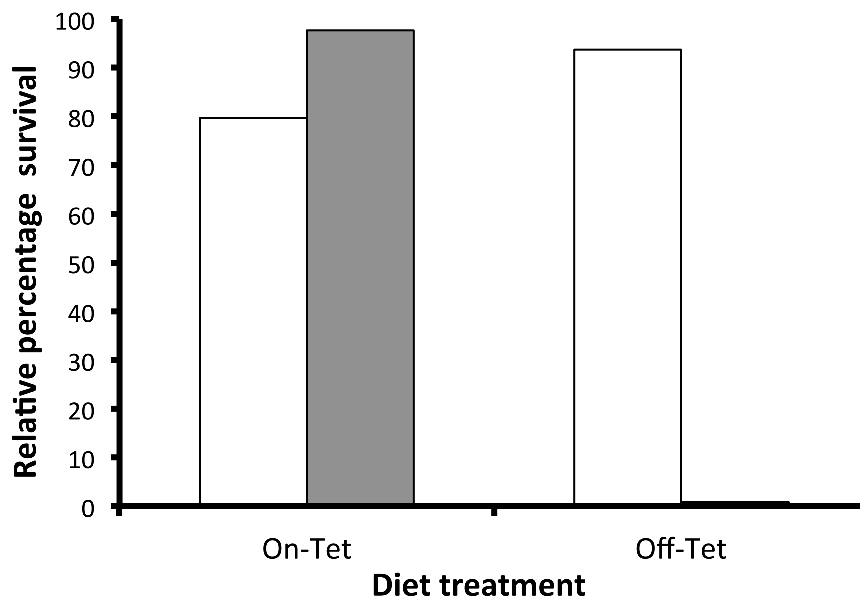


Figure 2.3: Results of test cross 1 (OX4319L heterozygous ♂ × wild-type homozygous ♀). Results are cumulative results from each transgenic cohort relative to its wild-type cohort. Males are represented in white, females in grey. On tetracycline, both transgenic sexes showed high relative survival (79.7% and 97.7%). Off tetracycline, male relative survival remained high (93.7%), however, female relative survival was very low (0.9%).

2.2.2.2 Test cross 2

In this test cross OX4319L-heterozygous males and OX4319L-heterozygous females were crossed together and their progeny reared on tetracycline diet.

Results:

Given an autosomal, dominant, non-deleterious gene showing Mendelian inheritance, we would expect this test cross to give a 1:2:1 genotype ratio and a 3:1 phenotype ratio. Thus, as 25% of the F_1 progeny in this test cross would be expected to be transgenic homozygotes, a reduction from the 3:1 phenotype ratio may imply a potential fitness cost of the transgene in its homozygous state. This is important as released RIDL insects are required to be homozygous for their insertion. At 71.3% of the total cohort, the percentage of transgenics was significantly less than expected (G = 4.16, n = 574, p = 0.041) (Figure 2.4).

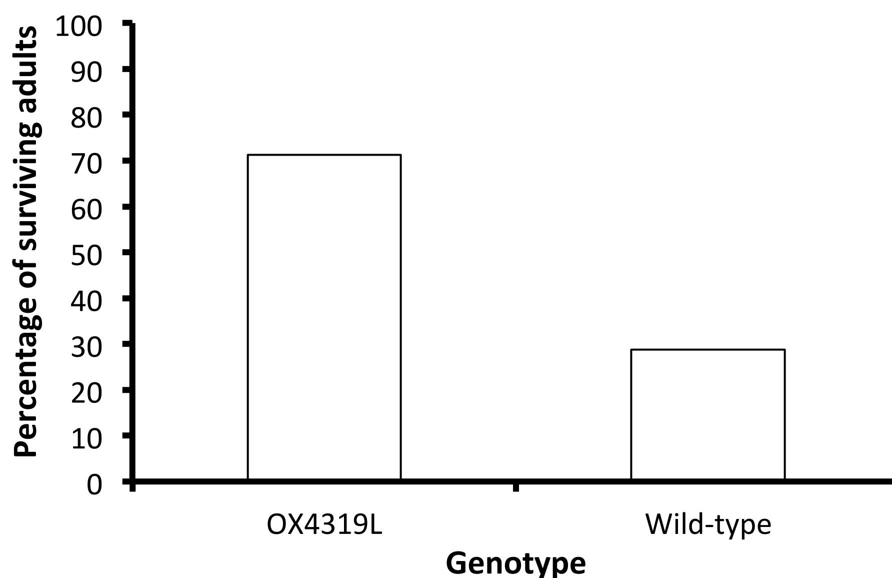


Figure 2.4: Results of Test Cross 2 (OX4319L heterozygous ♂ × OX4319L heterozygous ♀). Of a total of 574 F1 progeny, 71.3% were transgenic and 28.7% were wild-type.

However, this minor reduction in viability (3.7%) was not considered large enough to preclude further investigation of the line at this stage.

Taken together, these preliminary experiments signal that OX4319L displays the fundamental characteristics required of an effective fsRIDL line. That is, highly penetrant female lethality off-tetracycline, and relatively well-repressed lethality on-tetracycline. The line was therefore selected (along with OX4319N injected by a colleague, T. Marubbi) as a candidate for more detailed analysis.

2.2.3 Investigating the evolutionary conservation of *dsx* in Lepidoptera

The function of *dsx* in arthropods is almost universally conserved. The exception to this is the Lepidoptera, where *dsx* appears to play a converse role in sex-determination; constitutively inducing female development as opposed to male. However, investigations into the role of *dsx* and its up/down-stream partners have only recently begun in this order. Much of the research into this has been carried out on model organisms such as the silkworm, *Bombyx mori,* and it is unclear to what extent the unique function of this complexly regulated gene is conserved among the Lepidoptera. However, the OX4319 construct allows us to assess this. Accurate alternative-splicing of *dsx* pre-mRNA is dependent on both the correct splicing sequences present in the DNA and their recognition by endogenous splicing machinery. Both of these may have diverged over evolutionary time periods. In pink bollworm, the sex-alternate splicing of *Pgdsx* has been characterised in its endogenous state as well as when used artificially to drive fsRIDL (Jin et al., 2013). In this case, both splicing sequences and machinery are in taxonomic agreement. In OX4319L, however, the *dsx* DNA sequence is conserved with that in pink bollworm OX4319 lines (as the same minigene was transformed into each species) but the conservation of splicing machinery is unknown. Comparing the splicing of the OX4319 minigene in pink bollworm and diamondback moth fsRIDL lines thus allows investigation of conservation of function in *dsx* splicing machinery between these two relatively distantly related lepidopteran families (Gelechiidae and Yponomeutidae).

In addition, the recent publication of the diamondback moth genome (You et al., 2013) and the identification of coding and non/coding sequences within *dsx* important

for its correct splicing (Jin et al., 2013; Nagaraju et al., 2014) allow a comparative analysis of these highly important genomic areas across a large phylogenetic distance.

Results:

Analysis at the DNA level showed a high level of sequence conservation between the lepidopteran species assessed. In the diamondback moth, the repeated sequence blocks (AGTGAC/T) in the second female-specific intron previously found by Jin et al. (2013) to be highly conserved between silkworm, codling moth and pink bollworm, and vital for *dsx* splicing, were present. Additionally, analysis of the putative CE1 splicing suppressor binding site present on female-specific exon 2 (Nagaraju et al., 2014) showed a high level of homology between those species compared (Figure 2.5).

```
        5′                  3′
AA      TTAATAATATAAGTGGTGTA
AM      TTAATAATATAAGTGGTGTA
BMX     TTAATAATATAAGTGGTGTA
OS      TTAATAATATAAGTGGTGTA
PBW     TTAATAATATAAGTGGTGTA
DBM     TAAATAACATTAGTGGTGTA
CM      TAAATAATTTTAGTGGTGCT
```

Figure 2.5: DNA sequences from the 20-nt CE1 putative splicing suppressor region in the second female specific exon of lepidopteran *dsx*. Species are as follows; AA = Indian Golden Silkmoth *Antheraea assama*, AM = Tusseh Silkmoth *Antheraea mylitta*, BMX = Silkworm *Bombyx mori*, OS = Adzuki Bean Borer *Ostrinia scapulalis,* PBW = Pink Bollworm *Pectinophora gossypiella*, DBM = Diamondback Moth, *Plutella xylostella*, CM = Codling Moth *Cydia pomonella*. AA and AM sequences from (Shukla and Nagaraju, 2010), BMX, PBW and CM sequences from (Jin et al., 2013), OS sequence from (Sugimoto et al., 2010) DBM sequence from (You et al., 2013).

Where sequence differences do occur they appeared to concur with phylogenetic distance (Figure 2.6). This finding is important as previous research has implied high conservation of this sequence across lepidopteran families (Nagaraju et al., 2014).

However, while these authors contest that their sample group (AA, AM, BMX and OS) represents a large phylogenetic distance, most are relatively closely related within the Macroheterocera (the macro-moths) and their out-group, the Pyraloidea. With the inclusion of representatives from more distantly related families such as the Torticidae (codling moth) and Yponomeutidae (diamondback moth), it can be seen that sequence divergences have occurred over time, even in this supposedly highly conserved region.

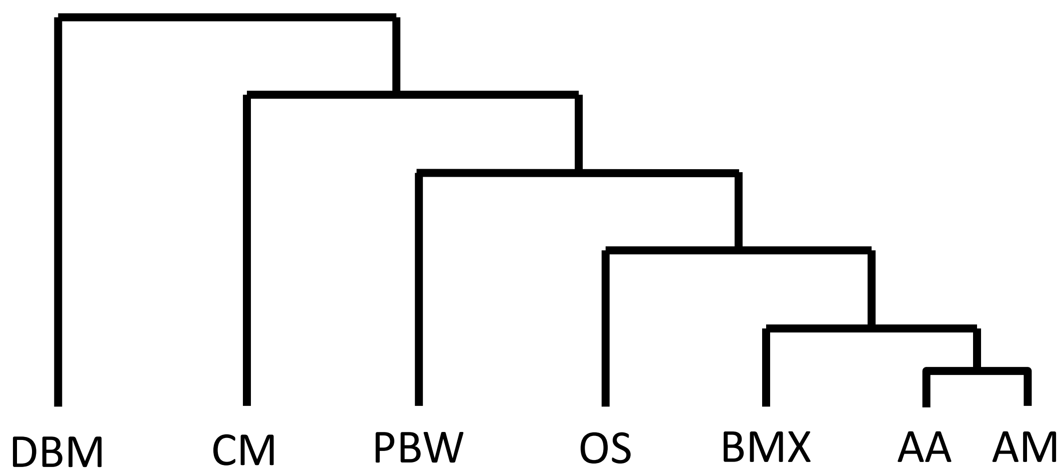


Figure 2.6: Simplified phylogeny (branch lengths not drawn to scale) showing the evolutionary relationships between species compared in Figure 2.5. Families are as follows; AA and AM = Saturniidae, BMX = Bombycidae. Both of these families are classified within the superfamily Bombycoidea. OS = Crambidae, PBW = Gelechiidae, CM = Tortricidae, DBM = Yponomeutidae. Data extracted from (Regier et al., 2013).

However, despite these divergences in DNA sequence, sex-specific splicing of the *Pgdsx* minigene in diamondback moth OX4319L was as expected, given our knowledge of *dsx* splicing in pink bollworm. Two-step RT-PCR using primers specific for the outermost *Pgdsx* exons (SE1 and SE2, Figure 2.1) amplified bands of the appropriate size in males (c. 200 bp) and females (c. 1.5 kb) (Figure 2.7). Female fragments are larger due to the inclusion of one or more female-specific exons as well as the tTAV coding sequence. Once these bands were sequenced (with the aid of a

colleague, Dr C. Harris) it was found that males produced a single transcript while multiple, larger, splice variants were present in females. This was expected as endogenous *Pgdsx* shows multiple splice variants in females. The single male (235 bp) and most prominent female (1,551 bp) transcript were found to match those previously amplified in pink bollworm OX4319 strains (Appendix Figure A.2).

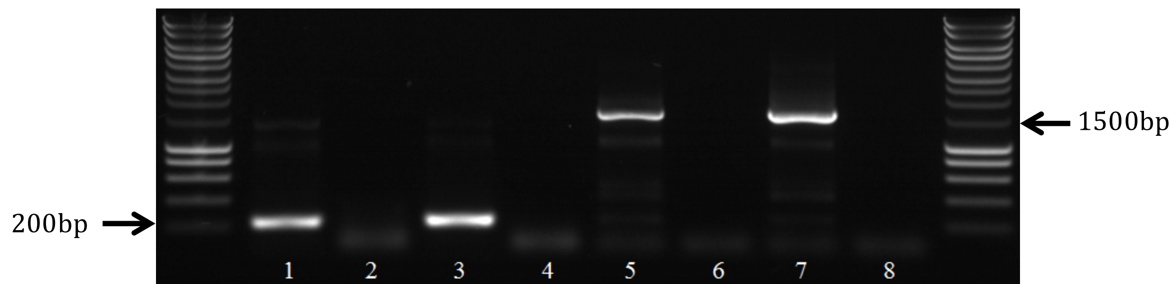


Figure 2.7: Gel photograph showing the products of Two-step RT-PCR on OX4319L. All lanes used the same primer combination designed to anneal to SE1 and SE2 (Figure 2.1) Lanes 1-4 used ♂ gDNA, lanes 5-8 used ♀ gDNA. Even-numbered lanes are no-RT controls.

These results show that the splicing of this *Pgdsx* minigene was maintained in both diamondback moth and pink bollworm, implying functional conservation of the *dsx* splicing machinery between these two species.

2.2.4 Characterisation of the transgene insertion locus

To characterise the OX4319L transgene insertion, the locus of insertion was identified and the endogenous areas flanking the insertion sequenced. The sequence flanking a transgene insertion site is useful in generating homozygous colonies of that line (by PCR) (Figure 2.9). In addition, as each insertion event is unique, the flanking sequence acts as a 'genetic fingerprint' for each line, enabling accurate distinction

between transgenic lines with the same central marker and acting as a redundant field monitoring tool (to differentiate by PCR between wild-type and transgenic individuals); this information is useful for characterising the line and is also likely to be required by regulators prior to field use, should this become desirable.

Results:

A preliminary PCR assay screened genomic DNA (gDNA) extracted from three OX4319L individuals for any evidence of plasmid backbone in the insertion. An antibiotic resistance gene, a hypothetical inserted remnant from the plasmid backbone, carried with the construct in a transformed strain might make future field releases with this line unacceptable from a regulatory perspective. No evidence of plasmid backbone was found in OX4319L (Figure 2.8).

Figure 2.8: Gel photograph displaying the results of a PCR screen using primers designed to amplify backbone-specific sequence. Lanes 1-3 are 3 OX4319L individuals. Lane 4 is a positive backbone control (OX4358 *Ae.aegypti* gDNA – remnants of backbone remain at the transformation locus in this line). Lane 5 is a negative backbone control (OX513 *Ae. aegypti* gDNA) and lane 6 is a H_2O control. Expected band size is 773 bp.

The endogenous areas flanking the OX4319L insertion site were then cloned through nested PCR and flanking primers within these areas designed (sequence presented in Appendix Figure A.1). These flanking primers were used to select OX4319L-homozygotes to found a homozygous colony (Figures 2.9 and 2.10).

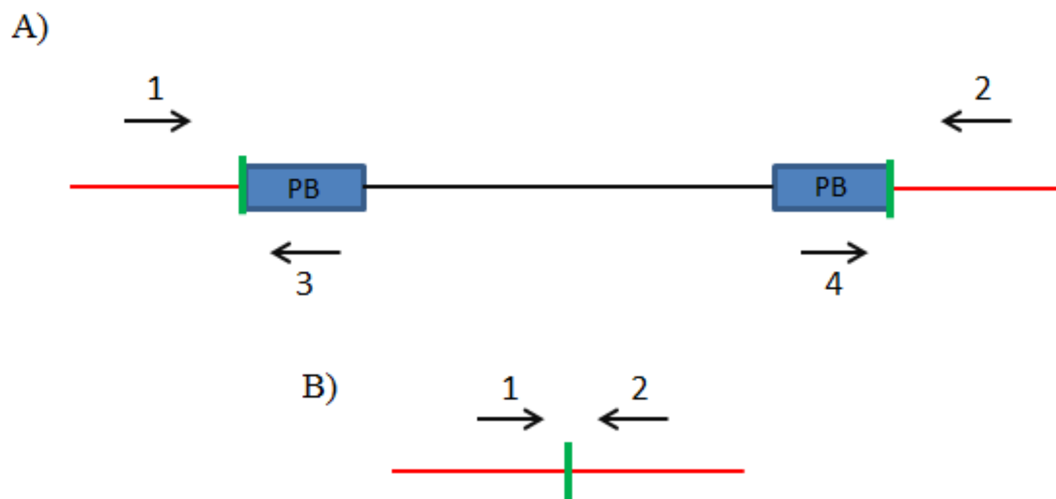


Figure 2.9: Schematic illustrating how flanking and *piggyBac* primers can be used to distinguish between different genotypes. Endogenous flanking sequences are shown as red lines, inserted construct sequence in black. *piggyBac* transposon regions (PB) are shown as blue boxes. Endogenous *piggyBac* recognition site (ttaa) is represented with a vertical green line. Primers are displayed as arrows with numbers representing different primers. A transgenic homozygote would have two copies of A), a heterozygote one copy each of A) and B) and a wild-type homozygote two copies of B). As the flanking primers (1 and 2) are not able to amplify across the entire insertion (the distance is too great), the absence of an amplified band when these primers are used indicates the presence of a transgene homozygote. Similarly, the presence of an amplified band when a flanking primer (1 or 2) is used in combination with a construct-specific *piggyBac* primer (3 or 4) indicates that at least one copy of the transgene allele is present. By performing two PCRs, one with the flanking primers and one with either of these primers and its complementary *piggyBac* primer, OX4319L-homozygous, OX4319L-heterozygous and wild-type homozygous individuals can be distinguished.

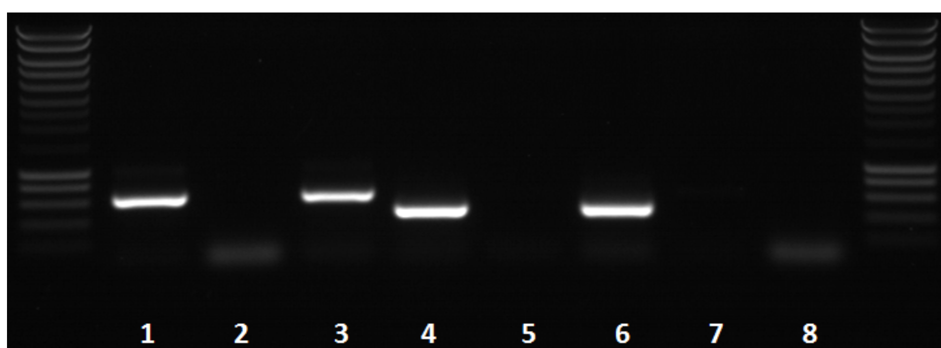


Figure 2.10: Gel photograph showing the distinction by PCR between transgenic genotypes and wild-type using PCR. Lanes 1 and 2 are DNA extracted from OX4319L-homozygous insects, 3 and 4 are OX4319L-heterozygotes, 5 and 6 are wild-type, 7 and 8 are no-template controls. Lanes 1, 3, 5 and 7 used the OX4319L 5′ flanking primer and a 5′ *piggyBac* primer, lanes 2, 4, 6 and 8 used the OX4319L 5′ and 3′ flanking primers. PCRs using the *piggyBac*-specific primer only amplified fragments in transgenic individuals, whereas combining the two flanking primers only produced amplicon in the heterozygote and wild-type.

Previous attempts to create homozygous colonies in this way with mosquitoes have been hindered by issues with polymorphic wild-type sequence in the area of insertion, resulting in potential non-amplification of some wild-type alleles with available 'wild-type' primers. To circumvent this potential issue, before the colony was PCR-screened for homozygotes, it was derived from heterozygotes in which the wild-type locus could be PCR-amplified (individuals from this line termed OX4319L-A). In this way, it was made certain that the only wild-type allele present in the next round of screening (for homozygotes) would amplify by PCR using the available flanking primers.

A total of 162 individuals were taken to form OX4319L-A. These were crossed and the resulting progeny screened for transgene homozygosity. 70 of these homozygotes were taken to form OX4319L-B. The sex ratio of collections made from OX4319L-B was monitored over two generations, with evidence of a substantial departure from an assumed 50:50 sex ratio taken as an indication of a fitness cost in OX4319L homozygous females. An overall ratio of 54:46 was observed, not significantly different to the expected ($G = 1.33$, $n = 217$, $p = 0.248$). From this point onwards, OX4319L refers to the line homozygous for the transgenic insertion, unless otherwise stated.

At this point, homozygous colonies for two OX4319 strains - OX4319L and OX4319N - were held in the laboratory. In order to determine which of these lines to take forward for further investigation they were compared in a series of experiments assessing any potential transgene-associated fitness costs which may compromise their performance in the field and/or suitability for mass-rearing.

2.2.5 Comparing fsRIDL phenotypes as homozygotes

All OX4319 lines possess identical transgene sequences as they have been transformed with identical constructs. However, the phenotype and fitness characteristics of different lines carrying the same construct will each be unique due to their independent transgene insertion sites (positional effects) (Lyman et al., 1996; Scolari et al., 2008; Ant et al., 2012; Jin et al., 2013; Yonemura et al., 2013). For example, transgenesis may confer a selective disadvantage to carriers through reduced survivability and a lower level of sexual competitiveness (Woodruff, 1992; Woodruff et al., 1999; Pasyukova et al., 2004). However, the magnitude of these effects will depend on factors including the level of coding sequence disruption at the locus of transposition (insertional mutagenesis) (Cooley et al., 1988), the presence and identity of nearby regulatory sequences (e.g. enhancer traps) (Bellen et al., 1989; Uchino et al., 2008), or the conformation of surrounding genomic architecture (e.g. heterochromatin silencing and gene modifiers) (Horn et al., 2002; Ahrens and Devlin, 2011). In the case of protein-coding transgenes such as OX4319, the levels and characteristics of transgene expression (e.g. temporal and/or spatial expression profiles) may also be affected by these latter points. Comparison between candidate lines was therefore necessary, in order to assess the degree to which positional effects were evident and to select the fittest line.

Both OX4319L and OX4319N had previously shown tightly controlled tetracycline-repressible, female-specific lethality in transgene-heterozygotes. However, the fitness costs associated with insect transgenesis may be exacerbated if two copies of the transgene are present, for example if fitness costs are recessive (Mackay et al., 1992). As fsRIDL moths will eventually be reared and ultimately, compete for females,

as homozygotes (at least in the generation of release) the rearing characteristics, survival and longevity of this genotype were compared between the two lines.

2.2.5.1 Survival to adulthood and rearing characteristics

Autocidal release strategies such as RIDL require the production of large numbers of insects. Diet and the associated economic cost of rearing these insects contribute significantly to the costs of a programme. Any fitness costs placed on these insects which impacts on their productiveness can therefore have major impacts on the economics of a release programme. As such, these experiments set out to compare the rearing characteristics of the two lines in their homozygous states. Samples of eggs from each homozygous transgenic line (OX4319L and OX4319N) were reared in synchrony with wild-type on and off tetracycline and proportional survival recorded.

Results:

Data from this experiment was first used to assess the fsRIDL phenotype of the construct, as in Test Cross 1 (Figure 2.11).

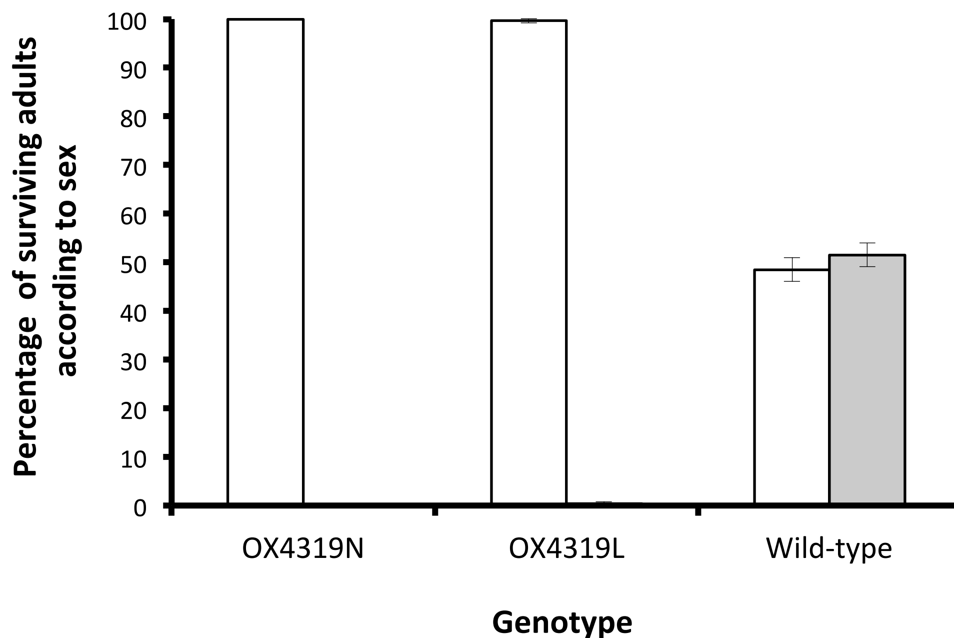


Figure 2.11: Graph showing the sex ratio (as a percentage) of homozygous individuals which survived to adulthood from three strains when reared off-tetracycline. Males are represented in white, females in grey. 100% of adult survivors in OX4319N were male. In OX4319L 99.7 ± 0.456% of adult survivors were males. In wild type, 48.7± 2.45% of adult survivors were male. Error bars = 1 S.E of the mean.

As expected, female lethality remained high when larvae were reared in the absence of tetracycline. For OX4319L, this effect was more pronounced than in Test Cross 1 (0.38% and 0.94% survival, respectively), possibly due to the presence of a second copy of the transgene allele. The wild-type control sex ratio was c. 1:1.

Once the baseline lethality of the homozygous lines had been confirmed, the data was used to investigate the overall viability of the lines. This first took the form of

comparing total survival of each line and wild-type when reared on tetracycline (Figure 2.12).

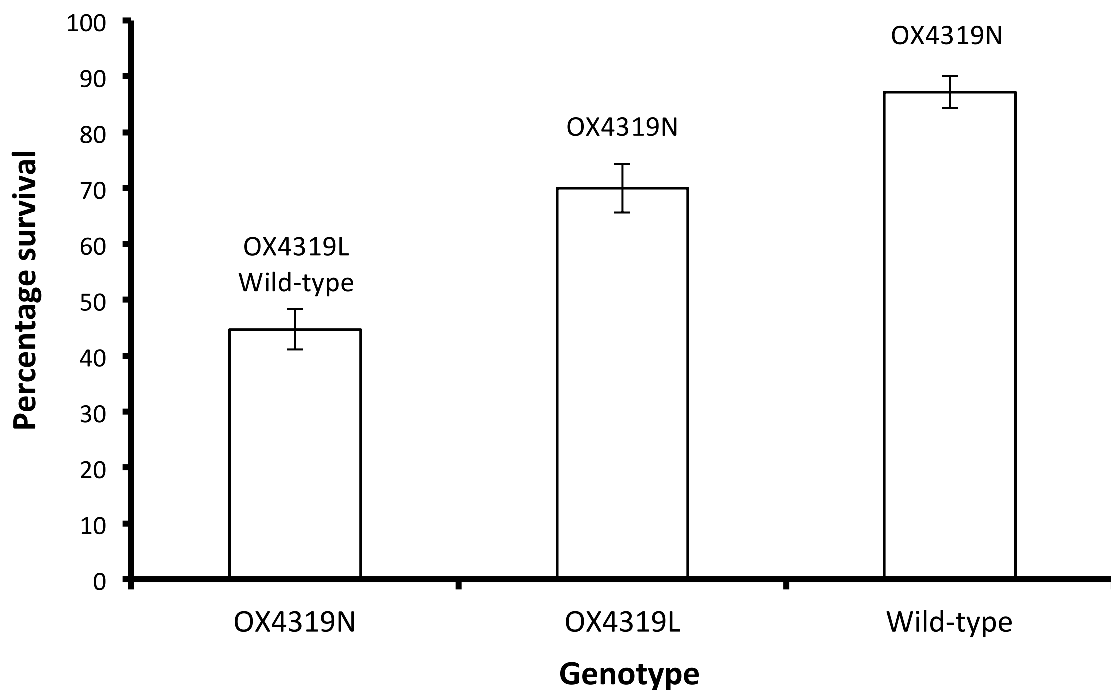


Figure 2.12: Graph showing average percentage survival to adulthood of three strains reared on tetracycline (mixed males and females). Lines are significantly different to those labelled above their column. OX4319N line survival = 49.7 ± 3.59 %; OX4319L line survival = 69.9 ± 4.35%; and wild-type survival = 87.1 ± 2.86%. Error bars = 1 S.E. of the mean.

OX4319N showed significantly reduced survival relative to both OX4319L ($Z = -3.08$, $p<0.001$) and wild-type ($Z = 4.74$, $p<0.001$). OX4319L survival, however, was not significantly different relative to that of wild-type ($Z = 2.16$, $p = 0.0766$). When corrected for control mortality (Abbott, 1925), OX4319N and OX4319L mean survival was calculated as 51.3% and 80.0%, respectively. These figures highlight the superior productivity, in terms of rearing, of OX4319L relative to OX4319N. The reduced survival of the transgenic lines, relative to wild-type, is possibly a result of the fsRIDL system not being fully repressed, leading to basal expression of tTAV and/or a fitness

cost associated with the transgene insertion site. Interestingly, OX4319N appears to suffer significantly greater fitness costs from these potential effects than OX4319L. This finding fits well with the previous data showing increased lethality in OX4319N females compared with OX4319L females when reared off-tetracycline. It is possible that, due to positional effects, the overall level of tTAV expression in OX4319N is higher than in OX4319L. Thus, when de-repressed, more tTAV is produced in OX4319N (leading to a higher proportion of female lethality) but conversely, when reared on tetracycline, a higher level of basal expression remains, leading to a reduced survival to adulthood overall. To test this hypothesis, the sex ratios of the strains, when reared on tetracycline, were compared (Figure 2.13).

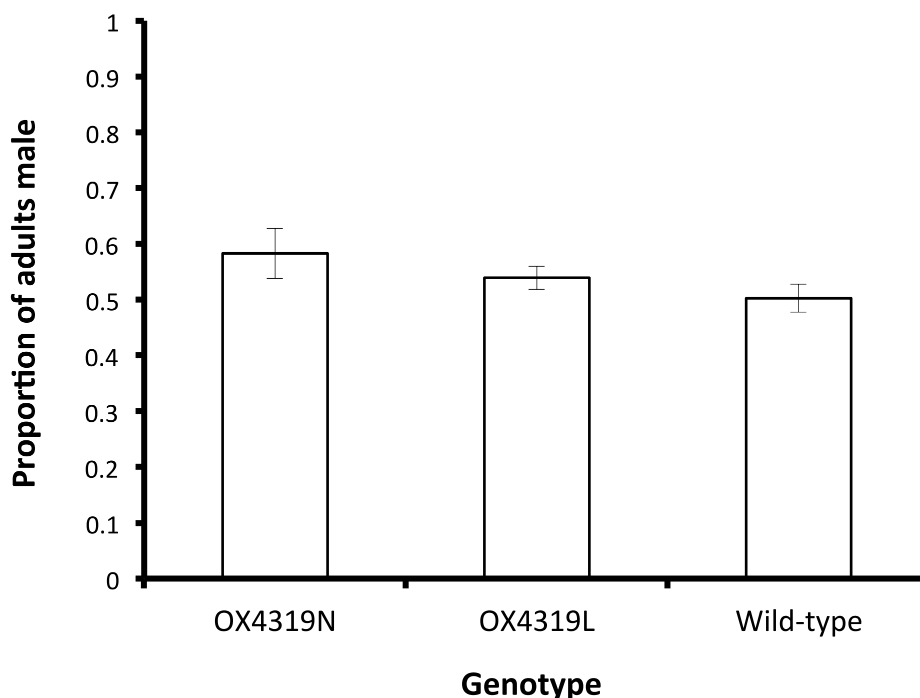


Figure 2.13: Graph showing the average proportion of homozygous adults surviving from three lines reared on tetracycline) which were male (♂ / total survivors). Average sex ratio for OX4319N is 0.583 ± 0.0451, for OX4319L is 0.539 ± 0.0207 and for wild type is 0.503 ± 0.0253. Error bars = 1 S.E. of the mean.

If the reduced survival to adulthood of the transgenic lines shown in Figure 2.13 were as a result of 'leakiness' in the tet-off system, and specifically in OX4319N, we would expect this to have a greater detrimental effect on female survival than male survival. While the mean sex ratios of the transgenic lines were slightly higher than wild-type these differences were not statistically significant, and do not fully explain the large, significant differences in survival to adulthood shown in Figure 2.12. As such, it is not only female transgenic survival which is being affected in these transgenic lines. Previous research (conducted by colleagues, unpublished) has shown that the sex-alternate splicing of OX4319 is not 100% sex-specific and, therefore, some tTAV translation does occur in males. Basal leakiness of the tet-off system may therefore also be responsible for reduced male survival. In addition, it is likely that the underlying genetics of each strain (positional effects of the insertion) are playing a large part in determining strain fitness.

In a final analysis, data from these experiments was used to estimate the impact of rearing off tetracycline on the males in each transgenic line, which would indicate their ability to produce adult males for a release programme. As female survival is close to zero in both transgenic lines when reared off tetracycline (Figure 2.11), the number of surviving females cannot be used to evaluate male survival (as in the on-tetracycline experiment – Figure 2.13). Instead, male survival was estimated by applying the average sex ratio observed in the off-tetracycline wild-type control replicates to the total number of first instar larvae known to have been in each transgenic off tetracycline replicate. This gave an estimate of the number of males which were placed into each diet cup at the beginning of the experiment. Using this value and the number of males known to have survived to adulthood in each cup, proportional survival of males off tetracycline could be estimated for each line (Figure 2.14).

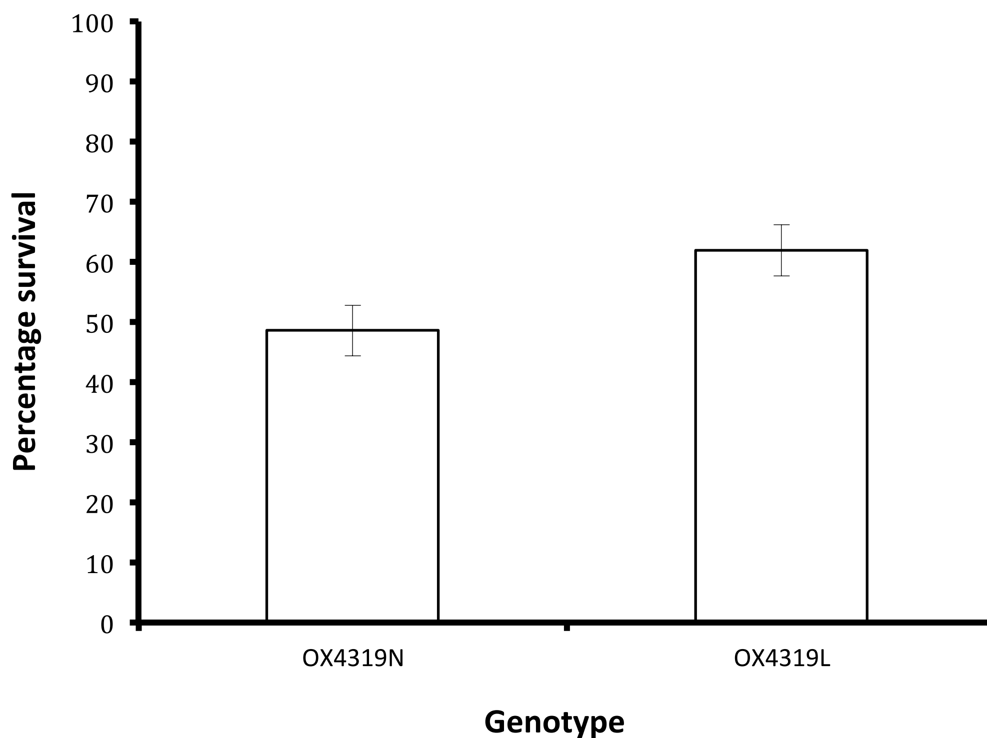


Figure 2.14: Graph showing the estimated mean survival of males to adulthood for two transgenic lines when reared off tetracycline. Survival in OX4319N was 48.6 ± 4.22%. Survival in OX4319L was 61.9 ± 4.26%. The two lines differed significantly. Error bars = 1 S.E. of the mean.

Male survival appears to be reduced, off tetracycline. This is not unexpected, considering the previous results on tetracycline as well as the lack of 100% splicing fidelity. When corrected for wild-type control mortality, mean OX4319N male survival was 66.6% and that of OX4319L was 84.8%. OX4319L male survival was significantly higher than that of OX4319N ($t = -2.607$, $p = 0.0184$). While these figures are not low enough to cause concern if these lines were potentially mass-reared (especially in the case of OX4319L), they agree with the results of previous experiments indicating incomplete fidelity of the *Pgdsx* minigene sex-specific splicing and presence of basal expression and/or deleterious insertion effects.

2.2.5.2 Longevity

Adult longevity is often used as an indicator of insect fitness (Saeed et al., 2010; Hasan and Ansari, 2011) and competitiveness in SIT programmes (Barry et al., 2007; San Andres et al., 2007; Gavriel et al., 2010). This is particularly important for species including lepidopterans which mate multiply, and where these matings may take place over a period of time. As such, the longevity of homozygous males from OX4319L and OX4319N was investigated on and off tetracycline and compared to their wild-type background. Longevity of individuals off tetracycline will impact their ability to compete over time in the field while longevity of the lines on tetracycline may provide insight into the results of the previous experiment.

2.2.5.2.1 On tetracycline

Results:

Due to the negative effects on survival to adulthood associated with transgenesis in the previous section, we might have expected reduced adult longevity in the transgenic lines, even on tetracycline. However, this was not the case. Survival curves generated for each strain differed very little (Figure 2.15). Mortality in each strain was low until around day 20, after which it increased dramatically, resulting in death of all individuals by day 41. Covariates included in the model were pupal weight of each insect, and genotype (wild-type, OX4319N and OX4319L). Model simplification resulted in no parameters being retained. Therefore, neither weight, nor genotype, nor the interactions between these two variables, had a significant explanatory effect on male adult survivorship. It is possible that competition with other larvae in the previous rearing experiments exacerbated any small fitness costs present, whereas in these

experiments, individuals did not compete for resources. Additionally, it may be that the fitness costs associated with transformation in OX4319, such as tTAV toxicity, are confined to the larval stages, and thus this is the main stage of selection.

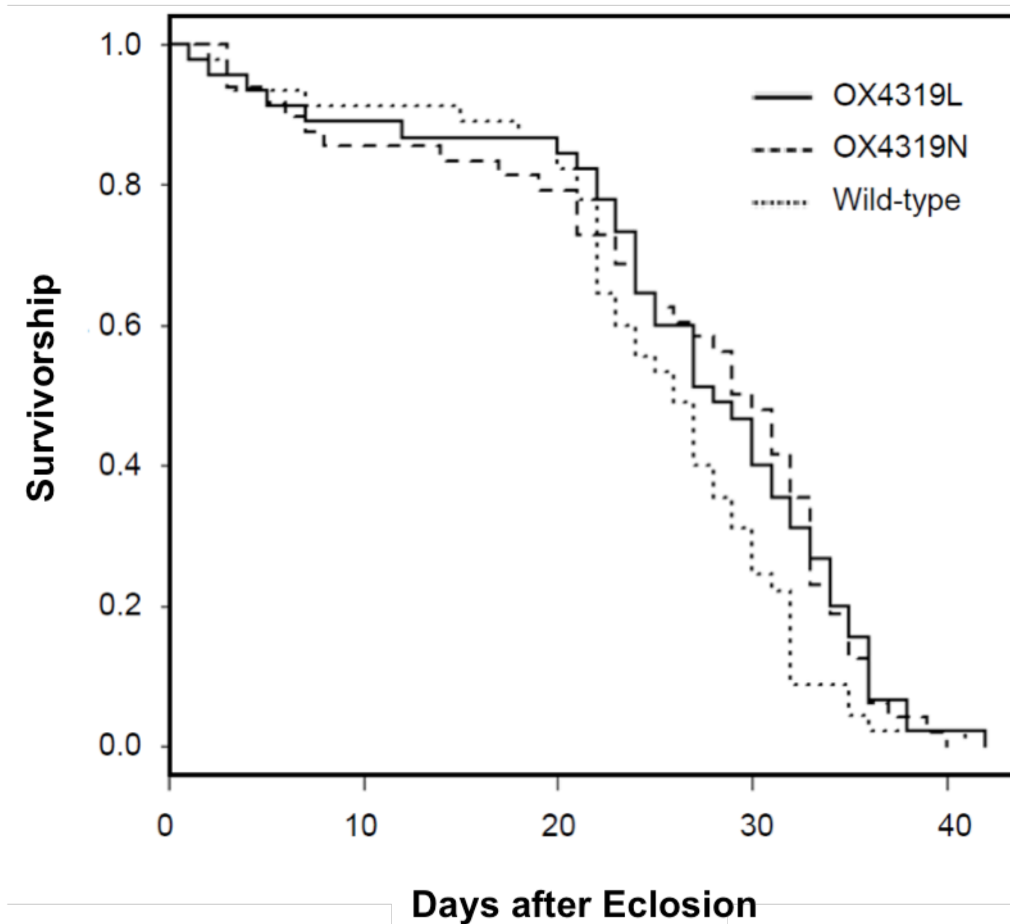


Figure 2.15: Kaplan-Meier object showing the survivorships of adult males, reared on tetracycline, from three genotypes (OX4319L, OX4319N and wild-type). Average longevity of OX4319N was 26.02 ± 1.51 days, OX4319L was 26.42 ± 1.49 days and wild type was 25.3 ± 1.79 days. Parameter estimates followed by 1 S.E. of the mean.

2.2.5.2.2 Off tetracycline

Results:

The wild-type strain displayed a similarly shaped survival curve in this experiment as in the on tetracycline experiment with mortality mainly confined to the period after day 20. However, differences between the survival curves generated for the transgenic lines when reared off tetracycline, in contrast to when reared on tetracycline, were immediately obvious (Figure 2.16).

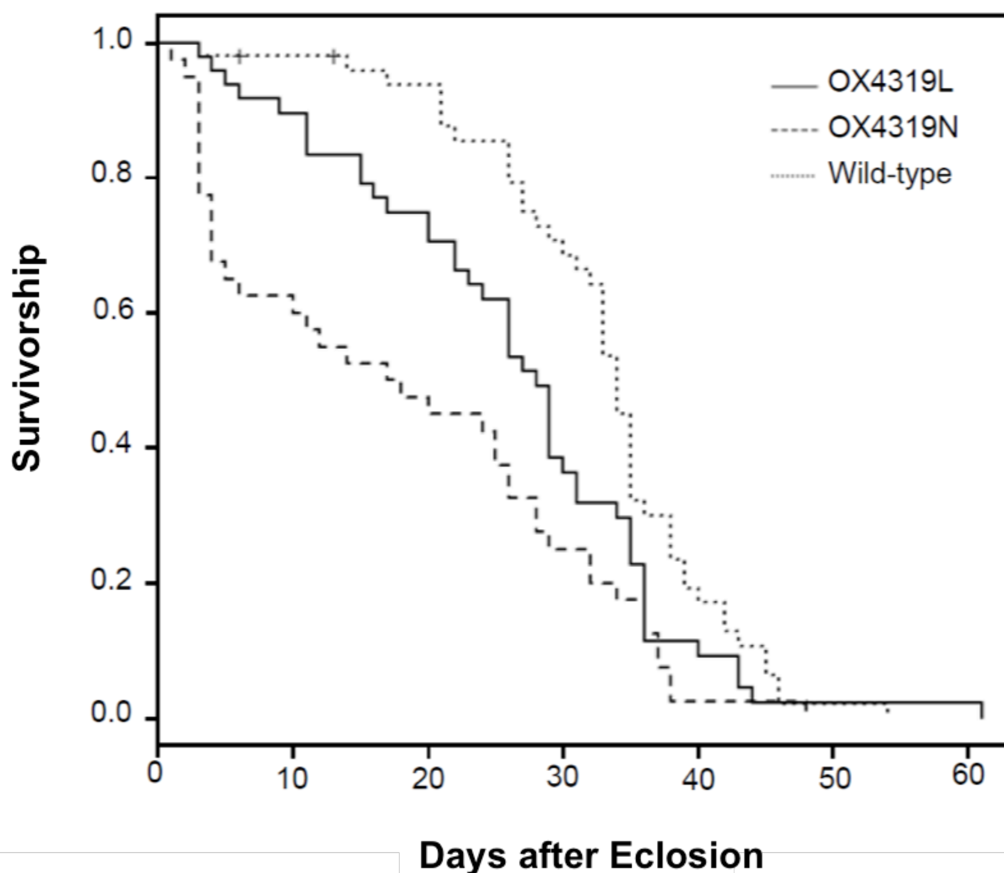


Figure 2.16: Kaplan-Meier object showing the survivorships of adult males, reared off tetracycline, from three genotypes (OX4319L, OX4319N and wild-type). Average longevity of OX4319N was 18.3 ± 2.22 days, OX4319L was 25.9 ± 1.729 days and wild-type was 31.7 ± 1.43 days. Replicate censoring symbolised by crosses on curves. Parameter estimates followed by 1 S.E. of the mean.

Prior to day 20 (approximately), both transgenic lines show an increased mortality relative to wild-type. Survivorship of OX4319L followed an almost linear trend, while that of OX4319N decreased at an above-linear rate. Although direct comparisons between the two experiments cannot be made, inferences can be drawn through statistical analysis of the transgenic lines against their wild-type controls. In a preliminary analysis, the interaction between weight and survival for OX4319N was found to be significant (Z = 2.266, d.f. = 132, p=0.0234). However, when post-hoc testing (Simultaneous Tests for General Linear Hypotheses) was used to investigate the interaction terms of the model, this significance vanished, presumably due to the correction factor applied. Disregarding the weight variable, the main effects of the model were analysed using the same post-hoc testing method. It was found that survivorship differed significantly between wild-type and OX4319N (Z = 3.692, df = 90, p < 0.001) but not OX4319L (Z = 1.59, df = 90, p = 0.249), while the difference in survivorship between OX4319N and OX4319L was not significant (Z = -2.191, df = 90, p = 0.073).

These results, taken with those of the rearing and larval survival experiments, suggested the greater fitness of OX4319L compared to OX4319N, specifically in the absence of tetracycline. This decrease in male fitness in the absence of tetracycline lends weight to the hypothesis (outlined in Section 2.2.5.1) that the *Pgdsx* cassette used in the OX4319 construct allows some leakage of female specific splice variants (and therefore tTAV) in males. When tetracycline is present it seems that this small amount of tTAV can be sufficiently suppressed to avoid significant fitness costs (at least in adults) but in the absence of tetracycline reductions in viability are apparent. Furthermore, it appears that the leakage of this system in males is greater in OX4319N than OX4319L, possibly due to transgene positional effects (for example the presence

of nearby silencing regions). As such, OX4319L was chosen as the line on which to base future investigations into this system.

2.2.6 Analysis of heterozygous fsRIDL phenotype

Analysis of the homozygous fsRIDL phenotype is important for determining mass-rearing efficiency and the penetrance of this system as a GSS. However, it is the characteristics of fsRIDL heterozygotes that will determine whether a particular line will be efficient as a means of population control (as the targets for fsRIDL selection in the field are transgene-heterozygotes). As such, the fsRIDL phenotype of OX4319L-heterozygous individuals was investigated in two experiments. These experiments sought to gain a more detailed understanding of unrepressed female-specific lethality in this line, temporally, on host plant material, and at varying concentrations of tetracycline.

2.2.6.1 Temporal investigation of fsRIDL penetrance on host plant material

Larval diet has been found to significantly impact the fitness of herbivorous insects (Awmack and Leather, 2002). Specifically, the choice of host plant can alter fitness costs associated with a range of “costly” alleles such as insecticide resistance, as well as affecting biological parameters such as growth and fecundity (Raymond et al., 2011). It has been found that artificial diets can provide a reduced quality of nutrition to insects compared with host material (Brinton et al., 1969; Greene et al., 1976), although this may not always be the case (Bailey, 1976). In the context of an fsRIDL system, the

potential increased nutritional quality provided to larvae developing on crops in the field may be problematic if it allows female heterozygotes to survive. Moreover, even if fsRIDL penetrance remains complete in the field, the economic viability of a crop protection programme may be compromised if heterozygous females survive to a late larval stage (where feeding damage increases significantly). To assess this, OX4319L-heterozygotes were reared in parallel with wild-type individuals on host plant material (Chinese leaf – *Brassica rapa*), with their survival and development characteristic recorded on a daily basis.

Results

Once corrected for control mortality, OX4319L-heterozygote mortality stood at 41%. This is less than the 50% we may have expected (given a 50:50 sex-ratio) from a female-lethal system. However, as the penetrance of OX4319L was complete (no OX4319L females survived to adulthood while the wild-type sex ratio was 41:55) this divergence from 50% is not a consequence of reduced construct effectiveness, but more likely a non-equal sex ratio in the initial larval collection. Cox Proportional Hazards analysis showed significant differences between the hazard functions of the two lines ($n = 220$, $Z = -5.566$, $p < 0.001$) (Figure 2.17). The instantaneous risk of death (the risk of dying between the specified time points, here being 24 hours) to wild-type individuals remained relatively low (not rising above 0.02 – approximately 2%), apart from a small rise to 0.04 during the eclosion period (day 11 onwards). An increase in mortality during this later period is not surprising as escape from pupal casing can be challenging. In the case of OX4319L heterozygotes, risk of death increased rapidly to a peak of 0.16 at day 6 and declined thereafter. Daily observations of individuals showed that, in many OX4319L replicates that did not survive this initial period, feeding ceased at

approximately day 3 (circa second instar) followed by a short period of wandering, then death (in concurrence with RIDL phenotypes observed previously in pink bollworm (Morrison et al., 2012)). In contrast, the small amount of mortality in wild-type individuals was more often instantaneous and due to stochastic effects, such as becoming trapped in leaf tissue. The amount of damage caused by larvae at the second instar is minimal and unlikely to significantly reduce crop quality or yield. These results concur with experiments conducted on pink bollworm RIDL lines reared on cotton bolls, where penetrance of the lethal phenotype was retained on this natural diet (Morrison et al., 2012).

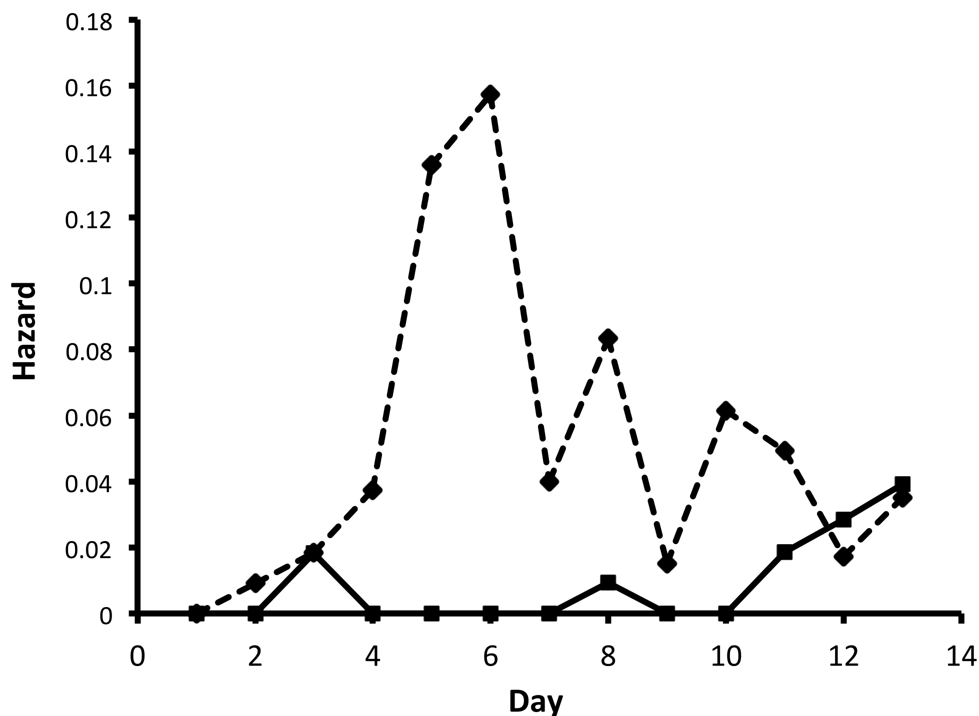


Figure 2.17: Hazard function graph showing the instantaneous risk of death of 100 heterozygote OX4319L (dashed line) and 100 wild-type (solid line) larvae reared from first instar to adult eclosion or death on Chinese cabbage leaves.

A second analysis of this data showed that the time to eclosion of OX4319L (mean 10.45 ± 0.096 days) and wild-type (mean 10.16 ± 0.11 days) males did not differ significantly (n= 110, W = 1269, p = 0.080). This is an improvement on F1 sterility in Lepidoptera, where time to adult eclosion can often be significantly delayed in the F1 generation leading to potential de-synchronisation of peak mating periods between wild and sterile moths (LaChance 1985, Bloem et al. 1999, Bloem, Carpenter & Hofmeyer 2003, Ayvaz, Albayrak & Tuncbilek 2007).

2.2.6.2 Tetracycline dose-response in heterozygotes

One of the primary concerns over the use of genetically modified insects in the field is that permissive conditions may be found, leading to unintended population persistence. In the case of RIDL this would take the form of heterozygote insects having access to environmental concentrations of tetracycline high enough to disengage engineered lethality. As tetracycline is amongst the most commonly found antibiotic contaminants this question of potential environmental exposure requires due consideration. However, as with the regulation of pesticides, the question is one of hazard and risk. While it is undeniable that environmental tetracycline could in some scenarios cause the breakdown in a RIDL control programme, the risk of this occurring will depend on the probability of insects encountering sufficiently high levels of this antibiotic. In order to evaluate this risk, a tetracycline dose-response experiment was performed on OX4319L-heterozygotes. The results of this experiment were then related to the available literature on tetracycline contamination in crucifer crops.

Results:

Relative percentage survival to adulthood of OX4319L-heterozygous males remained constant at around 80%, regardless of tetracycline dosage. This shows that in heterozygous males, fitness costs (potentially deriving from the lack of fidelity in *Pgdsx* sex-specific splicing) are not exacerbated by reductions in tetracyline (Figure 2.18), in contrast to those recorded previously for homozygous adults (Section 2.2.5.2). This was not the case with female survival. At the highest dosage (normal on-tetracycline rearing concentration for this line) no great difference was observed between the relative percentage survival of the two transgenic sexes (as assessed by 95% C.I overlap). However, a trend in reduced transgenic survival to adulthood was observed at tetracycline doses lower than this, with the highest dose at which transgenic female lethality was complete being 0.01 μg/ml

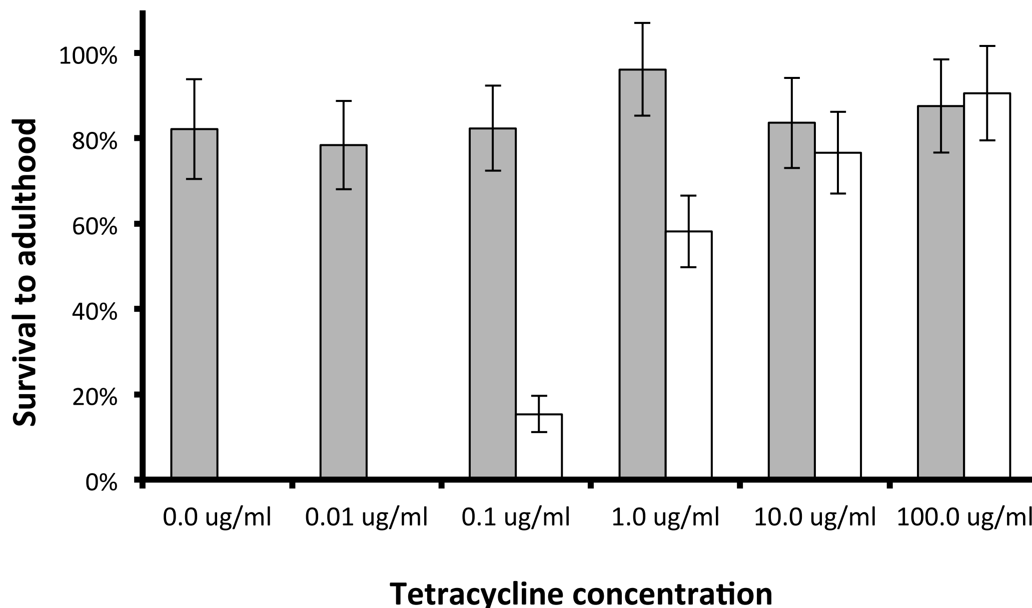


Figure 2.18: Graph showing the percentage survival to adulthood of male (grey) and female (white) OX4319L heterozygotes relative to their wild-type siblings at varying concentrations of tetracycline provisioned in the larval diet. Error bars represent 95% confidence intervals.

The literature on tetracycline uptake by crucifer crops is limited. The most detailed analysis was carried out by Kumar et al. (2005). In this study, the tetracycline uptake by cabbage crops grown in soil laden with manure from animals previously treated with this antibiotic and then spiked with tetracycline solution was measured. The highest concentration of tetracycline found in cabbage tissue was equivalent to approximately 0.0045 µg/ml. This is less than half the highest concentration found in this experiment at which no female heterozygotes survived. In addition, it is worth noting that the concentrations of tetracycline used by Kumar reached artificially high levels and uptake into plant tissue under "normal" field conditions is likely to be much lower. From this analysis it is possible to conclude that the likelihood of suppression of fsRIDL lethality in OX4319L by environmental tetracycline contamination is extremely low.

2.2.7 Transgene-associated fitness costs

The success of autocidal approaches such as RIDL depends on the field performance of engineered insects, especially in terms of finding and mating with wild counterparts. Additionally, the fitness of a transgenic strain will impact on its productivity and mass-rearing efficiency, with potential economic consequences. Estimation of fitness costs associated with transgenic insect strains, particularly those affecting mating performance and productivity, has therefore become an area of considerable research interest (Catteruccia et al., 2003; Irvin et al., 2004; Moreira et al., 2004; Marrelli et al., 2006; Marrelli et al., 2007; Lambrechts et al., 2008; Scolari et al., 2008; White et al., 2010; Harris et al., 2011a; Harris et al., 2012; Massonnet-Bruneel et al., 2013; Paton et al., 2013). In any transgenic organism, fitness can be negatively

affected by several factors. These may include expression of the transgene sequence, insertional mutagenic effects of the transgene insertion site, and inbreeding depression, genetic drift or selection related to laboratory adaptation and rearing (Cooley, Kelley, and Spradling 1988; Bellen et al. 1989; Horn et al. 2002; Uchino et al. 2008; Ahrens and Devlin 2011). In RIDL insects, expression of the transgene sequence comprises the intended expression, and off-target (basal or unrepressed) expression. For fsRIDL constructs, expression is intended to be lethal to females reared in the absence of tetracycline, while off-target expression might lead to negative effects on males. Similarly, females may be negatively affected by transgene expression even in the presence of tetracycline, if expression of the transgene is not repressed below a harmful level.

In order to analyse the potential fitness costs associated with the fsRIDL transgene insertion in OX4319L, its competitiveness was compared to its background wild-type strain in a number of experiments. These experiments sought to include aspects of competitiveness which act at the pre- and post-copulatory stages, as well as individual and population levels. In all cases, comparisons with the wild-type background colony was chosen over truly wild insects in order that any potential deleterious effects arising from transgenesis could be isolated from laboratory adaptation. The selection process which 'wild' insects undergo when brought into artificial laboratory conditions is severe (Zygouridis et al., 2014). As they may suffer fitness costs associated this period of maladaptation, it would be inappropriate, and potentially misleading, to use them as representatives of the competition that OX4319L males would face under field conditions. This would be best achieved by field cage or open release studies which require an extensive regulatory approval process not permitted within the timeframe of this thesis.

Prior to these assessments, an experiment was carried out to determine the degree to which the 'natural' timing of mating behaviour had been maintained in our laboratory-reared colony. The wild type strain was chosen for these tests as large numbers of individuals were readily available and the departure between OX4319L and its wild type background (approximately 10 generations) was deemed to be minimal relative to that between the laboratory reared colony and their wild progenitors (over 360 generations).

2.2.7.1 Circadian rhythm of mating

In the wild, diamondback moth females begin producing sex pheromone at dusk, and the majority of mating takes place on the undersides of leaves within the first 4 h of scotophase (Talekar and Shelton, 1993; Lee et al., 1995). This minimises the exposure of the vulnerable mating pair (as well as the ovipositing female) to predators such as birds and other insects. This behaviour should be characterised in the laboratory-adapted diamondback moth strain that is utilised in this thesis for a number of reasons. Firstly, prior to mating competitiveness assays being performed in the lab, the peak mating period should be identified to maximise output from these experiments and to ensure the most realistic results. In addition, any major divergence from natural mating behaviour could potentially impact the effectiveness of transgenic lines derived from this background strain if they were to be deployed in the field, for example by desynchronising their mating behaviour with wild females and/or placing them at higher risk of predation.

Results:

The mating behaviour of our laboratory-reared diamondback moth strain closely followed what we would expect, judging from literature on wild moths in the field (Talekar and Shelton, 1993) (Figure 2.19).

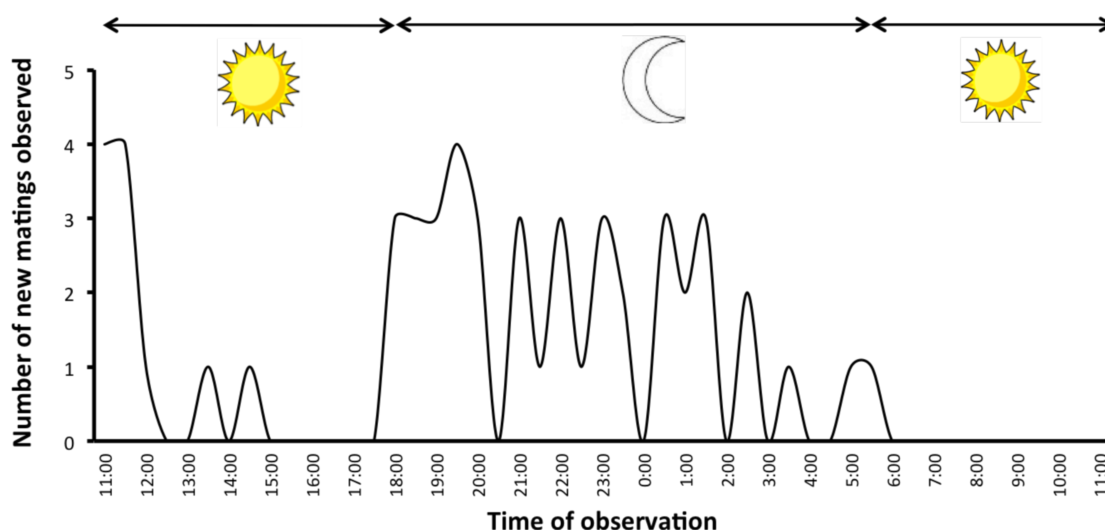


Figure 2.19: Graphic representing the mating behaviour of a laboratory adapted wild-type diamondback moth strain over a 24-h period. Number of matings in scotophase = 42 and photophase = 14. In total, 56 matings were observed from 40 mating pairs. The average length of copulation in scotophase was 75 ± 8.75 min and photophase was 72.6 ± 6.48 min. Estimated parameters followed by 1 S.E. of the mean.

While a relatively large number of copulations occurred immediately after males and females were combined (as would be expected after being isolated for 48 h), this rapidly diminished and by 15:00 was reduced to zero. However, at the onset of scotophase (18:00) a large number of copulations were observed. This behaviour persisted at a high level for around 3 h. After this period, the number of new copulations observed remained high but became more erratic, with the level starting to diminish by 02:30 and ceasing completely with the onset of photophase at 06:00. Significantly more

copulations took place within scotophase than photophase (diff = 0.3125, df = 1, p = 0.0012). The length of copulations, however, did not differ significantly between scotophase and photophase (W = 322, df = 56, p = 0.59). Our wild type diamondback moth strain was found to exhibit a higher degree of remating than previous studies (Figure 2.20). While 30% of females in this study showed some degree of remating, Wang, Fang et al., (2005) found that <18% mated more than once, with no females mating more than twice.

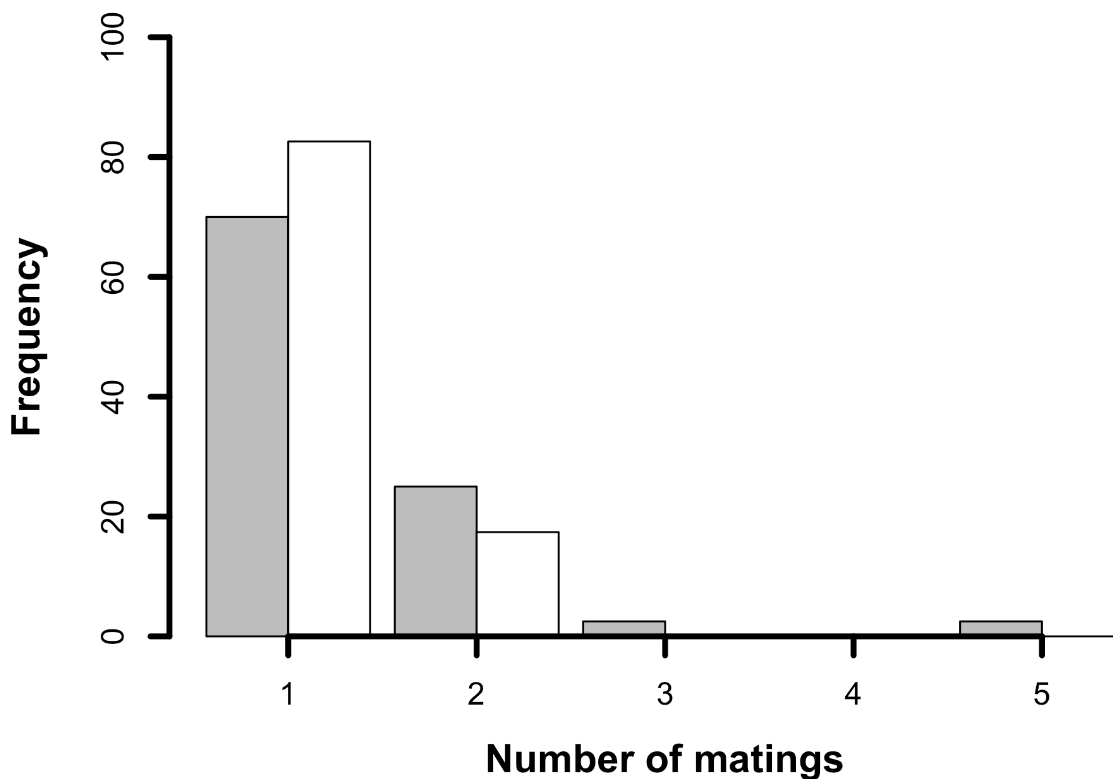


Figure 2.20: Comparison of the frequency of remating shown by female diamondback moth between this study and that by Wang, Fang et al, (2005). Females in that study were taken from a recently colonised laboratory strain (10 months), whereas the strain used in this study has been reared in the laboratory for over 15 years. Grey bars represent this study, white bars represent Wang, Fang et al., (2005)

Taken together, these results suggest a potential small departure from ‘natural’ mating behaviour in our laboratory-adapted strain. While copulation is primarily initiated at the onset of scotophase, some mating does take place during the ‘daytime’. Those matings that do take place during ’daytime’ are not kept as brief as possible, which might be expected for predator avoidance (although no such predatory stimuli were included here), but are as long in duration as those during the ‘night time’. In addition, the potentially hazardous act of mating, during which the mating pair is vulnerable to predation, is committed more frequently than might be expected from a wild population. However, it is important to note that in this study (as in Wang, Fang et al., (2005)) female remating occurred with the same male and thus it provides an estimate of multiple mating propensity rather than polyandry. These findings may therefore differ from those in the field, when multiple males are present. It is possible that, after being reared for so long in the absence of predator selection pressures, these moths have relaxed their predator avoidance behavioural mechanisms, such as those exhibited by other nocturnal mating lepidopterans (Acharya and McNeil, 1998). While the effects of this possible loss of defensive behaviour on the longevity of RIDL moths in the field have yet to be quantified, the general retention of ‘natural’ mating time initiation implies that released males will at least be responsive to females at the correct time of day, an issue which has severely impeded SIT programmes in other insects, such as the olive fly (Zervas and Economopoulos, 1982). Having established that this behaviour has been retained, mating competitiveness assays will be performed during scotophase.

2.2.7.2 Relative sterility index

The Relative Sterility Index (RSI) (McInnis et al., 1996) measures the relative mating competitiveness of one insect strain against another. The value of the index is equivalent to the proportion of total matings observed by males of the strain of interest, given an equal initial number of competitors from each strain. Within the context of autocidal control, RSI represents a preliminary indication of a strain's ability to induce sterility within a target population. RSI is the most widely used measure of irradiated insect sexual competitiveness in SIT at the laboratory stage and thus represents a highly standardised method with which to test OX4319L (Calkins and Parker, 2005; Allinghi et al., 2007; Orozco-Davila et al., 2007).

Results:

The average RSI value for OX4319L males was 0.41 ± 0.036 (a value of 0.5 represents equal competitiveness) (Table 2.1). Although this was significantly lower than the wild-type strain ($\chi^2 = 6.40$, df = 1, p = 0.0114), this result represents an encouraging level of performance. In SIT programmes for medfly, an RSI of 0.2 (where irradiated males are 25% as competitive as wild ones) is considered the minimum value acceptable for effective suppression (FAO/IAEA/USDA, 2003).

Table 2.1: Summary of experiments estimating the Relative Sterility Index (RSI) of OX4319L versus its wild type background strain. In each given replicate, matings represents the number of copulations 'won' by that genotype.

Replicate No.	Wild-type matings	OX4319L matings	Total matings	% ♀ mated	RSI wild-type	RSI OX4319L
1	35	21	56	56	0.625	0.375
2	19	14	33	66	0.576	0.424
3	15	18	33	66	0.455	0.545
4	11	6	17	57	0.647	0.353
5	24	10	34	68	0.706	0.294
6	25	22	47	94	0.532	0.468
Mean						0.410 ±0.036 (1 S.E of mean)

2.2.7.3 Lifetime mating performance

While an RSI score is a highly transferable measure of first copulation initiation success, it provides only a very brief window of insight into total mating competitiveness. In reality, whether an allele is inherited is influenced by a number of interrelated factors including sperm competition and male longevity (of special importance to orders which mate multiply such as Lepidoptera). Recent research in the Mediterranean flour moth *Ephestia kuehniella* Zeller suggests that there is a positive correlation between perceived competition by adult males and the amount of sperm transferred to females with which they mate (in response to increased sperm competition from other males). This increased reproductive effort in the face of competition has the dual result of shortening longevity, and decreasing the number of females inseminated (Xu and Wang, 2014). However, the degree to which fitness costs

suffered by transgenic individuals such as OX4319L will interact with such competition effects is unknown. For example, if transgenic males suffered from extra fitness costs compared to wild-type males this could cause their longevity to decrease at a faster rate in response to competition. This in turn could lead to reduced numbers of transgenic males relative to wild-type counterparts in the latter stages of a generation and a subsequent reduction in mating pressure from transgenics. Additionally, if fitness costs affecting sperm transfer are associated with a transgene insertion (as has been found with irradiated lepidopterans (Koudelova and Cook, 2001), these individuals may not be able to compete as effectively at the post-copulatory stage, or may not be able to induce re-mating refractoriness in females to the same degree, even if the overall numbers of matings were similar between genotypes. In a second mating test these factors were explored by including post-copulatory effects, reduction in male performance over time and re-mating effects within a highly competitive mating environment. The experiment used was a derivative of a classic assay developed by Fried (1971) modified so that heterozygous mortality could be controlled for, and so that output could be compared to previous estimates of RSI.

Results:

Two experiments were run simultaneously, one to assess mating competitiveness and another to act as a control for heterozygote mortality. The correction factor calculated for heterozygote mortality was estimated at 1.08. When applied to the mating experiment, an average RSI score equivalent of 0.374 ± 0.046 was estimated (Table 2.2). This was significantly different to wild-type (χ^2 =422, df = 1, $p < 0.001$).

Table 2.2: Summary of modified Fried test experiments estimating the mating competitiveness, and Relative Sterility Index (RSI) equivalent value, of OX4319L versus its wild-type background strain.

Replicate No.	No. OX4319L heterozygous progeny	OX4319L heterozygous progeny corrected for mortality	No. wild-type progeny	RSI equivalent
1	579	625	1233	0.336
2	630	680	1085	0.385
3	799	862	878	0.496
4	393	424	1090	0.280
Mean				0.374 ± 0.046 (1 S.E of mean)

Although lower, this value does not differ substantially from that estimated by the RSI mating assay. This is the case despite the fact that embryonic mortality of heterozygotes was not controlled for by the mortality control factor applied in this experiment. In addition, to keep the experiment manageable, heterozygote mortality was estimated in low-density rearing cups. It is likely that heterozygote mortality versus that of wild-type was considerably higher in the high-density rearing arena of the larger pots.

In a second analysis of this data, the RSI equivalent of OX4319L was assessed on each collection day (relative to wild-type) (Figure 2.21).

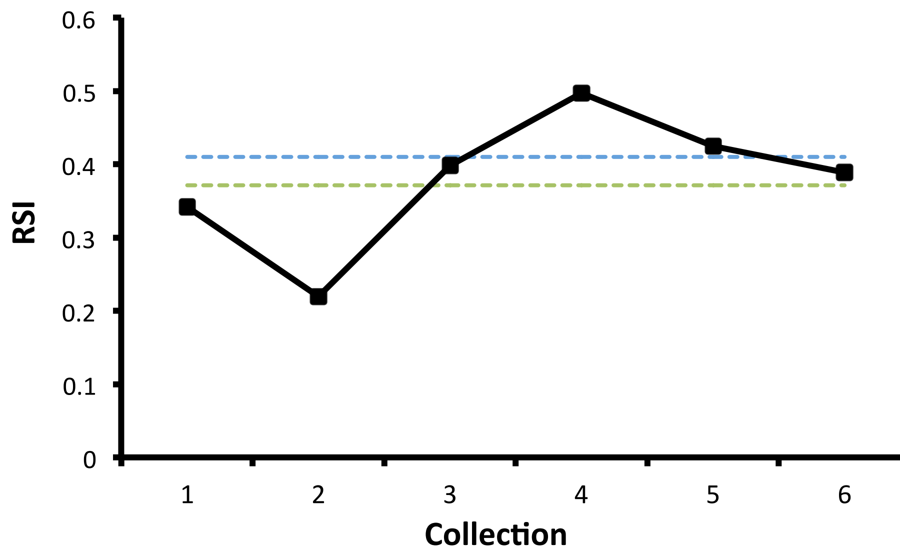


Figure 2.21: Graph showing the RSI equivalent calculated for OX4319L on 6 consecutive 2 day collection periods (solid black line); the average RSI equivalent over this time period (green dotted line) = 0.37, and the RSI estimated for this line previously (blue dotted line) = 0.41.

These results indicate that the share of progeny produced by OX4319L does not appear to decrease over time even when under intense competition for mates. This is encouraging as it implies that fitness costs affecting the lifetime mating success of OX4319L males, and their response to long periods of mating competition, are not substantial.

2.2.7.4 Population genetics of transgene-associated fitness costs

The two previous experiments have focussed on fitness costs which affect the mating behaviour of homozygous adult males. This is appropriate as it is primarily this cohort that will introduce the lethal fsRIDL alleles into the target population. However, the fitness of other transgenic cohorts such as females, heterozygotes and non-adult stages is also of importance, for example in mass-rearing colonies or in the F1 hybrid field generation. In order to gain a more complete understanding of the cumulative

impacts of transgene associated fitness costs acting across a population these issues were explored in a final series of multi-generational cage experiments. In these experiments, the evolution of fsRIDL allele frequency was tracked over time when transgenic individuals were reared in competition with wild-type counterparts in mixed populations. This allowed the estimation of population-level fitness costs for individual transgenic genotypes and provided parameter estimates useful in modelling the dynamics of transgene insertion alleles under a variety of scenarios.

Two experiments were conducted: the first under permissive (on-tetracycline) conditions, and the second under restrictive (off-tetracycline) conditions. Mass-rearing of fsRIDL insects would be conducted under permissive conditions, whereas released insects and their progeny would face restrictive conditions. Fitness costs under permissive conditions therefore affect ease and efficiency of rearing, and also inform consideration of the likely fate of any hypothetical wild-type allele that entered a tetracycline repressed transgenic population, or vice versa. This information is useful both in predicting the likely outcome were a wild-type allele to invade a homozygous OX4319L stock colony (a major issue for current GSS strains in medfly) and the probability of transgene persistence in the field (if permissive conditions are encountered). This latter point is important to fsRIDL insects from a regulatory perspective but is also interesting in the context of alternative transgenic pest management systems based on the spread of synthetic disease refractory alleles which are designed to persist in the field (Sinkins and Gould, 2006). Fitness costs under restrictive conditions inform models of the rate of loss of the transgene from a wild population were releases to cease, and can be used to quantify heterozygote male competitiveness. The starting frequencies of the transgenic allele in each experiment were thus chosen to reflect those expected in extreme examples of these two scenarios:

(1) wild-type contamination in a mass-reared colony (fsRIDL allele frequency = 0.75) (and rearing under permissive conditions), and (2) after cessation of inundative releases of fsRIDL insects into the field (fsRIDL allele frequency = 0.25) (and restrictive conditions). Under permissive conditions, the significance of transgene frequency trends over time (selection acting on the transgene) was assessed using a variety of statistical tests described in the materials and methods section (2.4.7.4.1 – Analysis).

Results

2.2.7.4.1 Selection on transgene under permissive conditions:

OX4319L transgene allele frequency declined by 63.3% over 10 generations, a trend which was significantly non-neutral (tau = -0.956, $P < 0.01$) (Figure 2.22). Frequency Increment Testing (FIT) showed a significant departure by the transgene allele frequency from the null, neutral drift distribution, which would have been expected in the absence of selection ($t_{FITT} = 2.32$, $\alpha = 0.05$).

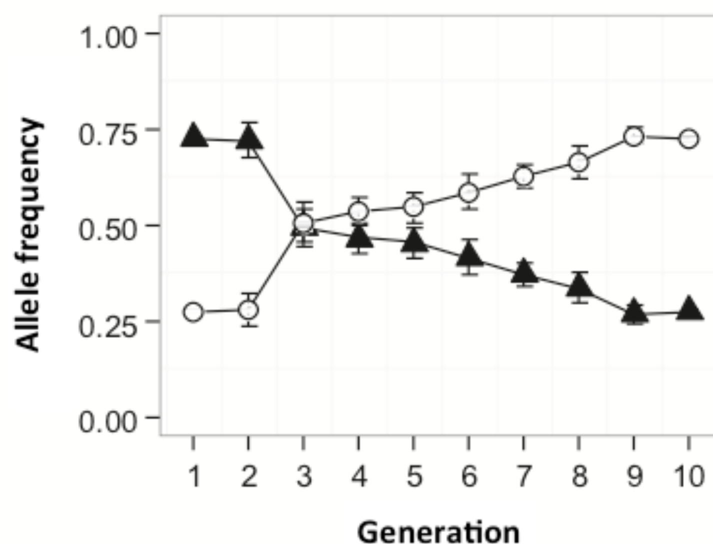


Figure 2.22: Graph showing mean transgene and wild-type allele frequencies (± SE) tracked over 10 generations in three mixed populations of diamondback moth containing the OX4319L transgene insertion and wild-type alleles. Triangles and circles represent the mean frequencies of the transgene and wild-type allele recorded in each generation, respectively.

The homozygous wild-type genotype (-/-) showed the highest average increase in frequency, from 0.07 (±0.02 SE) in Generation 2 (the first generation expected to represent genotypes at Hardy-Weinberg equilibrium) to 0.47 (±0.02 SE) in Generation 10 (Figure 2.23A). Non-stationary trends were suggested for -/- (tau =0.944, $P < 0.01$) and homozygous transgenic (R/R) (tau = -0.889, $P < 0.01$) genotype trajectories, but not for the heterozygous genotype (R/-) (tau = 0.056, $P > 0.1$).

Relative fitness values (compared against the wild-type allele) for the R/- and R/R genotypes were calculated to be $W_{R/-} = 0.736 \pm 0.07$ SE, and $W_{R/R} = 0.477 \pm 0.20$ SE, respectively (Figure 2.23B), with significant differences in Corrected Rate of Increase (CRI) calculated between R/R and R/-, -/- genotypes (Stat = -3.509, $P < 0.01$, Stat = -4.968, $P < 0.01$;) but not between R/- and -/- genotypes (Stat = -2.068, $P > 0.1$).

Relative fitness parameters estimated from these empirical data were used to predict changes in genotype frequency over time (Figure 2.23C) using a recursive general selection model (Haldane, 1931). Due to high selection pressure against both heterozygote and homozygote transgenic genotypes, this model predicted wild-type allele fixation, and the concomitant loss of the transgene within 30 generations (where the modelled number of transgenic individuals falls to <1, and assuming identical starting conditions to experimental populations).

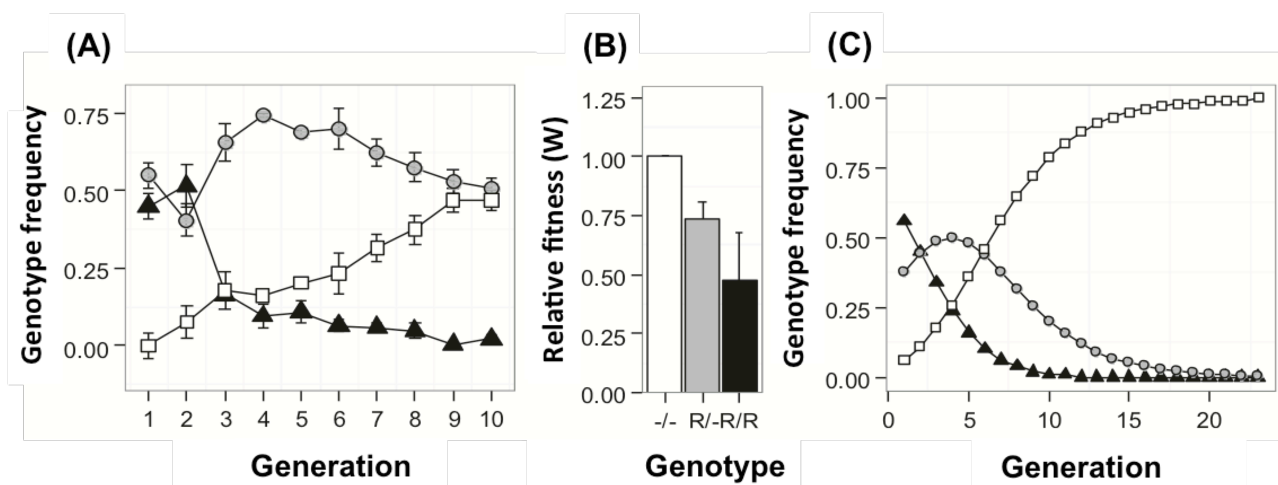


Figure 2.23: (A) Mean frequencies (± SE) of transgenic homozygous (R/R, black triangles), heterozygous (R/-, grey circles) and wild-type (-/-, white squares) frequencies in three mixed-genotype populations observed over 10 generations. Populations were established with 100 homozygous transgenic male and 100 heterozygous transgenic females. (B) Relative fitness values for -/-, R/- and R/R genotypes (±SE) of OX4319L calculated from Corrected Rate of Increase (CRI) parameters generated from (A) with values relative to the -/- genotype. Relative fitness values were $W_{R/R} = 0.477 \pm 0.20$ SE, $W_{R/-} = 0.736 \pm 0.07$ SE, and $W_{-/-} = 1$. Significant differences in Corrected Rate of Increase (CRI) were calculated between R/R and R/-, -/- genotypes but not between R/- and -/- genotypes. (C) Results of a deterministic population genetics model illustrating theoretical genotype trajectories in a mixed-genotype population begun and held under the same conditions as those in this experiment and using the experimentally derived mean estimated relative fitness values from (B). Note that model outputs represent more generations than the cage experiment, to illustrate longer-term dynamics. Genotypes represented by identical symbols as in (A).

2.2.7.4.2 Selection on transgene under restrictive conditions:

In order to assess any potential fitness costs affecting transgene heterozygotes, the rate of transgene frequency decline in three experimental populations reared off tetracycline was compared with the rate of decline predicted by a stochastic model. In this model, the effects of engineered dominant female-lethality (off-tetracycline) on population transgene allele frequency, but no other transgene-associated fitness costs, were included. As such, significant departure between the observed and modelled results would be attributable to transgene-associated fitness costs not included in the model (Materials and Methods 2.4.7.4.2 – Analysis). Given an initial transgene allele frequency of 0.25 in a closed population of 200 individuals under restrictive conditions,

the model predicted extinction of an fsRIDL transgene within nine generations in approximately 95% of iterations, with a mean and maximum number of generations until allele loss of 6.5 (±1.8 SD) and 15, respectively (Figure 2.24). OX4319L allele frequency decay in experimental populations fell well within the variation predicted by this stochastic model. Mean number of generations until disappearance was 6.0 (± 0.58 SE), with the most persistent replicate taking 7 generations. On average, OX4319L allele frequency decreased in each generation by 50% (± 6.0 SE) while modelled mean fsRIDL allele frequency fell by 47% (± 7.2 SE). These estimates of transgene allele loss were not significantly different (t = -0.260, $P > 0.1$).

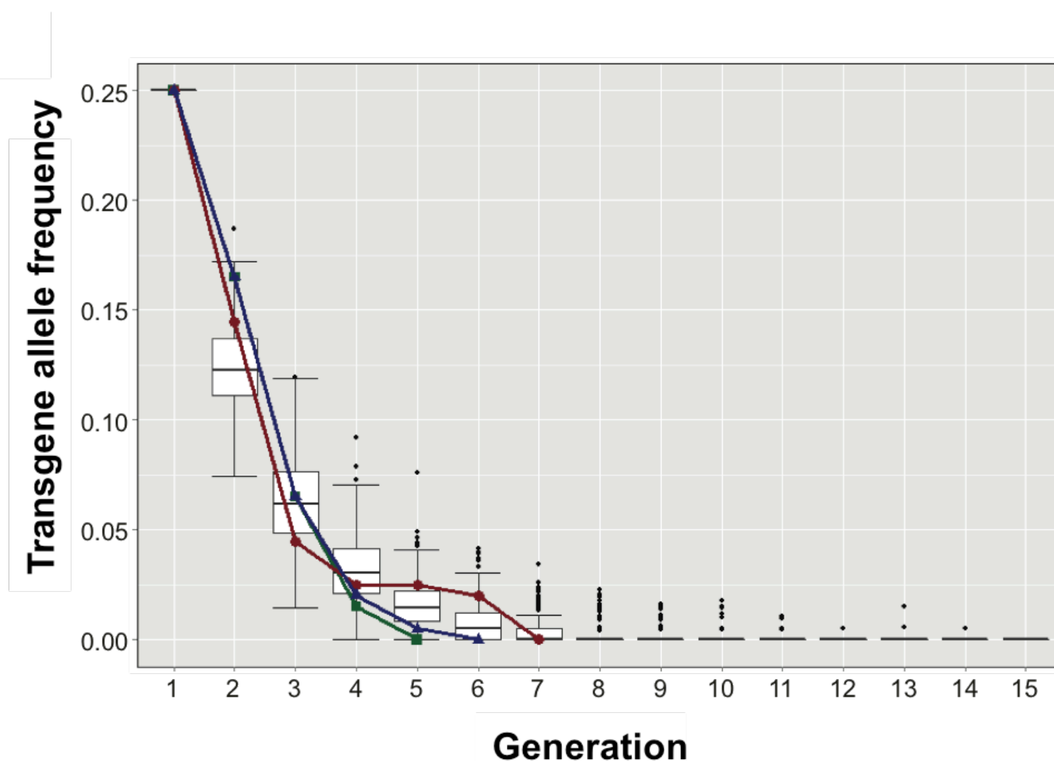


Figure 2.24: Boxplots showing results from 250 iterations of a stochastic model simulating engineered female-specific selection on an fsRIDL allele in a panmictic, closed population of constant size over 15 discrete generations. Starting population consists of 200 individuals and an initial fsRIDL allele frequency of 0.25 (f = 0.25) propagating in the absence of the transgene repressor (under restrictive conditions). Horizontal bold lines represent generational medians; upper and lower box lines represent first and third quartiles, respectively; outer horizontal lines represent 1.5× the interquartile range; and dots represent data points over 1.5× above or below the first and third quartiles, respectively. Overlaid onto the boxplots are lines (red, blue and green) showing allele frequency changes from three replicates of caged experiments tracking OX4319L allele frequencies in mixed populations of diamondback moth reared under analogous conditions to those used in the model (initial fsRIDL allele frequency of 0.25, restrictive conditions).

2.2.7.4.3 Discussion of population-level fitness costs

Even under permissive conditions, significant negative OX4319L allele frequency trends were recorded over the 10 generations of this experiment. These frequency changes could be attributed to selection against the transgene as opposed to neutral drift. As the experimental population size (*N*) was substantially larger (20×) than the number of generations observed (200 v 10), it is assumed in any case that genetic drift (which changes allele frequencies over periods of approximately *N* generations) is unlikely to have significantly affected results in this experiment (Illingworth et al., 2012). This selection against the transgene was primarily directed against R/R individuals as this was the only genotype to show both significant negative frequency trends as well as CRI values significantly different from -/- (Figure 2.23A and 2.23B).

As this experiment took into account a wider range of factors than previous studies of OX4319L competitiveness (in terms of behaviours, life-stages and sexes included), a lower estimate of overall fitness might have been anticipated. In particular, fitness costs for a female-lethal strain, even under permissive conditions, might be significantly higher in females than in males. To a certain extent this hypothesis is justified by the results of this experiment. The estimate of relative R/R fitness in this study, 0.477 (Figure 2.23B), was slightly lower than that calculated for homozygous OX4319L males (0.374/0.626 = 0.597; Table 2.2). This equates to a reduction in the relative fitness of homozygous OX4319L individuals of approximately 12% when females and immature stages are included in the analysis. Nonetheless, even this conservative estimate of relative fitness remains well above the converted minimum value of 0.25 for an efficient autocidal release program (FAO/IAEA/USDA, 2003).

Beyond potential impacts on suppression efficacy, the transgene-associated fitness costs present in OX4319L have a number of implications at the population-genetics level. Using the best estimate of the fitness costs associated with this line (derived from this experiment), population modelling suggests that even under conditions where engineered lethality is suppressed, competition between transgenic and wild-type alleles will result in the eventual extinction of the transgene (Figure 2.23C). However, the relatively slow rate at which this would occur in an OX4319L colony (approximately 6% reduction in transgene allele frequency per generation) suggests that a wild-type allele contaminating a homozygous mass-rearing colony would spread only slowly. This is a significant improvement over classical translocation-based sexing strains, for example the Medfly *tsl* strains, which have severe fitness costs associated with their chromosomal aberrations (Fisher, 2000). In these *tsl* strains, even prior to behavioural competition with wild-type individuals being factored in, 50% of embryos produced by *tsl* males each generation will die due to aneuploidy. Homozygous females, too, will suffer fitness reductions as they possess two copies of the *tsl* and *wp* mutations, while heterozygous females will no longer be susceptible to removal by temperature or visual (*wp*) selection (Morrison et al., 2009).

Although no significant fitness costs associated with R/- were evident under permissive conditions, these may have been more marked under restrictive conditions (as seen in Section 2.2.5). This was assessed by comparing fsRIDL allele evolution between modelled populations (in which the sole selection force on fsRIDL insects was engineered female-lethality (see Materials and Methods 2.4.7.4.2 *Analysis*) and empirical data collected for OX4319L under analogous conditions. Divergence between these two data sets might have implied extra transgene-associated fitness costs not factored in to the modelled populations dynamics. However, experimental OX4319L

allele decay fell well within that predicted by the stochastic model (Figure 2.24) and average generational OX4319L allele frequency reduction did not differ significantly from that predicted by modelling. This indicates that fitness costs to OX4319L heterozygote males (as carriers of fsRIDL alleles under restrictive conditions), if present, have little effect on allele frequency evolution under these conditions. As predicted, the OX4319L transgene went extinct in all caged populations within a small number of generations, implying that transgene persistence in the field would be relatively transient in the absence of ongoing releases of transgenics. Even if some individuals had access to permissive conditions, i.e. high levels of tetracycline, during larval development - implausible in the field for these insects (Kumar et al., 2005; Hu et al., 2010; Seo et al., 2010 - this thesis Section 2.2.6.2) - most would likely not, and the fitness costs of the transgene identified here under both restrictive and permissive conditions would in any case ensure its rapid disappearance from the population.

Fitness costs due to transgene expression would be expected to be dominant or semi-dominant. Interestingly, the inheritance of fitness costs associated with the OX4319L transgene insertion appear to differ according to sex. When females are included in the analysis (permissive conditions), fitness costs appear to be semi-dominant, with heterozygote fitness intermediate between wild-type and transgenic homozygotes. However, when only male heterozygotes are assessed (restrictive conditions) no significant fitness reductions are observed, implying that, if they exist, they are substantially recessive. This is somewhat surprising for a conditional lethal transgene and suggests that basal expression of the lethal effector in OX4319L, at least in males, is relatively harmless (as observed in Section 2.2.2 and 2.2.5). In contrast, taking a similar multi-generational approach, Paton et al (2013) identified strong selection against a synthetic (non-lethal) malaria-refractory transgene (EVida3) in the

mosquito *Anopheles gambiae*, resulting in extinction of the allele in all replicate populations in 10 generations or less. Significant fitness costs to the larval stages of these transgenic mosquito strains were identified, possibly due to unintended background transgene expression. The much longer predicted persistence times of the OX4319L allele under permissive conditions suggests that it is possible to design constructs and generate transgene insertion sites where such background fitness costs can be minimised. This is encouraging both for the mass-production and field application of this strain, and also for the development of 'self-sustaining' strains and strategies in which, unlike fsRIDL, the transgene is intended to persist in the environment for many generations or indefinitely after releases cease (Sinkins and Gould, 2006), for example to block the malaria parasite life-cycle in the disease vector (Corby-Harris et al., 2010). In particular, it suggests that it may be possible to deploy such transgenic technologies effectively, at least on trial scales, by inundative release. Even though the transgenes would eventually disappear (due to associated fitness costs), and so the approach is self-limiting, the transgene may persist in closed populations at a useful frequency for many generations (Gould et al., 2008; Rasgon, 2009). This would allow disease-refractory effector genes and molecules to be tested in the field without requiring coupling to synthetic gene-drive systems, which are more challenging from both technical and regulatory perspectives.

2.3 Conclusion and Summary

The aim of the work described in this chapter was to generate an fsRIDL line of diamondback moth and test it for the characteristics necessary in an autocidal release strain. Such a line was successfully created utilising the sex-alternative splicing from *Pgdsx* to drive conditional tTAV lethality in females in OX4319L. Engineered female-specific lethality in this line was tightly controlled on tetracycline, and highly penetrant off tetracycline, both as homozygotes and heterozygotes. As such, this line shows strong potential for use both as a genetic sexing strain, and as a population suppression tool.

The fsRIDL characteristics of heterozygous OX4319L individuals were assessed in two scenarios relevant to field use. These experiments confirmed the penetrance of fsRIDL lethality to females on host material (and demonstrated that economic damage to the crop is unlikely to be caused by this cohort) and showed that tetracycline levels required to disengage this lethality are unlikely to be sequestered by crucifer crops in the field. These results are important for regulators tasked with assessing the safety of using this and other fsRIDL lines in the field.

Molecular analysis of OX4319L allowed investigation into the conservation of *dsx* splicing in the Lepidoptera across a previously untested phylogenetic range. Results showed that splicing directing sequences believed to be highly conserved across the Lepidoptera show some divergence at broader phylogenetic levels than those previously tested. This, however, did not impact on the ability of endogenous splicing machinery to recognise and splice these exogenous regions (at least in diamondback moth). These results are an interesting contribution to our knowledge of the regulation and evolution of this complex sex determination mechanism.

Finally, as a preliminary assessment of the potential fitness costs suffered by OX4319L, its competitiveness was explored in four experiments. The first two of these focussed on the mating competitiveness of homozygous males with the aim of assessing the efficacy of this strain as a population suppression tool in the field. The second two experiments took an evolutionary approach and explored the population-level consequences of these fitness costs over multiple generations under both permissive and restrictive conditions. In concert, these experiments provided a robust and complementary assessment of the fitness costs affecting OX4319L. A modest reduction in competitiveness relative to wild-type was recorded in all experiments (with the exception of heterozygote males off tetracycline) most likely the result of insertional mutagenesis and/or basal tTAV toxicity. Nonetheless, OX4319L males when reared off tetracycline displayed a relatively high level of mating performance for cage testing. On tetracycline, the fitness of individuals carrying this transgene puts it at an advantage compared with current translocation-based GSS methods in the context of mass-rearing programmes. Models using these fitness estimates suggest that, even if high levels of tetracycline are available, field persistence of this transgene insertion is highly unlikely due to competition with wild-type alleles.

While this work provides an initial indication of the performance of OX4319L, the true field competitiveness of an insect strain will be influenced by a wide range of biotic and abiotic factors not easily tested in the laboratory (Pérez-Staples et al., 2013). For a more accurate estimate of this strain's competitiveness, further testing under more realistic field conditions is required (Calkins and Parker, 2005). However, the results described in this chapter indicate that this line is highly appropriate for the next stage of investigation: large-scale cage population suppression and resistance management trials.

2.4 Materials and Methods

2.4.1 General experimental details

2.4.1.1 Laboratory conditions and insects used

All laboratory experiments were performed within a temperature controlled CT room (25°C) with a 16:8 light:dark cycle. The diamondback moth wild-type background strain used for transformation and bioassays was provided by Syngenta plc. (Jealott's Hill, UK) and originated in Vero Beach, Florida, USA. Insects were reared on an artificial beet armyworm diet (Bioserv, U.S.A). For tetracycline diet, chlortetracycline was added at a concentration of 100 µg/ml; non-tetracycline diet contained no chlortetracycline.

2.4.1.2 Statistics

All statistical analysis was performed in R (v. 3.0.02) (R Core Team, 2013). Modelling was performed in R and Excel (Microsoft, USA). Data was checked for normality and non-significant difference of variances using Shapiro-Wilk test and F-test/Bartlett box tests. If data did not satisfy these assumptions, transformation was attempted. If this failed, non-parametric analysis was employed. Comparisons with multiple factors were first assessed using the relevant omnibus test and significant differences analysed using *post hoc* testing. Individual experimental statistics are discussed in each section.

2.4.1.3 Micro-injection

Injections used a micro-manipulator 5171 (Eppendorf – Hamburg, Germany). Treatment of eggs, injection protocol and rearing of potential transformants followed the methods of Martins, Naish et al, (2012).

2.4.2 Characterisation of the female-lethal phenotype

2.4.2.1 Test cross 1

Ten OX4319L heterozygous males and 20 wild-type homozygous females were crossed together in plastic diet cups. Eggs were collected on cabbage extract-baited Parafilm every 2 days and divided equally into two diet treatments (tetracycline and non-tetracycline). Larvae were fed every 2 days and, once eclosed, were scored for sex and genotype. Transgenic survival to adulthood was calculated as a proportion of that of wild-type counterparts. Observed numbers of male and female transgenics were compared to their respective wild-type expected numbers using a G-test.

2.4.2.2 Test cross 2

Ten OX4319L heterozygous males and 20 OX4319L heterozygous females were crossed and reared in the same manner as in test cross 1. However, only tetracycline diet was used, theoretically allowing individuals of both sexes and genotypes to survive. The number of transgenic individuals surviving to adulthood was compared to the expected 3:1 (transgenic:wild-type) phenotype distribution using a G-test.

2.4.3 Investigating the evolutionary conservation of *dsx* in Lepidoptera

RNA was extracted from OX4319L males and females (Qiagen RNeasy mini kit). Two-step RT-PCR was performed on these RNA samples (SuperScript II Invitrogen) using primers specific to the shared *Pgdsx* exons in the OX4319 minigene (primer 1855 3816RTEX1 + primer 1377 RTPBWMEXONR). Primers were designed to amplify sequence specific to the minigene in order to prevent amplification of endogenous *dsx* transcripts. Amplified fragments were purified using an agarose gel and ligated into pJET plasmids. Plasmids were transformed into competent *E. coli* cells and colonies allowed to grow overnight. Twelve colonies were selected and sent for sequencing (GATC Ltd). Returned sequences were analysed for divergence with previously sequenced pink bollworm transcripts using Vector NTI Advance 11.5 and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Divergences at the genomic sequence level were analysed by comparison of data sets (origins of each given in results section) in ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

2.4.4 Characterisation of transgene insertion locus

Genomic DNA (gDNA) from three OX4319L individuals was extracted (PureGene DNA purification kit). A primer specific to the backbone sequence (primer 250 MI3F), in combination with one specific to the 3′ *piggyBac* region (primer 218 PB3) was used to test for the presence of plasmid backbone (carrying antibiotic resistance gene) and no evidence of its presence was found.

Flanking sequence of OX4319L was identified through a series of nested PCRs. Extracted OX4319L gDNA was digested using five restriction enzymes (*Cla*I, *Nar*I, *Bgl*II, *Bst*BI and *Bc*II). Digested fragments were ligated with known flanking adaptors with termini matching those generated by the restriction enzyme used. Ligated fragments were then subjected to a series of PCRs using primers specific to the adaptors and the 5′ and 3′ *piggyBac* areas respectively. Amplified bands were taken as an indication that the restriction enzyme used had cut in an area close enough to the insertion to allow a PCR product to be amplified. Fragments of the expected size were purified, ligated into pJet plasmids (Fermentas) and then cloned into competent *E. coli* cells. Colonies were screened using PCR (pJEt flanking primers) and a diagnostic digestion (*Bgl*II); four colonies were selected for sequencing. Of these four colonies, the 5′ and 3′ flanking sequences were identified accurately from two. Nucleotide BLAST analysis (http://blast.ncbi.nlm.nih.gov/) of the flanking sequence showed no significant similarity to known sequence. This is an indication that the insertion will not cause a deleterious effect, for example by disrupting the coding sequence of an important gene.

As a final test, two primers each were designed for both the identified 5′ and 3′ flanking regions. These forward and reverse primers were used in all combinations on wild type diamondback moth gDNA in a PCR screen. Similarly, these flanking primers were combined with the relevant *piggyBac* primers in a PCR screen of OX4319L gDNA. Bands of the expected sizes were observed from both PCRs indicating the utility of these primer combinations in distinguishing OX4319L genotypes from each other, and from wild-type. For primer details see Appendix Table A.2

These flanking primers, in combination with those specific to the construct (*piggyBac*) were then used to generate both OX4319L-A and OX4319L-B. In both rounds of screening, gDNA was extracted from a hind leg of OX4319L individuals using a NaOH

leg extraction technique (developed by Dr Tarig Dafa'alla). Individuals were anaesthetised using CO_2 and had one of their hind legs removed. To extract the gDNA, each leg was placed in 25 µl 100 mM NAOH and heated to 99°C for 30 min. After this point, 5 µl of 500mM HCl + Tris was added to each sample to neutralise the solution. PCR screening was conducted on extracted gDNA and individuals screened as heterozygous or homozygous for the insertion were taken and crossed to their respective genotypes to form two separate colonies.

Sex ratio of the OX4319L-B line was analysed by taking approximately 100 pupae at random from collections over the subsequent 2 generations after line formation, sexing them and comparing to an expected 50:50 ratio using a G-test.

2.4.5 Comparing fsRIDL phenotypes as homozygotes

2.4.5.1 Survival to adulthood and rearing characteristics

Three strains were compared in these experiments, wild-type, OX4319L and OX4319N. Eggs from each strain were collected on Parafilm sheets, counted out into batches of approximately 100 and allowed to hatch in plastic diet cups. Diet cups were arranged in a blocked experimental design so as to minimise possible bias in larval survival based on position within the room. Between 18 and 24 replicates were performed for the transgenic lines and 12 repeats performed for the wild-type strain. These replicates were divided between those fed non-tetracycline diet and those fed tetracycline diet. All replicates were fed from the same respective blocks of diet, on the same days. Once eggs had hatched (approximately 4 days post start of experiment), egg sheets were removed from cups and the number of unhatched eggs counted. Subtracting the number of unhatched eggs from the number of eggs in each cup gave the exact

number of first instar larvae placed onto diet in each cup. This method is preferred to counting out first-instar larvae as it minimises deaths due to handling. Larvae were allowed to develop and, once eclosed, the proportional survival and sex ratio of each cup was recorded. Data was analysed using a logistic regression GLM and binomial quasibinomial errors with subsequent post-hoc analysis using Generalised Linear Hypothesis Testing where required.

2.4.5.2 Longevity

2.4.5.2.1 On Tetracycline

OX4319L, OX4319N and wild-type eggs were reared on tetracycline in plastic diet pots. Once pupated, 50 males were selected from each strain via manual sexing. Each pupa was individually weighed (OHAUS Explorer microscales) and assigned a random number between 1 and 150. Each pupa was placed in an individual Petri dish with a sugar water source (renewed every 2 days). Petri dishes were placed in chronological order. Date of eclosion and death were recorded for each individual. Once mortality was complete, data were analysed using parametric survival analysis (ANCOVA). Explanatory variables included strain as a fixed treatment variable, weight as a random control variable and the interaction between these two variables. Weibull errors were used, as the hazard appeared to be non-constant and modelling showed that this provided the minimum error deviance. No data was censored.

2.4.5.2.2 Off Tetracycline

OX4319L, OX4319N and wild-type eggs were reared on off-tetracycline diet in plastic diet pots. Once pupated, 50 males were selected from the wild-type strain by manual sexing. Fifty individuals (all males) were also taken from each transgenic line.

Experimental protocol and statistical analysis follow that of the on-tetracycline experiment, apart from censoring during the analysis.

2.4.6 Analysis of heterozygous fsRIDL phenotype

2.4.6.1 Temporal investigation of fsRIDL penetrance on host plant

Male OX4319L homozygotes were mated to wild-type females to give OX4319L heterozygotes. One hundred newly hatched first-instar larvae from each of wild-type and these heterozygotes were randomly chosen and placed singly in Petri dishes onto 3.5 × 3.5 cm cabbage leaf disks (Chinese leaf, *Brassica rapa* supplied by Dr Steven Foster, Rothamsted Research). Petri dishes were sealed with Parafilm to prevent leaf desiccation, and all dishes were checked after 1 h for evidence of larval mining behaviour to ensure that all replicates were undamaged by handling. Leaf disks were replaced every 3 days. Mortality was scored daily until all replicates had either eclosed or died, with sex of pupae, and date of pupation/eclosion also recorded. Hazard functions calculated from daily survival data were compared between OX4319L and wild-type insects using Cox Proportional Hazards Survival Analysis. Time to eclosion in males was compared using a Wilcoxon Signed-Rank test.

2.4.6.2 Tetracycline dose-response in heterozygotes

OX4319L heterozygote males were produced by crossing homozygous males to wild-type females and rearing off tetracycline. Heterozygote males were themselves crossed to wild-type females (giving an expected 1:1 offspring genotype ratio) and the progeny reared on artificial diet containing varying quantities of tetracycline (0.01

μg/ml, 0.1 μg/ml, 1.0 μg/ml, 10.0 μg/ml, 100 μg/ml and 0.0 μg/ml). Survival to adulthood of heterozygotes was calculated relative to their wild-type siblings, of the same sex, reared in the same pot. Between 8 and 10 replicates were performed per diet treatment. Data collection was aided by Adam Walker, Thea Marubbi, Debs Granville and Nerys Humphrey-Jones.

2.4.7 Transgene associated fitness costs

2.4.7.1 Circadian rhythm of mating

Wild-type males and females were sexed as pupae and allowed to eclose over 24 h. Pupae that did not eclose during this period were discarded. Once eclosed, sexes were kept separate for a further 24 h in order to allow the observational period to coincide with the peak reproductive period (48-62 h) (Maa, 1986; Pivnick et al., 1990). Individual male and female adults were combined in Petri dishes to make 66 pairs. Pairs were provided with a sugar water source and cabbage extract-painted arena to induce mating. Pairs were observed every 30 min for 24 h (beginning at 11:00 am) with number of new copulations and length of copulation (to nearest 30 min) recorded. Data were analysed to test whether length of copulation time (Mann-Whitney U test) and/or number of copulations (McNemar's paired Chi-squared test) differed significantly between scotophase and photophase. Level of remating was also calculated and compared to literature for relatively wild insects. Due to space limitations, these experiments were conducted in a pink bollworm insectary with a 12:12 light:dark cycle as opposed to one with a 16:8 cycle. However, as it was the initiation of mating

behaviour with regards to scotophase which was being investigated, rather than the total 24-h circadian rhythm, this is unlikely to have had an effect on the conclusions drawn.

2.4.7.2 Relative sterility index

OX4319L and wild-type eggs were reared off tetracycline. Wild-type pupae were sex-sorted by hand, while OX4319L produced only male pupae. Adults were collected within 24 h of eclosion and equal numbers of males from each strain placed within a 1 m × 1 m × 1.5 m insect cage (Bugdorm) along with a Parafilm 'artificial leaf' to act as a mating stimulant. Five hours later, at the onset of scotophase, a number of female wild-type adults equal to half the total number of males were released into the cage. Cages were checked every 15 min and mating pairs isolated and removed with the aid of a red-filter light. The genotype of the male within each mating pair was inferred through fluorescence microscopy. Six replicates were performed with a total of 220 mating pairs observed. To determine significance of RSI values, Chi-square analyses were performed using a likelihood framework (Morrison et al., 2009). One replicate in which OX4319L males eclosed with visible deformities was recorded (RSI = 0.15) but not included in the final analysis due to possible issues with rearing confounding the result (adult deformities are most often traced to inadequate larval rearing conditions). However, a subsequent analysis including this replicate was conducted. Inclusion caused a very slight decrease in RSI from 0.41 to 0.388. However, the two data sets (included and non-included) did not differ significantly (Welch two-sample t test).

2.4.7.3 Lifetime mating performance

In the mortality control test OX4319L pupae were out-crossed to wild-type in pooled crosses, producing OX4319L heterozygous eggs. Concurrently, wild-type eggs were collected at low density. All eggs were counted into batches of approximately 100, and placed into individual plastic diet cups (exact number of eggs in cup recorded). Eggs were provided with tetracycline diet (replaced every 2 days) and unhatched eggs counted 4 days after setting up the experiment. Subtracting the number of unhatched eggs from those counted into the cup gave the exact number of first instar larvae in that replicate. Larvae were allowed to pupate and the number of pupae in each cup counted. Proportional survival to pupation in each cup was calculated and averaged across each genotype. The difference in survival to pupation between the two genotypes was used to correct for heterozygous mortality in the mating experiment. Twelve replicates were performed per genotype.

In the mating competitiveness test, OX4319L and wild-type eggs were reared off tetracycline. OX4319L eggs produced only male pupae, and wild-type pupae were sexed by hand. Once eclosed, 20 males from each genotype were placed in a 30 cm × 30 cm × 30 cm insect cage (Bugdorm) along with a Parafilm artificial leaf and a non-tetracycline sugar water source. Five hours later, 20 wild-type females were introduced. Parafilm and sugar water were replaced every 2 days until egg-laying ceased (6 collections). Eggs were reared on tetracycline diet in plastic pots. Once each collection had pupated, individuals were scored for fluorescence and this raw data corrected for heterozygote mortality. Corrected proportions of each genotype (heterozygotes and wild-type) reaching pupation were calculated and summed for each replicate then a value equivalent to an RSI score generated. Four replicates were performed. Analysis performed was identical to that in the previous section. Due to the differences in

experimental protocol, however, this estimate of RSI was not compared statistically to that previously produced for this line. Subsequent to this analysis, the changes in RSI calculated for OX4319L were plotted over the collection period in order to identify any potential great reduction in competitiveness over time.

2.4.7.4 Population genetics of transgene-associated fitness costs

In each experimental generation, the adult insects were housed for 1 week in a 30×30×30 cm netted cage (Bugdorm). Prior to introduction of these insects into the cages, they were sexed and screened as pupae for fluorescence and maintained as separate cohorts until eclosion. When all adults had eclosed, cohorts from each population were placed in their respective cages, with males being introduced first and females 2 h later. Two egg collections were made from each cage during this period, placed on diet and resulting pupae used to found the next generation. As egg collections were taken within approximately the first week after adult eclosion, these experiments did not seek to measure adult fitness costs which manifest after this point. In a mass-rearing setting, adults are rarely kept beyond the first week, when reproductive productivity is highest, and in the wild mean adult lifespans are expected to be less than 5 days (Furlong et al., 1995).

2.4.7.4.1 Selection on transgene under permissive conditions

Experiments: Insects were reared on tetracycline diet and adults given access to tetracycline laced sugar water (transgene permissive conditions). Initially, three independent cage populations (replicates) with a known transgene allele frequency (0.75) were established by crossing OX4319L heterozygous males with OX4319L

homozygous females. Two hundred of the resulting progeny from each cage were selected at random as pupae to found the following generation. After egg collections, each cage was frozen and dead adults collected. Of these dead adults, 96 were randomly selected and their gDNA extracted using the following method. Decapitated bodies were placed in individual wells of a 96-well PCR plate, each containing 75 µl of 100 mM NaOH. Plates were heated to 99°C for 30 min in a PCR block and then 15 µl of a second solution [250 mM Tris-HCl (pH 8.0) and 0.04% Phenol Red] was added to each well. Samples were genotyped by PCR for presence of the transgene and of the corresponding no-insertion wild-type allele using reactions analogous to those described in section 2.2.4. Genotyping results provided estimates of the number of individuals from each of the three genotypes (R/R, R/- and -/-), which was used to calculate generational transgene and wild-type allele frequencies. The experiment was run for 10 generations.

Analysis: Under neutral conditions it is assumed that, having reached Hardy-Weinberg equilibrium, allele and genotype frequencies will remain relatively stable, albeit subject to genetic drift. To test for significant trends in frequency change (a non-stationary process potentially resulting from selection), Mann-Kendall tests were performed. Where these implied that selection was occurring, two further analyses were performed. To test whether significant allele frequency trends could be statistically attributed to selection (rather than drift) a Frequency Increment test (FIT) was performed on mean generational transgene allele frequencies (Feder et al., 2014). The fitness of individual genotypes was also analyzed by comparing Corrected Rate of Increase (CRI) parameters using a Kruskal-Wallis Rank sum test and subsequent post-hoc testing using Tukey contrasts. CRI parameters compare observed and expected (under Hardy-Weinberg given the previous generations allele frequencies) genotype frequencies, with

the difference between these values indicating the direction and magnitude of selection against a particular genotype. The mean of these parameters provided an estimated rate of change for each genotype per generation. This allowed for the calculation of mean genotype-specific fitness values (*W*) relative to homozygous wild-type (Hamilton, 2009). As our best estimates of the fitness costs associates OX4319L, these genotype fitness values were subsequently used to predict longer-term fsRIDL allele frequency evolution for this line. Projected trajectories were calculated using a recursive version of the general selection model (Haldane, 1931) with initial simulated population genotype frequencies analogous to those used in experimental cages. The general selection model is a deterministic model of natural selection which allows the prediction of changes in genotype frequencies over time for a diallelic locus where the relative fitnesses of those genotypes are known. The model assumes an infinite population size, random mating, constant selection and no migration. For calculating times to extinction of modelled fsRIDL alleles, a hypothetical population size of 200 individuals was used to generate a minimum threshold allele frequency of $(1/ (2 \times 200)) = 0.0025$.

2.4.7.4.2 Selection on transgene under restrictive conditions

Experiments: Transgene restrictive conditions were created by rearing larvae and adults without access to tetracycline sources (diet or in sugar water). Three replicate populations were analysed with initial transgene frequencies of 0.25, established by crossing OX4319L heterozygous males with wild-type females; this represents the maximum starting allele frequency for a female-lethal transgene in the absence of artificial releases of transgene homozygotes. Replicate populations were observed until the transgene was no longer detected, and for one further generation to confirm

extinction. After pupation, 200 insects from each replicate were randomly selected, then scored for the fluorescent marker and sexed. Once eclosed, these adults were placed in new cages to found the next generation. Due to the high level of penetrance of the female-lethal trait in OX4319L under restrictive conditions (>99), and the relatively low initial transgene frequency, it was assumed that all transgenic individuals observed from Generation 2 onwards were heterozygous for the fsRIDL transgene.

Analysis: Under restrictive conditions it is hypothesized that the trajectory of fsRIDL transgene frequency change will be most prominently directed by dominant lethality in females, and the directional selection that this confers. Theoretically, this effect will result in a 50% reduction in fsRIDL allele frequency in each generation relative to the previous. However, this trajectory may be influenced by population-level stochastic effects as well as hypothetical transgene-associated fitness costs. A discrete-generation, stochastic model was developed (with advice from Dr Hongfei Gong) to simulate the potential trajectories of fsRIDL allele decay under situations analogous to our experimental populations (population size of 200, initial fsRIDL allele frequency of 0.25, restrictive conditions). This model allows for random variation in the sex ratios of the 200 individuals selected each generation. As it is assumed that only males can carry the fsRIDL allele, this also created variance in the number of individuals inheriting the fsRIDL allele each generation. No fitness costs other than those imparted by female-specific lethality were included and thus modelled trajectories represent the potential distribution of trajectories given stochastic variation in population sex ratio and subsequent fsRIDL allele inheritance alone. This was achieved by first estimating the probability that a male in a given generation *(t)* was transgenic *p(t)*, where the ratio of the number of transgenic males in the previous generation *M(t -1)* and the number of

total males in the previous generation *N(t -1)* is halved, representing the halving of the allele frequency each generation due to female-specific lethality (1).

$$p(t) = 0.5 \times \frac{M(t-1)}{N(t-1)} \tag{1}$$

This probability was then used to calculate the fsRIDL allele frequency in generation *t* *γ(t)*. Here N(t), the number of males in generation *t*, was generated using a Binomial (200, 0.5) distribution. The product of N(t) and p(t) was then divided by 200 to represent the proportion of transgenic males in the total population and halved to give the allele frequency (as transgenics are assumed to be heterozygotes) (2).

$$\gamma(t) = 0.5 \times \frac{N(t) \times p(t)}{200} \tag{2}$$

In Generation 1, fsRIDL allele frequency and sex ratio (male:female) were set at 0.25 and 0.5, respectively, to match the known starting conditions in the experimental populations. 250 independent populations were simulated and allowed to persist until fsRIDL allele extinction. Modelled results were compared to empirical data collected for each species in order to assess whether observed fsRIDL allele frequency decay fell within predicted variation in trajectories, given the assumptions of the model. Subsequently, the mean fsRIDL allele frequency reduction per generation was calculated for both the model and experimental data sets and compared using a Welch two-sample t-test. Modelling was performed in R and Excel.

Chapter 3

Chapter 3: Proof-of-principle cage studies

3.1 Introduction

Proof-of-principle studies are an important concept in scientific investigation. Their primary purpose is to provide an indication of whether a hypothesis will hold under a set of defined, preliminary conditions, usually prior to more realistic assessment. In terms of applied research, this model of incremental testing is vital, both to accurately identify faults in procedure or product and because full-scale testing is usually economically and politically unfeasible at an early stage. In applied entomology, proof-of-principle experiments often take the form of cage studies. In these cages, populations can be established under highly controlled conditions allowing the accurate assessment of treatment effects. Biotic factors such as parasitism, predation and disease can be avoided (or included) while climatic factors can be introduced along a gradient running from laboratory, through glasshouses to open field.

In SIT, cage studies form an integral part of the research process when developing a strategy for a new pest insect (Kuriwada et al., 2012; Gato et al., 2014), including lepidopterans (Hoa and Tien, 2001; Hight et al., 2005), and are recommended as part of the quality control process for commercial-scale release programmes in medfly SIT (FAO/IAEA/USDA, 2003). In developing fsRIDL strains, cage trials have been implemented to link observed female lethality off tetracycline to the population level; that is, to demonstrate the feasibility of population suppression (de Valdez et al., 2011; Ant et al., 2012). Cage studies have also been used for other genetic pest management strategies including those where transgenes are engineered to persist and spread within populations, for example X-shredding (Galizi et al., 2014), driving

homing endonuclease (Windbichler et al., 2011) and Maternal Dominant Embryonic Arrest (MEDEA) (Chen et al., 2007) genes. These enclosed, controlled studies are particularly relevant to genetic pest management strategies as in the majority of cases the testing of these technologies needs specific permits for field experiments beyond those required for contained use. Furthermore, regulatory agencies may require data from cage studies as part of the case for open field experiments.

Cage studies thus represent a highly appropriate next step in the testing of the fsRIDL allele in OX4319L. In this chapter the two primary pest management effects of an fsRIDL system will be tested in OX4319L, that is, its ability to suppress target pest populations and provide insecticide resistance management.

3.2 Population suppression

Efficient management of pest populations requires an understanding of their ecology and population biology. In the case of autocidal release programmes such as RIDL or SIT this is particularly pertinent, as management is achieved through the mating behaviour of the pest insect. This allows a good approximation of the efficiency of a release programme through established population biology models.

At its simplest, an autocidal release programme can be summarised by (1).

$$N_{g+1} = N_g R_g H_g \quad (1)$$

Here, the population size (in this case, of females) in generation N_{g+1} is a function of the number of females in the previous generation (N_g) the reproductive rate of those females in that generation (R_g), which will show density dependence, and the suppressive effect of released males (H_g), that is their progeny share (assuming a bisex

lethal mechanism). H_g is itself a function not only of the ratio of wild:sterile males in the population (release ratio) but also the respective sexual performances of those males. Of these two factors, modelling studies have shown that the competitiveness of males is of greater importance than the release ratio in determining suppression efficiency (Barclay, 1982; Ito and Yamamura, 2005). When competitiveness of released insects is equal to that of those in the wild, release rates as low as 1:1 are theoretically able to cause local population extinction, while overflooding ratios as high as 112:1 and 311:1 have in some empirical cases been unable to cause population suppression (Rhode et al., 1971; Cirio and Murtas, 1974).

The use of fsRIDL represents a special case of autocidal control where only the female cohort of the population is targeted directly. This has been found to be amongst the most efficient means of genetic population management (Schliekelman et al., 2005.; Thresher, 2007). In this case, the estimation of H_g is more complex as it must also take into account the reproductive contributions and relative fitness of heterozygous male transgene carriers. As accurate estimation of H_g and R_g together are necessary to calculate an effective release ratio, this calculation is thus more difficult for fsRIDL than bisex lethal systems.

In this experiment, four stable experimental wild-type populations of diamondback moth were established in glasshouse cages. Two of these were selected as control cages, while the other two were treated with weekly introductions of OX4319L males. Population dynamics were compared between treatment and control populations. Transgenic males were released into treatment cages at a rate of 10:1 (10 OX4319L males released for every wild-type male eclosing – also known as the recruitment rate). This 10:1 ratio is in keeping with release rates used in previous cage suppression studies investigating the use of transgenic female targeting alleles - 3:1 (Galizi et al., 2014),

10:1 (Ant et al., 2012; Leftwich et al., 2014) and 8.5-10:1 (de Valdez et al., 2011), and much lower than the sterile:wild ratios that successful SIT programmes have aimed to achieve against other Lepidoptera: pink bollworm, (60:1) (Walters et al., 2000); codling moth, (40:1) (Proverbs et al., 1982); and painted apple moth, (100:1) (Suckling et al., 2002; Wee et al., 2005). As such it should provide an effective comparative test of this strain's population suppression capacity.

3.2.1 Results and Discussion

Releases of homozygous OX4319L males into the two experimental cages began 9 weeks after the initial wild-type introductions into the cages. At this point consistent egg counts between weeks indicated that the population size in each cage had reached equilibrium (Figure 3.1A). The first re-introductions of transgenic larvae into the two treatment cages took place 2 weeks later (Figure 3.1C), indicating successful mating by OX4319L males. The proportion of re-introduced pupae that were transgenic increased as releases continued into the treatment cages, eventually reaching 100% 7 and 9 weeks after OX4319L releases began.

The increasing introgression of the female lethal transgene into these treatment populations had a substantial effect on population sex ratio and reproductive capacity. By week 15 (6 weeks after OX4319L releases began) the number of dead adult females collected in each of the treatment cages had decreased considerably relative to that of control cages (Figure 3.1B), concurrent with a reduction in the reproductive output of these cages (Figure 3.1A). As the generation time (egg to egg) of the insects in these experiments was approximately 3 weeks, these time periods fit the hypothesis that introductions of the OX4319L transgene into the treatment populations were causing

reductions in the number of females reaching adulthood, and thus the number of eggs being laid. Ten weeks after OX4319L releases began (week 19) the reproductive output of both treatment cages had dropped to 0, and no dead female adults were collected after this point. In this experimental protocol this equates to approximately 3 generations. Cages were continued for a further 2 weeks after egg-laying ceased, indicating that their populations were extinct.

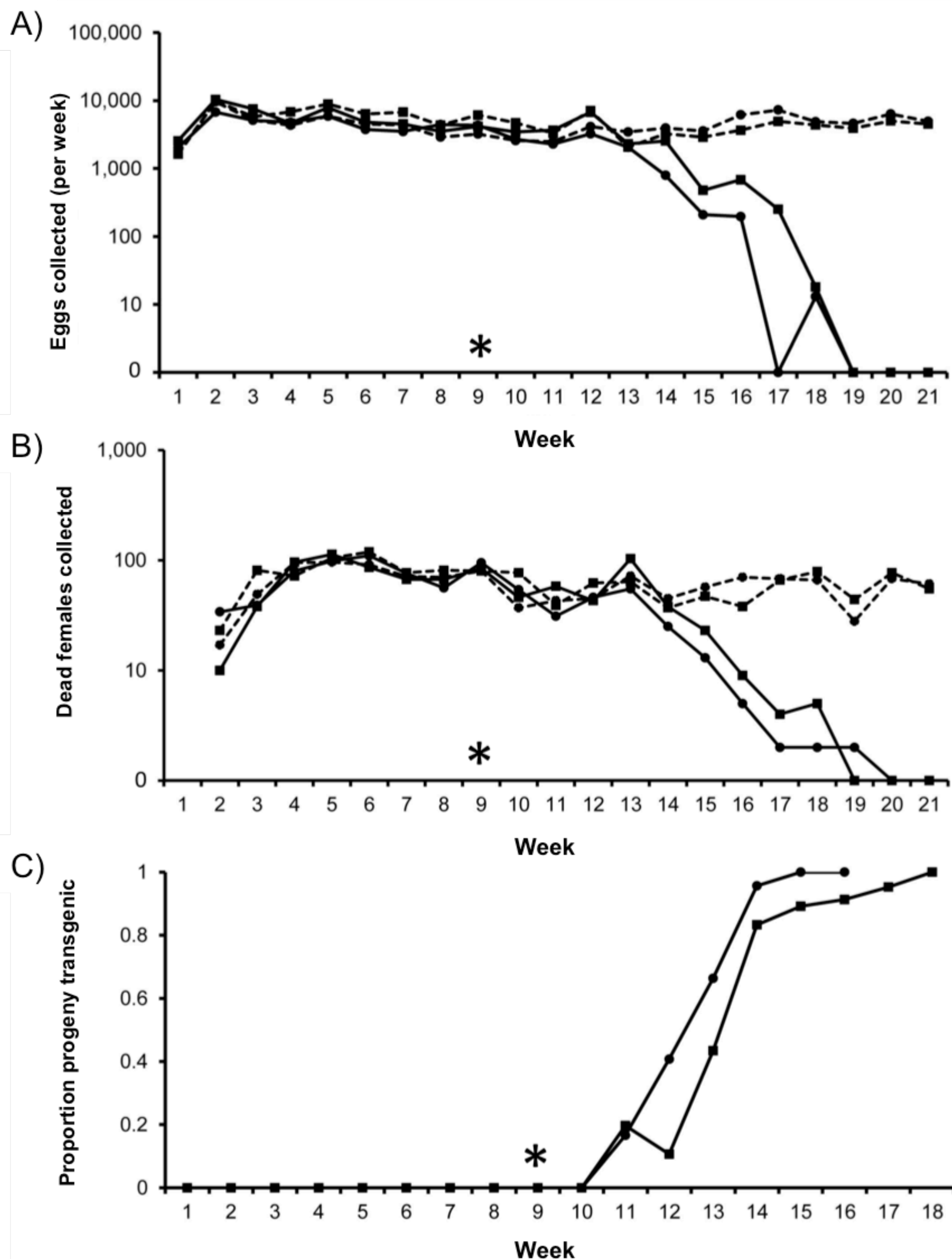


Figure 3.1 Suppression of caged populations of diamondback moth by weekly introduction of OX4319L males. Graphs showing (A) number of eggs collected, (B) number of dead females collected weekly from cages, and (C) proportion of cage progeny that were transgenic re-entering the cages, over the experimental period. Solid lines represent OX4319L treated populations (Cages 1 and 2, circular and square data-points, respectively). Dashed lines represent untreated control populations (Cages 3 and 4, circular and square data-points, respectively). In week 9, return of pupae into control cages and treatment cages was made proportional and release of OX4319L males into treatment cages began (marked by asterisk).

The results of this experiment allow an assessment of the relative competitiveness of this strain by comparing the times to extinction (here defined as the generation where egg production ceased) with those previously conducted on other transgenic female-specific suppression strains (Table 3.1)

Table 3.1: Comparison of the number of generations required to cause extinction calculated from four different cage population studies involving the release of transgenic insects carrying female-specific transgenes. Generations were given in the publication (Galizi et al., 2014) or calculated from raw data based on experimental protocol (others). Number of replicates noted in brackets behind generations to extinction. The female flightless system causes deleterious transgene expression in the wing muscles of females off tetracycline. The X chromosome-shredder strain contains an autosomal insertion of a homing endonuclease gene which cleaves a sequence specific to the X chromosome. Thus, male carriers of this insertion produce only Y bearing gametes. The three other studies utilised a positive feedback tTAV expression loop under the regulatory control of sex-alternative splicing introns. Collectively, female flightless and the three female lethal systems fall under the acronym fsRIDL.

Publication	Species	System	Release ratio	Average generations to extinction
(de Valdez et al., 2011)	*A. aegypti*	Female flightless	8.5-10:1	4.66 ± 0.88 (3)
(Galizi et al., 2014)	*Anopheles gambiae*	X chromosome - shredder	3:1	4.33 ± 0.88 (3)
This study	*P. xylostella*	Female lethal	10:1	3 ± 0.0 (2)
(Ant et al., 2012)	*B. oleae*	Female lethal	10:1	3 ± 0.0 (2)
(Leftwich et al., 2014)	*C. capitiata*	Female lethal	10:1	3 ± 0.0 (2)

The time to extinction in OX4319L fits well with those previously displayed by other strains. This implies that the OX4319 system is comparable to other transgenic suppression technologies which are currently being considered. This is the case even though the experimental protocol for treatment cages in this study (tri-weekly releases of insects back into cages with only a single OX4319L release per week) would have proved a sterner and more realistic test of a strain's mating ability (due to the constant

presence of newly eclosed wild-type males in these cages) than the other experiments where transgenic and wild-type insects were introduced together, once per week or, in the case of the X-shredder system, once per generation. One point which should be noted, however, is the relative effectiveness of the X-shredding system compared with the female lethal (or effectively sterile in the case of female flightless) fsRIDL strains. This strain was competitive at release rates approximately 3× lower than the other strains compared. This discrepancy highlights the potential advantages of using a transgenic system which acts at the pre-zygotic stage as opposed to those which are expressed in the inheriting individual (Figure 3.2), at least in pests whose populations do not show high degrees of density-dependence. In these cases it may be preferable to use later-acting systems in order to maximize the population limiting effects of negative density-dependence (Phuc et al., 2007b). Nonetheless, in the X-shredding strain, all male carriers of the construct (including heterozygotes) will produce only male gametes and thus produce only male offspring (Figure 3.2A). In the other systems compared here, heterozygote carriers will pass on the female-specific transgenes to only 50% of their offspring (and thus only half of their female progeny will be non-viable) (Figure 3.2B). Thus the sex-ratio of the population is distorted to a greater extent per X-shredding allele released compared with those other systems. This issue can be mitigated to a certain extent by the release of fsRIDL strain males carrying multiple independently segregating copies of the transgene, although this is likely to involve increased transgene-associated fitness costs (Figure 3.2C). Conversely, however, due to the current non-repressible design of the published X-shredder system it is impossible to create transgene homozygotes (as carrier males cannot produce X bearing sperm – even in stock colonies). As such, currently, twice as many transgene alleles can be carried into target populations per fsRIDL insect released than in the X-shredding system.

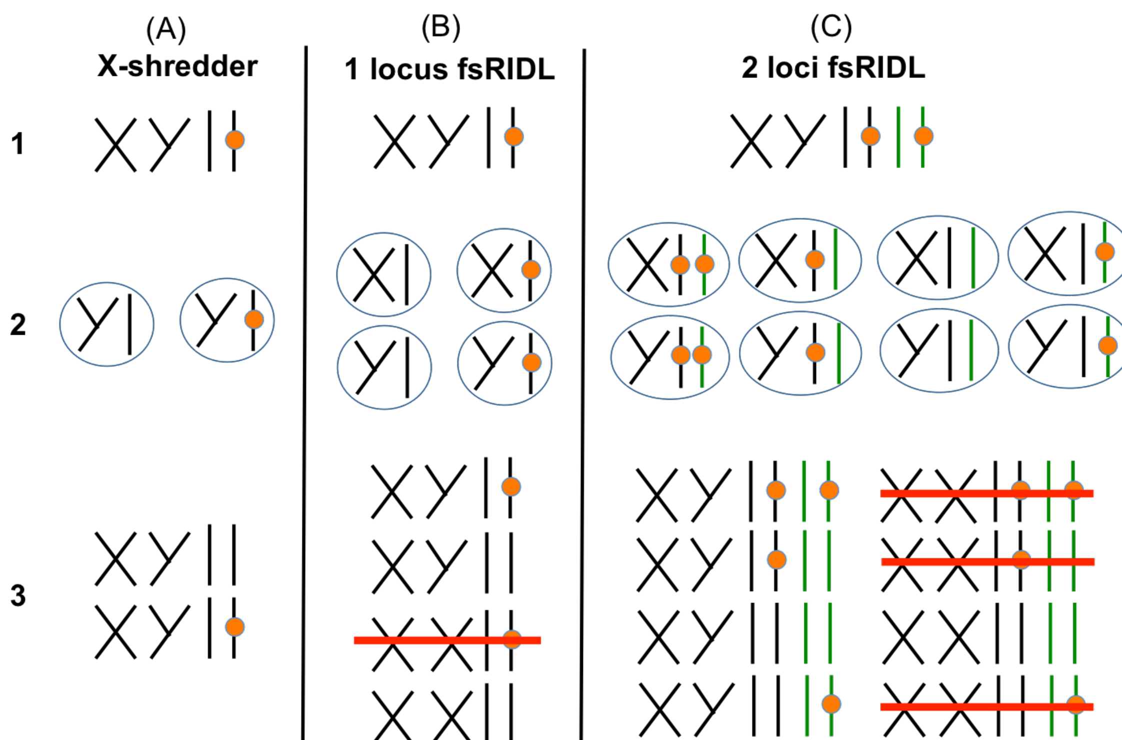


Figure 3.2: Diagram comparing the consequences of three female-specific suppression strategies (A: autosomal X-shredder endonuclease, B: Single insertion female-lethal gene and C: Two non-linked copies of B) on the sex ratios of the F2 generation of a release programme. X and Y represent sex chromosomes and vertical bars represent autosomal chromosomes. Transgene insertions are represented by orange circles. For simplicity, males are assumed to be heterogametic although this is not the case for lepidopterans. Non-viable progeny are those which do not survive, or are unable to reproduce and are represented by red horizontal bars through genotypes. 1: the F1 generation of a release programme. In all strategies, transgenics are male heterozygotes resulting from the mating of released homozygous males and wild-type females. 2: Genotypes of the gametes produced by these males. (2A) As cleavage occurs during spermatogenesis, all X bearing sperm are affected regardless of whether they carry the autosomal transgene. Thus only Y-bearing sperm are functional. (2B and C) sperm are produced in mendelian ratios. 3: The progeny produced from the fertilization of a wild-type female by the sperm in 2 (the F2 generation). (3A) Only males are produced (sex ratio = 100:0), 50% are transgenic. (2B) 50% of progeny are transgenic, with a 50:50 sex ratio this results in the death of 50% of females and 25% of total progeny (post-selection sex ration of 66:33). (3C) 75% of progeny are transgenic and only 25% of females survive. 37.5% of total progeny do not survive (post selection sex ratio of 80:20).

It should be noted that the point of this experiment and the comparisons made above was not to directly quantify fitness costs associated with OX4319L, but rather to demonstrate its suppression potential. A recent modelling study by Robert et al. (2012) explored the experimental design of cage studies such as that used here and previously for mosquitos (de Valdez et al., 2011). It found that at high release ratios (10:1 and above), fitness costs are unlikely to result in increased times until population extinction

unless the costs are relatively large (transgenic male RSI equivalent of 0.25 or less) and replication is high. These results are interesting as they contradict those indicating the relative importance of insect fitness over release rates (Barclay, 1982; Ito and Yamamura, 2005). Taking the results of Robert et al. into account, it is unlikely that a significantly increased time to extinction would have been observed in this experiment (as the release ratio was 10:1, in keeping with previous experiments using this protocol). However, it is possible from these results to state that the fitness costs associated with this line do not appear to be large under these conditions (eg. resulting in an RSI of 0.25 or less).

As a proof-of-principle study these results display the ability of the OX4319 female-lethal allele to cause substantial reductions in a target population. With such transgenes applied against isolated populations, as in the caged experiments reported here, local extinction of the target pest by this means is achievable. However, as in any proof-of-principle study, a number of sacrifices were made in terms of biological realism. These limitations include both biotic and abiotic factors which may cause variations from the results described here if this line were to be employed in the field. These can be divided into three main components: i) genotype-level interactions in competitiveness; ii) density-dependent population factors; and iii) effects of immigration.

i) **Genotype-level interactions in competitiveness**. These experiments were conducted in a glasshouse under controlled conditions. In the field, conditions will vary considerably. Released insects will be exposed to greater abiotic variations in temperature, humidity, precipitation, photo-intensity, wind, day length, geography and biotic variations including local sex-pheromone components and reception, flight

ability, predator presence, and sugar resource availability. It is unknown to what extent these factors will interact at the genotype level, but it is likely that released insects, with their laboratory-adapted genetic backgrounds, will be at least somewhat maladapted to such conditions. Additionally, how these factors will affect the genetic hybrids formed when released and wild insects mate in the field is unknown. All of these components may affect the relative competitiveness of transgenic insects and thus reduce field H_g. The level to which H_g of OX4319L might be affected in the field is difficult, if not impossible, to predict at this stage and can only be accurately assessed under more realistic biological settings.

One conclusion that can be drawn regarding competitiveness, however, is on the larval diet used to rear released insects for this experiment. Due to issues with larval rearing, OX4319L males were reared on tetracycline diet and sexed by hand prior to release. This controlled for the deleterious effects of manual handling (as reintroduced populations were similarly handled) but may have increased the fitness of these released insects (as it would have potentially suppressed off-target tTAV expression in males). While certainly a limitation of this study, the results described in Chapter 2 and Section 3.3 (where OX4319L released males were successfully reared off tetracycline and suppressed target populations) suggest that, at least qualitatively, this did not affect the results of this experiment.

ii) **Density-dependent population factors**. Many insect populations (Stiling, 1988), including lepidopterans (Underwood, 2010), show some degree of density-dependent regulation. These factors can have substantial effects on the dynamics of these populations and can significantly alter R_g and, consequently, suppression efficiency (Robert et al., 2012). While this experiment did introduce an initial carrying

capacity (K) into the experimental design through the tight control of pupal reintroductions into cages, true density dependence was not evident. Negative density dependence (overcrowding) would have resulted in a release from intraspecific competition at lower pest population densities, a subsequently higher reproductive potential per female, and thus a reduced suppression efficiency. However, during the suppression phase, in the treatment cages, the contribution of a single female to population size in generation g^{+1} (in terms of the number of pupae reintroduced) was theoretically equal, regardless of population size in the preceding generation (g). This contribution was reduced compared to that which would have occurred if all pupae reared from collected eggs were reintroduced instead of a ratio set around 200. The negative "density dependence" in this experiment thus consists of a flat rate of population reduction for each and every female across time rather than the varying behavior of population increase we would expect at low and high densities (Figure 3.3).

Similarly, the effects of positive density dependence (undercrowding or Allee effects) were absent from this experiment. In the wild, an insect population might suffer from decreased population growth rates at lower densities if this made mates more difficult to find, reduced their genetic heterogeneity, making them more vulnerable to plant secondary compounds or insecticides, or prevented the satiation of natural enemy complexes (Courchamp et al., 1999; Tobin et al., 2011). In the relatively small and homogenous cages used in this experiment it is unlikely that any of these factors would have had a significant effect at low population sizes (Figure 3.3). In fact, it is likely that these spatial conditions increased the frequency of multiple mating compared to what would be expected under field conditions.

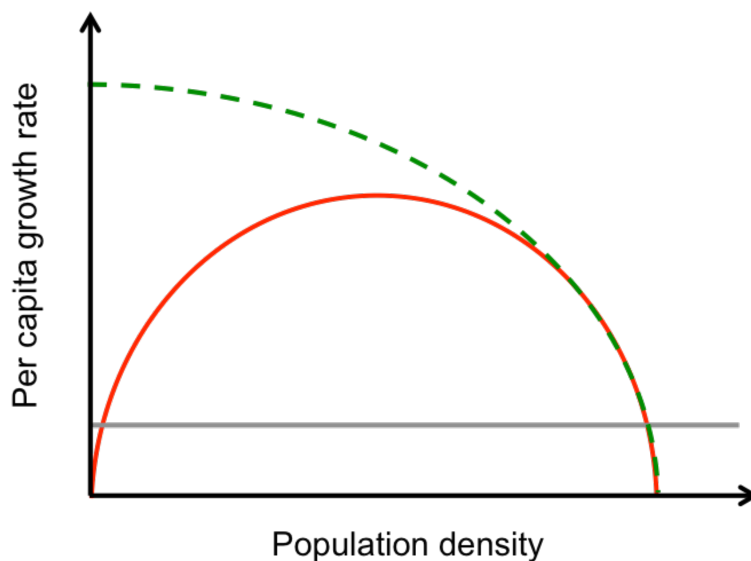


Figure 3.3: Relationship between population density and the per capita growth rate (hereby R_0) of that population for two forms of density dependence (green and red lines) and the present study (grey line). If negative density dependence is present (green line), R_0 is high at low densities but asymptotes at the population carrying capacity. If both positive and negative density dependence are acting, R_0 at small and large population sizes will be low (red line). In this experiment R_0 was independent of population density and was set at a low level (grey line).

Diamondback moth populations are known to exhibit negative density dependence, primarily due to intraspecific competition and parasitism (Karimzadeh et al., 2004; Wang et al., 2004). The role of positive density dependence, as is often the case, is more difficult to assess. However, it is generally thought that the frequency of encountering receptive mates decreases at lower population densities. This has been used to explain the non-establishment of introduced parasitoid species in classical biological control efforts (Stiling, 1988). It is difficult to predict what effects these processes will have on suppression efforts in the field. However, a characteristic of autocidal control strategies is that, as a target population is reduced, the suppression pressure on that population will increase (given a constant release rate) (Knipling, 1979). It is thus unlikely that negative density dependence will pose a challenge to the use of OX4319L in the field, assuming that initial release rates are set at a suitably high level.

iii) **Immigration.** Immigration is a major concern in autocidal release programmes. Even small numbers of immigrants or non-sterile mated females can cause significant increases in eradication times, if not the failure of suppression programmes altogether (Prout, 1978; Lance and McInnis, 2005; Robert et al., 2012). In this experiment, each population was closed prior to OX4319L transgenes being introduced. However, in an extreme scenario the introduction of a single wild-type female each week into treatment cages would theoretically have made eradication impossible. Thus, it is likely that the suppression effects observed for this strain in these cage trials would be lessened when applied in the field against non-isolated pest populations or when pests migrate between areas. However, even though the diamondback moth is known to travel large distances in some circumstances (Smith and Sears, 1982; Harcourt, 1986; Honda et al., 1992; Chapman et al., 2002), it does not typically travel far when host plants are available (Cameron et al., 2002; Mo et al., 2003). Although complete isolation of diamondback moth populations would be unlikely in most scenarios (with the possible exclusion of early-season populations which have overwintered), efficient control of target populations is likely achievable with some degree of population isolation and/or with sufficient scale of application to reduce edge effects. Moreover, the SIT has previously demonstrated efficient control of other lepidopteran pests - codling moth and pink bollworm - that are able to travel relatively large distances (Bariola et al., 1973; Mani and Wildbolz, 1977).

3.3 Insecticide resistance management

Insecticide resistance poses a major threat to global food production, as well as the management of disease vector populations (Whalon, 2008). Loss of action in insecticides not only has economic and social, but ecological consequences, as pest managers resort to increased spraying regimes and older, less specific compounds. Lepidopterans in general, and the diamondback moth in particular, are highly adept at developing resistance to these substances.

Resistance is a particular concern if it forms in response to novel technologies. These newer compounds and strategies have been developed to reduce grower reliance on older, more harmful substances and to increase the sustainability of agricultural systems. Their loss would be of considerable concern to these efforts. An example of such a novel technology is *Bt* crops. *Bt* crops are engineered to express insecticidal Cry toxin proteins from the soil bacterium *Bacillus thuringiensis*. A major advantage of these crops is their low environmental impact, with the effects of the toxin limited to target species both taxonomically (due to the high species-specificity of Cry toxins), and ecologically (as toxin expression is limited to crop tissue, which needs to be ingested to take effect). Cry toxins cause lethality by binding to receptors within the apical membranes of midgut cells and causing cell lysis through pore formation. Prior to this, ingested Cry proteins (prototoxins) must go through a complex series of chemical reactions inside the midgut as well as interactions with multiple other midgut membrane proteins in order to become activated (Pardo-López et al., 2013). The complexity of these interactions makes Cry toxins highly specific. Cultivation of *Bt* crops has increased rapidly over the past two decades, primarily targeting lepidopteran and coleopteran pests. In 2013, total global cultivation reached 75 million hectares, making

it the most rapidly adopted crop technology in recent history (James, 2013). The rapid adoption of *Bt* cotton, for example, by growers worldwide since the mid-1990s has resulted in sharp reductions in the volumes of conventional insecticides applied, the resurgence of natural enemy communities, and increased yields (Shelton et al., 2002; Cattaneo et al., 2006; Lu et al., 2012).

Resistance in pest populations is an ongoing threat to transgenic *Bt* crop efficacy (Tabashnik, Brevault et al. 2013). The most widely applied resistance management method is the high dose/refuge strategy (Bates et al., 2005). Here the Cry toxin is expressed at sufficiently high levels for resistance to be functionally recessive and a proportion of the crops grown are non-*Bt* varieties (known as the refuge) allowing *Bt* susceptibility alleles to persist within the field pest population. If the refugia comprise a sufficient proportion of the overall crop, and mating occurs between pests from *Bt* and refuge areas, this results in the maintenance of a pest population that is heterozygous for *Bt* resistance, and therefore *Bt*-susceptible. Although the high-dose/refuge strategy has been largely successful in delaying *Bt* resistance, the development of *Bt*-resistant populations has now been reported in the field (Tabashnik et al., 2013). Recommendations on refuge size vary considerably between crop species and the number of engineered *Cry* genes, but may be as high as 50% of the total crop, potentially exposing large areas to economic levels of damage and making grower compliance unlikely (Tabashnik and Gould, 2012). As such, new means of managing *Bt* resistance, especially those which would function as effective pest control measures in their own right, would be of great economic and ecological benefit.

Modelling studies suggest that the release of insects carrying insecticide susceptibility alleles (s) can provide a resistance management effect analogous to that of the refuge in the high dose/refugia strategy (Alphey et al., 2007; Alphey et al., 2009).

Mating of released (ss) and resistant (rr) insects in the field results in the production of susceptible heterozygotes (rs), which are selected out of the population. As male fsRIDL heterozygotes are not subject to engineered lethality, their survival in the field will result in the introgression of susceptibility alleles into the target population. Results from these studies suggest that both the release of wild-type insects and fsRIDL males can have powerful resistance management effects at release rates significantly lower (<1:2, e.g. one released male for every two wild-type eclosing males) than those currently employed in radiation-based SIT programmes (Alphey et al., 2007). The simulated release of wild-type insects was predicted to prevent the spread of resistance to a greater degree than release of fsRIDL males (as susceptibility alleles are passed down through both sexes), however, fsRIDL males were more effective at limiting population growth rates.

In practice, the release of fertile male and female pest insects is unlikely to be acceptable to growers and thus, much of this modelling work has focused on the potential benefits of fsRIDL releases versus refuges in delaying resistance. Simulations predicted that minimum refuge size requirements could be significantly decreased if low-rate weekly fsRIDL releases were made into a *Bt* crop area (Alphey et al., 2007; Alphey et al., 2009). For an initial field *Bt* resistance allele frequency of 0.001 [as observed for tobacco budworm *Heliothis virescens* (Fabricius) (Gould et al., 1997), european corn borer *Ostrinia nubilalis* Hübner (Stodola et al., 2006) and diamondback moth (Ahmad, 1999)] and an fsRIDL male release ratio of 1:4, refuge requirements could theoretically be reduced to as little as 2-3% of those required if no releases were made. The authors suggest that with an initial 10% refuge, this would require only 0.2-0.3% of a field to consist of non-*Bt* plants which, in some cases, may be provided naturally by non-crop host plants. Their results also highlight the advantage of using

fsRIDL releases in driving down the levels of resistance (as opposed to limiting its spread). This could be useful as a remedial action if resistance was already widespread (in which case refuges can have limited effectiveness).

As this resistance management strategy has only ever been modelled, it remains unknown whether it will translate into reality, and to what extent real-world divergences from modelled parameters will impact its effectiveness. In particular, the relative fitness of resistant homozygotes on *Bt* plants was set well below 1 (0.1 (Alphey et al., 2007) to 0.6 (Alphey et al., 2009) and the fitness costs associated with the fsRIDL transgene were predicted to be fairly minimal (0.1 in both studies). Changes in either of these parameters could potentially affect resistance management both quantitatively and qualitatively. The experiments conducted here set out to investigate as a proof of principle the ability of sustained fsRIDL transgene introgression to delay insecticide resistance using real-world parameters. In doing so, a model system utilizing *Bt*-engineered broccoli (Metz et al., 1995; Cao et al., 2002) and a *Bt*-resistant diamondback moth strain was employed, which has been used effectively in the past to explore *Bt* crop resistance management strategies (Shelton et al., 2000; Tang et al., 2001; Zhao et al., 2003; Zhao et al., 2005).

This experiment sought to investigate the potential synergy that exists between *Bt* transgenic crops and insects carrying fsRIDL transgenes. Four treatments were assessed in multi-generational glasshouse cage experiments: 1) *Bt* broccoli plants only; 2) *Bt* broccoli plants + low-rate OX4319L release; 3) wild-type broccoli plants + low-rate OX4319L releases; and 4) wild-type broccoli plants + high-rate OX4319L releases. Release rates of OX4319L males were informed by a continuous deterministic population density model (see Materials and Methods 3.4.2.5). Using this model, the low OX4319L release rates chosen (3-5:1) were predicted to keep population density

relatively constant when combined with *Bt* selection (*Bt* broccoli plants), but unable to prevent population growth on their own. High release rates (20-40:1) were predicted to be able to cause population suppression in the absence of other control measures. Thus, treatments were designed such that each pest control method on its own (*Bt* and OX4319L release at low levels) would be expected to be insufficient for population control. The effects on both population regulation and resistance management when both treatments were combined could then be compared with that of each one applied singly. Each treatment was seeded with adult moths from a wild-type hybrid strain with a predicted Cry1Ac (*Bt* toxin) resistance allele frequency of 0.25. Population density and fluorescence ratios were tracked in each generation and resistance levels were assessed in the final generation of each treatment.

3.3.1 Results and Discussion

In cages with *Bt* broccoli and no OX4319L releases (Treatment 1), populations were well-controlled in Generation 1 (Figure 3.4A), presumably associated with the initially high frequency of genotypes susceptible to the *Bt* toxin. The subsequent populations in these cages, now highly *Bt*-resistant, then increased rapidly until Generation 4. Similarly, low-level releases of OX4319L males into populations reared on wild-type broccoli (Treatment 3) were not effective at preventing population growth, and this treatment was terminated at Generation 3. When *Bt* broccoli and low OX4319L releases were combined, however, the caged populations were well controlled throughout, with density increasing only slowly at each generation (Treatment 2). In Generation 3, the mean peak population density recorded for the *Bt* broccoli only and the low-rate OX4319L only treatments did not differ significantly (Contrast 1 – see

below). Concurrently, the mean peak population density in the combined OX4319 + *Bt* broccoli treatment was significantly lower than the low-rate OX4319L only treatment (Contrast 2), but not significantly different to that of the *Bt* broccoli only treatment (Contrast 3) (Contrast 1: diff = 337, p = 0.133; Contrast 2: diff = -545, p = 0.0272; Contrast 3: diff = -207, p = 0.315). By Generation 4, however, populations in the *Bt* broccoli only treatment were significantly larger than those where *Bt* broccoli was combined with low-rate OX4319L releases (t = -4.84, p = 0.0084). The high rate of OX4319L releases (Treatment 4) performed similarly to the combined OX4319L + *Bt* broccoli treatment, but by Generation 4 had started to reduce the caged population in the last remaining cage. Due to the lack of replication in this treatment in Generation 4, it was excluded from statistical analysis.

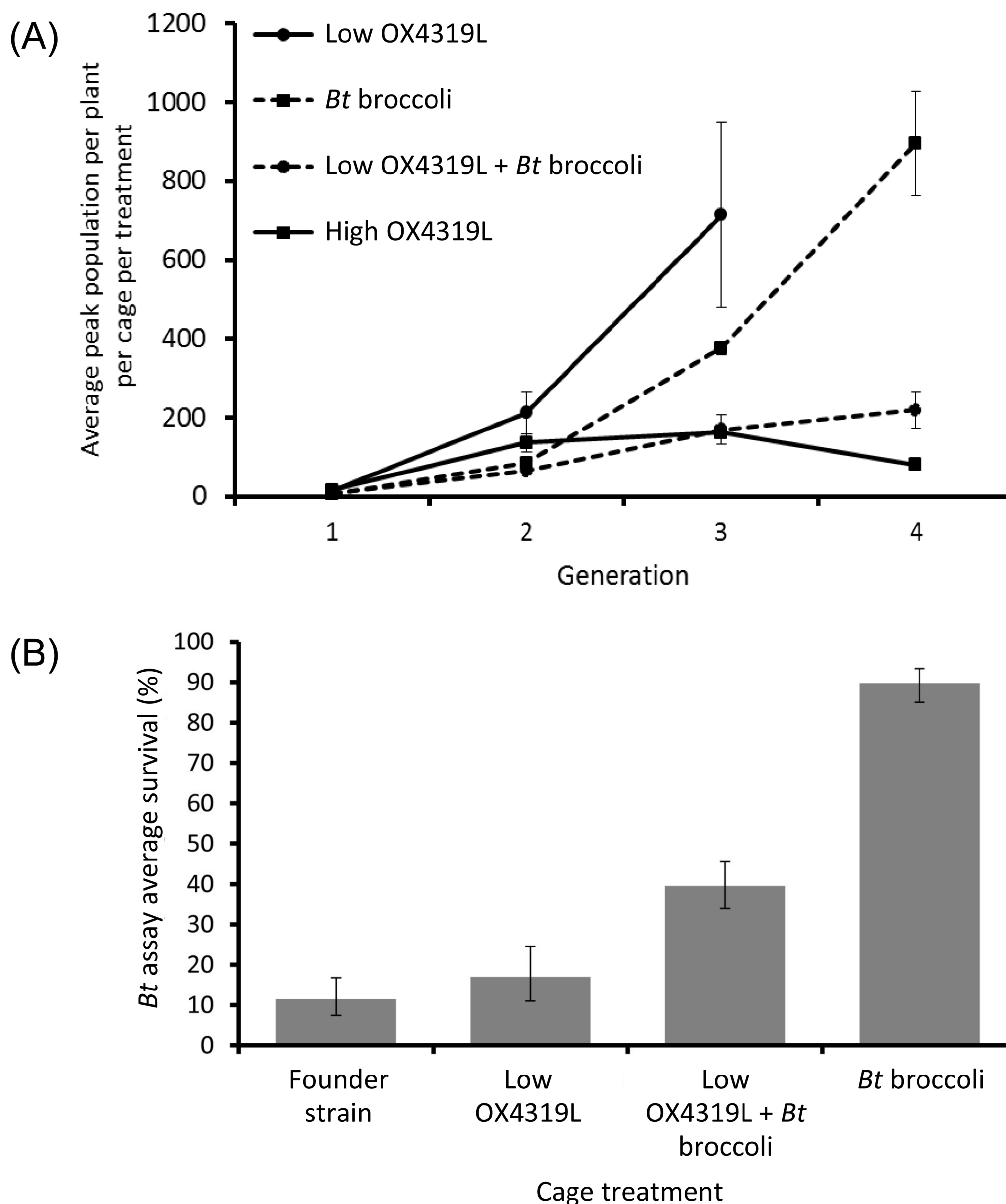


Figure 3.4. Graphs summarising the effects of *Bt* broccoli and OX4319L male releases on caged diamondback moth populations over multiple generations. (A) Graph shows the mean peak population size per plant, per generation, in four experimental treatments over the experimental period: Treatment 1, *Bt* broccoli plants, no OX4319L release; Treatment 2, *Bt* broccoli plants, low-rate weekly OX4319L releases; Treatment 3, wild-type broccoli, low-rate weekly OX4319L releases; and Treatment 4, wild-type broccoli, high-rate weekly OX4319L releases. Means were calculated from three experimental cage replicates, with the exception of Treatment 2 and Treatment 4, which were reduced to two and one cage replicates in Generation 3, respectively. Error bars represent standard error of the mean. (B) *Bt* survival assays. Mean survival of third-instar larvae from three experimental cage treatments and the founder strain used to begin these experimental treatments when exposed to a discriminating dose of *Bt* in artificial diet assays. For each cage, two *Bt* assays and one control assay were performed. *Bt* assays in each cage were summed and means represent averages of each set of treatment cages corrected for control mortality. The assays took place using individuals from the final generation in which each treatment was run or, in the case of the founder strain, in the generation prior to the start of the experiment. Survival was corrected for control mortality prior to analysis and error bars represent Pearson's Exact Confidence Intervals. The low OX4319L release (Treatment 2) was not significantly different from the founder strain; other pairwise comparisons are significantly different (Table 3.2).

Larval *Bt* survival assays conducted in the last generation of each treatment, and on the founder strain used to seed each cage in Generation 0, showed a significantly reduced proportion of resistant individuals in the combined OX4319L + *Bt* broccoli treatment relative to *Bt* broccoli only (Figure 3.4B). Survival rates on *Bt* were low and not significantly different between the founder strain and the low-rate OX4319L only caged populations (Table 3.2). Both treatments in which populations were exposed to *Bt* selection displayed significantly higher levels of survival on *Bt* compared to those where wild-type broccoli plants were used, indicating high levels of selection against susceptible genotypes in these treatment cages. However, significant differences between *Bt* treatments were apparent. In the *Bt* broccoli only treatment, *Bt* resistance rose to a high level in the population, as indicated by the high percentage survival rate (89.7% - 4.7, + 3.6 Pearson's Exact C.I). In the combined OX4319L + *Bt* broccoli treatment, the survival rate was significantly lower (39.5% - 5.6 + 6.0, Pearson's Exact C.I), implying a significantly higher frequency of *Bt*-susceptible individuals (and thus *Bt* susceptibility alleles) in these populations.

Table 3.2. Pairwise comparisons of survival assay data (from experiments described in Figure 3.4B). Treatment numbers given in brackets after names. Output was generated using an omnibus logit model for categorical data analysis, followed by post-hoc analysis using Generalised Linear Hypothesis Testing.

Treatments compared		**Z Value**	**P Value**
Founder strain	Low OX4319L	1.212	0.605
Founder strain	Low OX4319L + *Bt* broccoli	5.016	< 0.001***
Founder strain	*Bt* broccoli	8.557	< 0.001***
Low OX4319L	Low OX4319L + *Bt* broccoli	3.323	0.0048**
Low OX4319L	*Bt* broccoli	-6.682	< 0.001***
Low OX4319L + *Bt* broccoli	*Bt* broccoli	-5.594	< 0.001***

The results of these *Bt* assays provided an opportunity to estimate the final resistance allele frequency in each of the *Bt* treatments. This was complicated, however, as only around 90% of larvae from the *Bt* only cages survived. After four generations of strong *Bt* selection, and an assumed 0% survival of heterozygotes, it would have been expected that 100% of this population would have been able to survive a discriminating dose. There are two possible explanations for this discrepancy: 1) That susceptibility alleles were able to survive at relatively high levels in *Bt* cages, i.e. that selection by *Bt* broccoli plants in these cages was incomplete; or 2) that a proportion (approximately 10%) of homozygote larvae were unable to survive the *Bt* assay. The latter is more likely. Every *Bt* plant used in this experiment was tested for toxicity prior to being used. This was done using large numbers of homozygote susceptible larvae. Only plants which showed complete lethality at this stage were used in the experiment. Thus, survival of this genotype on these plants can be discounted. Additionally, the lethality of these transgenic plants to heterozygotes was tested in leaf assays prior to this experiment (and has been conducted multiple times by researchers in the lab of Prof. Tony Shelton) and was found to be complete. If the 90% survival figure was due to the survival of heterozygotes alone, this is highly likely to have been observed during these preliminary assays. Finally, it has been reported that this assay tends to underestimate resistance levels (personal communication, Prof. Tony Shelton). Assuming that 10% of homozygote larvae do not survive such a "discriminating dose" it is possible to correct the survival rates of the low OX4319L + *Bt* broccoli treatment assays. With a survival rate of 39.75% in this treatment it is expected that 43.73 % [(39.75/100) × 10 + 39.75] of larvae in this assay were resistant homozygotes. The remainder of the larvae in this assay (56.27%) would have been susceptible heterozygotes (arising from the mating of OX319L males and wild-type females) and therefore the frequency of resistant alleles in

this population would have been 0.72 [43.73 + (56.27/2)]. These figures fit well with those estimating the relative fitness of OX4319L males in these cages from fluorescence levels (Figure 3.5). In the fourth generation it can be seen that approximately 56% of eggs sampled in the OX4319L + *Bt* broccoli treatment were transgenic (heterozygotes).

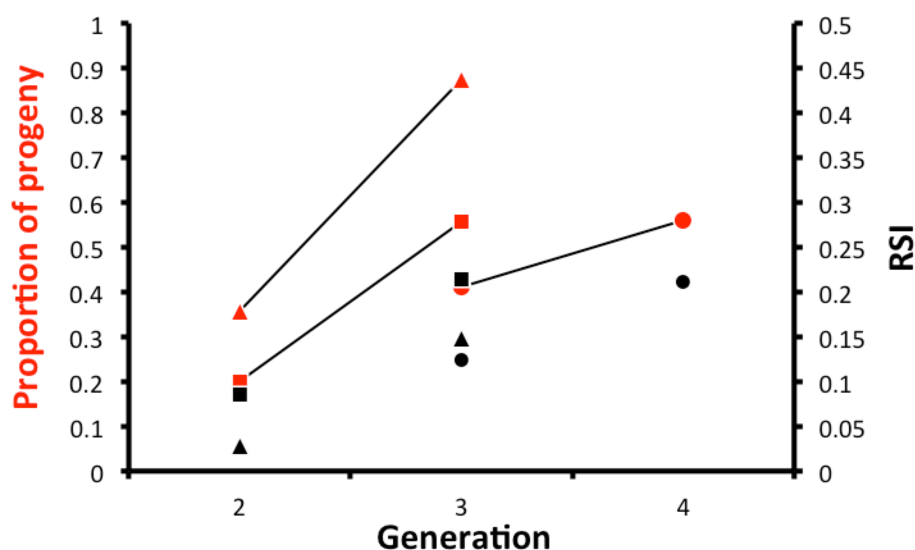


Figure 3.5: Graph showing the fluorescence ratio of progeny (scaled on left Y axis – red symbols and connected by lines) and the RSI (Relative Sterility Index) calculated from these ratios and taking into account release rates (scaled on right Y axis – black symbols) observed in samples of larvae taken from 3 generations in 3 OX4319L cage treatments. Triangles represent the high OX4319L release ratio, squares the low OX4319L release ratio and circles the combined OX4319L + *Bt* broccoli treatment. Due to issues with egg collections there are no data for the combined OX4319L + *Bt* broccoli treatment in the second generation.

As previously discussed, the calculation of competitiveness scores from fluorescence ratios in generations other than the first is complicated in fsRIDL strategies. In subsequent generations, the fluorescent progeny observed will have been contributed not only by released males, but also by their male heterozygous progeny from previous generations. The exact contribution of the heterozygous males is difficult to predict as they are likely to be more competitive than their homozygous, artificial diet reared forefathers, but will be competing against both wild-type and transgenic males.

The interpretations of the current results are complicated by two other factors. Firstly, improvements in rearing were made during the experiment. This is likely to have resulted in an increased released insect competitiveness between generations. Secondly, there were issues with the sampling in the first generation leading to an absence of data for one treatment (low OX4319L + *Bt* broccoli) and a lack of replication and small/unequal sample sizes in other treatments. These results were therefore not compared statistically and the inferences that can be drawn from them are relatively weak.

Generally, the RSI values calculated here are lower than those previously calculated for this line. For the two OX4319L-only treatments, only the first generation (Generation 2) is appropriate for calculating RSI scores. Both treatments showed RSI scores below 0.2, with the high release rate showing a score of less than 0.1. It is interesting that the RSI value calculated for the lower release ratio was higher than that of the high release ratio. This may imply that, within the confines of these caged populations, competition between released individuals (intra-genotypic competition) began to limit the efficiency of released insects when they were present at high densities. Even at these relatively low competitiveness scores, however, the overflooding ratios in these cages were sufficient for 55.6% and 87.5% of progeny (low and high release rate, respectively) to have been 'won' by transgenics in the next (3rd) generation. Assuming that all heterozygote larvae were subject to *Bt* selection, both the 3rd and 4th generations of the OX4319L + *Bt* broccoli treatment can be used to calculate RSI values. These increased from 0.12 to 0.21 during this period, likely as a result of increased quality of released insects.

Taken together, these RSI scores may imply that this line suffered additional fitness costs not measured in previous experiments. Released insects in these cages were subject to much harsher biotic conditions (temperatures in the glasshouse reached in excess of 35°C) than in previous studies and did not have access to water or sugar sources, although these conditions were also shared by their competitors. Furthermore, they were competing against insects which had been reared on broccoli plants as opposed to artificial diet (as in previous experiments) and which were substantially larger than them (observed). It is also likely that the recruitment rates of caged populations were underestimated, leading to an overestimation of release ratios (Materials and Methods 3.4.2.4 *Male recruitment rate estimation*) and hence an underestimate of RSI scores. In light of these factors the (tentative) estimates of progeny share 'won' by this line remains promising. What can be taken from these results is that RSI scores and other estimates of 'competitiveness' or 'fitness' are far from set in stone for any particular line. Rather, they are fluid and will vary depending on a large number of factors which act during the life-span of released insects, from their rearing conditions, to the climate in the field. Even in the relatively controlled conditions of these cage trials, different release rates and generations showed substantially different competitiveness scores. In the field, this variance is likely to be even higher as individual insects are subject to individual microclimates and combinations of competitors. While complicating the calculation of appropriate release rates, this also represents an opportunity for improving the efficacy of release programmes as it seems that there are multiple paths to increasing the competitiveness of released insects.

The simulations of Alphey et al., (2007, 2009) predict that even very low fsRIDL release rates (in the veritable absence of a refuge) can effectively delay

resistance if initial resistance alleles frequencies are sufficiently low. They define an effective delay as preventing the frequency of resistance alleles in the population rising above 0.5. The results of this experiment are difficult to compare to those from simulations because of the additions of refuges to their models, the much lower initial allele frequencies modelled (maximum frequency of 0.1) and the longer periods simulated (minimum of 200 generations). For the highest initial allele frequency (0.1) and highly competitive fsRIDL males (fitness cost of 0.1), their simulations predicted that release ratios of up to 100:1 would be unable to prevent resistance allele frequencies rising above 0.5 in the complete absence of a refuge, even when the fitness costs of resistance were relatively high (resistant homozygote relative fitness on *Bt* plants = 0.1, on refuge plants = 0.4, both relative to non-homozygotes). It is thus unsurprising that with a much higher initial resistance frequency, more realistic representations of transgene-associated fitness costs and the relative fitnesses of homozygous resistant individuals (no fitness costs have yet been observed for the *Bt* resistance allele used in these experiments (Tang et al., 1997)) that higher levels of resistance were recorded in this study. Here the frequency of resistance alleles in the OX4319L + *Bt* broccoli cages rose from approximately 0.34 to 0.72 in four generations. However, there remained significantly more individuals in these cages that were subject to *Bt* selection than in cages where no OX4319L releases had taken place. Thus, as a proof of principle, this experiment was successful in demonstrating the powerful resistance management effect that the release of insects carrying an fsRIDL allele can have. Furthermore, results show that combining effective insecticide resistance management with female-specific lethality can have a synergistic effect on pest management, to an extent controlling populations that were uncontrolled when either *Bt* or fsRIDL were used in isolation.

As in all proof-of-principle studies, these experiments made a number of assumptions for simplicity. The majority of these will be similar to those listed in Section 3.2.1 for the suppression experiment, as both were cage trials conducted under relatively controlled glasshouse conditions. Two assumptions that relate specifically to resistance management, however, are the dominance and fitness costs of *Bt* resistance.

The primary mechanism of resistance management in both the high/dose refuge and fsRIDL strategies is the production of heterozygotes which, theoretically, will be subject to *Bt* selection. However, resistance may not always be fully recessive. The dominance of resistance to *Bt* toxins can be measured in the parameter *h*, where $0<h<1$, and h=0 is completely recessive resistance, and h=1 is where resistance is dominant. *h* has been found to vary in both field and laboratory-colonised strains of lepidopterans, rising as high as 0.83 in the corn earworm *Helicoverpa zea* and 0.69 in the sugarcane borer *Diatraea saccharalis* (both in the USA), based on artificial diet, and plant assays, respectively (Tabashnik et al., 2008; Ghimire et al., 2011; Tabashnik et al., 2013). In general, variations in *h* away from 0 will reduce the effectiveness of resistance management as a proportion of heterozygotes produced by refuges or fsRIDL releases will survive on *Bt* crops. However, while high *h* values are severely detrimental to the high dose/refugia strategy (effectively no longer making it 'high dose'), at sufficiently high release ratios it can actually be beneficial to an fsRIDL resistance management programme (if not a population control programme). Here the majority of heterozygous fsRIDL males will survive even on *Bt*, thus further diluting the frequency of the resistance allele in the population (Alphey et al., 2009). This scenario is not desired from a pest control perspective, but does highlight one of the differences between these two resistance management methods. Additionally, unlike the high/dose refugia strategy, the release of fsRIDL insects will make fixation of the resistance allele

impossible as there is a constant influx of susceptibility alleles into the population each generation. In the experiments conducted here, resistance management was demonstrated using a model system where resistance to the engineered Cry1Ac toxin was fully recessive. If used against field populations displaying variations in *h,* this situation is likely to become far more complex.

Despite the potentially disastrous consequences a partially dominant resistance allele could have on the high dose/refugia strategy (and the apparent existence of such alleles in field populations), *Bt* crops remain largely effective (Tabashnik et al., 2013). One potential reason for this is the fitness costs associated with resistance alleles which may themselves range from recessive to dominant ($0<g<1$). These fitness costs will manifest in homozygote and/or heterozygote resistant individuals (depending on *g*) within *Bt* crops and/or refuges (depending on *h*). Additionally, some researchers have suggested that incomplete resistance (where homozygote resistant individuals survive to adulthood on *Bt* plants but are then at a mating disadvantage) may exist and this could fit within the definition of a fitness cost to the resistance allele (Carriere and Tabashnik, 2001; Carriere et al., 2010). Indeed, if such fitness costs did exist this could, in principle, provide a partial explanation of the increasing competitiveness of transgenic males in the low OX4319L + *Bt* broccoli treatment over the course of this experiment. Like resistance itself, there is great variation in the magnitude and dominance of fitness costs associated with resistant alleles. The estimation of these costs and their behaviour is complicated by the fact that they will be fluid and likely influenced by a range of environmental factors which will vary considerably in the field – as with RSI (Bergelson and Purrington, 1996). For example, it has been hypothesised that changes in climatic variables may influence the dominance and/or magnitude of fitness costs associated with Cry1Ac resistance in Arizona populations of pink bollworm (Carriere

and Tabashnik, 2001), while resistance to the insecticide dieldrin in the Australian sheep blowfly *Lucilia cuprina* varies from significant and dominant to moderate and additive depending on the season (Mckenzie, 1990). Generally, increasing magnitude and dominance of resistance fitness costs has the opposite effect of dominance of resistance, allowing the control of resistance alleles with lower refuge areas and/or fsRIDL release ratios (Alphey et al., 2009).

In the model system used for these experiments there are no reported fitness costs associated with the *Cry1Ac* resistance allele. If they did exist, they would possibly have had two impacts on the results of the experiment. Firstly, the resistance allele is likely to have decreased in frequency in the wild-type broccoli treatment, possibly to extinction. Instead it increased (albeit non-significantly) over the course of four generations. Secondly, if the mating competitiveness of *Bt* resistant individuals was affected (especially whilst under *Bt* selection), this is likely to have increased the rate of introgression of fsRIDL transgenes into the population. This latter point highlights a potential synergy between fRIDL and insecticides which has not previously been identified. If insecticide resistance is recessive (or at least not largely dominant) and significant fitness costs are associated with it, insecticides will select for genotypes that are less competitive against fsRIDL males, which will subsequently increase the efficiency and longevity of those insecticides. Depending on the magnitude and dominance of resistance and fitness costs in the field, this could represent a powerful positive feedback system to drive down insecticide resistance, and is an area which warrants further research.

3.5 Conclusion and Summary

The work described in this chapter set out to investigate the potential of the female lethal system in the OX4319L diamondback moth line as a pest management tool. This was shown both in its ability to cause outright population suppression, and to combine powerfully with *Bt* transgenic crops in limiting the spread of pesticide resistance and population growth.

In both sections of this chapter, OX4319L males remained competitive enough to exert a level of control over target populations. However, evidence from fluorescence data collected in the insecticide resistance management experiment suggests that there may be additional fitness costs to this transgene insertion which will become apparent under more stressful, realistic circumstances. The potential for laboratory-based studies to underestimate fitness costs in transgenic insects is well-known (Scott et al., 2006) and has been observed empirically in RIDL strains of mosquitoes (Harris et al., 2011b; Facchinelli et al., 2013). Thus, it is no surprise that the estimates of mating performance in this chapter were reduced compared to those in Chapter 2. As such, further testing of this line under field conditions, where both transgenic and wild insects will be subject to the most realistic range of fitness modifiers, is required.

Unfortunately, due to restrictions placed on the release of GMOs in Europe this next level of testing was not possible during the course of this thesis. However, full-scale field releases of this line designed to test its longevity, sexual competitiveness, flight capacity and pheromone response in the field are currently planned in the United States (Cornell University). The results of these experiments are eagerly anticipated.

3.4 Materials and Methods

3.4.1 Population suppression

3.4.1.1 General conditions and summary

Experiments were conducted in quarantine facilities at Rothamsted Research Station, Hertfordshire, UK. Larval rearing took place in a temperature-controlled room and experimental cages were housed within a temperature-controlled glasshouse (both 25°C and 16:8 light:dark photoperiod). Larval rearing methods followed those of Martins et al., (2012). All experimental populations were reared on non-tetracycline diet. Homozygous OX4319L males were produced on tetracycline diet (100 µg/ml) and sexed manually prior to introduction. OX4319L sexing on non-tetracycline diet did not take place due to issues with diet quality which caused severe reductions in insect flight ability during the course of the experiment. Experimental cages measured 120 × 100 × 120 cm (width, depth and height), with a zipped entrance at the front. Insects were added to the cages as pupae.

The experiment comprised two phases: Establishment and Suppression. During Establishment, stable mixed-age populations of wild-type diamondback moth (Vero Beach strain) were established in each of the four cages. During Suppression, weekly introductions of homozygous OX4319L males were made into two of the cages to investigate whether engineered female-specific lethality resulted in suppression of these populations. Throughout the experiment, cabbage extract-baited Parafilm (Bemis Company Inc., Oshkosh WI, USA) was hung from the roof of the cage to act as an artificial leaf oviposition substrate. These were replaced three times per week (Monday, Wednesday and Friday) and eggs collected on each sheet were counted. During each

egg collection, dead adult moths and uneclosed pupae were also collected from the cages, sexed and counted. Eclosed adults were provided with sugar water-saturated cotton wool reservoirs, changed every 2-3 days. The methods of this experiment were based upon those used previously to explore fsRIDL population suppression in the dengue vector *Ae. albopictus* (de Valdez et al., 2011) and the olive fly (Ant et al., 2012). However, a tri-weekly reintroduction schedule (as opposed to once per week) was chosen to more realistically simulate the continuous recruitment of individuals into a wild population.

3.4.1.2 Establishment phase

Cage populations were initiated by placing 200 unsexed wild-type pupae into each cage. Stable non-expanding populations were maintained in each cage to mimic the stabilizing effects of predation and other limiting factors in the wild. This was achieved by introducing a constant number of pupae back into the cages each week. A total of 200 first-instar larvae were selected each week to carry on the population, taken from the three weekly collections (67 larvae chosen from Monday collection, 67 from Wednesday and 66 from Friday). These larvae were reared in plastic beakers on non-tetracycline diet, and after pupation were sexed and transferred back into the cage from which they had been collected as eggs, 2 weeks earlier. Tri-weekly egg collections (and subsequent tri-weekly pupal reintroductions) maintained stable, mixed-age populations as might be expected in the field, where adult moths would be continuously entering the population. The first two introductions of wild-type moths (weeks 1 and 2) originated from an independent laboratory colony. From the third week, reintroduced pupae

originated from eggs from these cages, so thereafter each cage population was self-sustaining.

3.4.1.3 Suppression Phase

Once egg counts had stabilized (indicating stable populations) in week 9, two of the four cages were chosen as OX4319L treatment cages (Cages 2 and 4) and two as control cages (Cages 1 and 3). Cages were designated in a blocked design to minimise bias caused by uncontrolled abiotic factors. In non-treatment control cages the protocol from the establishment phase was continued. Each OX4319L treatment cage was randomly paired with a control cage for the remainder of the experiment (Cages 1 and 2; Cages 3 and 4). The reintroduction of pupae into each OX4319L treatment cage followed the same protocol as for the establishment phase; however, the total number of early-instar larvae selected for rearing that week was made proportional to the ratio between the number of eggs collected for that treatment/control pair of cages for the week when these larvae were collected as eggs. This method ensured that the population suppression effect of female death of transgenic larvae, reflected later by reduced number of eggs collected, resulted in reduced numbers of adults re-entering the OX4319L treated cages relative to an untreated population.

After cages had been paired, weekly introductions of homozygous OX4319L males into the treatment cages began. The target over-flooding ratio (OX4319L to wild-type males entering the population) was set at 10:1. This over-flooding ratio was calculated as 10 × the mean male weekly recruitment rate for the 3 weeks preceding OX4319L male introduction, and the numbers released per week were held constant for

the remainder of the experiment (Cage 2: 990 OX4319L males; Cage 4: 980 OX4319L males). Males were released as pupae into the cages once per week (Wednesday).

Pupae reintroduced into treatment cages were screened for the DsRed fluorescent protein transformation marker. Each treatment cage's end-point – extinction of each population – was pre-defined as 2 consecutive weeks of zero eggs collected.

During the suppression phase aid with egg counting and rearing were provided by Adam Walker, Thea Marubbi, Debs Granville and Nerys Humphrey-Jones.

3.4.2 Insecticide resistance management

3.4.2.1 General conditions and summary

Experiments were conducted at Cornell University, New York State Agricultural Experimental Station, Geneva NY, USA. Eleven cages, each 1.83 m × 0.91 m × 1.83 m (length × width × height), were placed in a temperature-controlled glasshouse with supplementary lights (23-27°C, 16:8h light:dark photoperiod, and uncontrolled relative humidity). The 11 cages were assigned to four treatments: Treatment 1, *Bt* broccoli plants, no OX4319L release; Treatment 2, *Bt* broccoli plants, low-rate weekly OX4319L releases; Treatment 3, wild-type broccoli plants, low-rate weekly OX4319L releases; and Treatment 4, wild-type broccoli plants, high-rate weekly OX4319L releases. Treatments 1-3 were assigned three cages (replicates) while Treatment 4 was assigned two cages.

3.4.2.2 Experimental diamondback moth strains

Two strains were used: a hybrid *Bt*-resistant/wild-type strain used to generate starting populations in the cages (founder strain), and OX4319L. To generate the founder strain, 25 males from a homozygous Cry1Ac *Bt* toxin-resistant stock colony (Zhao et al., 2003) were crossed to 25 females from the homozygous-susceptible 'Geneva 88' colony. Fifty-nine of the F1 male progeny from this cross were then mated to 100 females from the Geneva 88 strain. A total of 250 males and 250 females from this cross were then mated to produce the founder strain. These crosses provide an expected resistance allele frequency of 0.25 and an expected homozygous-resistant frequency of 0.0625. At all stages of founder strain production and maintenance, progeny were reared on wild-type broccoli plants in large numbers (>500 pupae collected per generation) to minimize the effects of inbreeding and genetic drift. However, *Bt* survival assay results (Figure 3.4b – Founder strain) suggest that this frequency had increased to 0.34 ($r = \sqrt{0.115}$) by the start of the experiment.

3.4.2.3 Experimental broccoli cultivars

Two strains of broccoli (*Brassica oleracea* L.) plants were used: a wild-type cultivar (Packman) and a transgenic strain engineered to express high levels of the *Bt* toxin Cry1-Ac (Metz et al., 1995). Together, this transgenic plant cultivar and the Cry1-Ac-resistant diamondback moth strain comprise a well-established model system used to study the dynamics of transgenic crops and their resistance management (Zhao et al., 2003; Zhao et al., 2005). Cry1-Ac toxin production was verified by screening the plants (4-5 weeks old) with susceptible Geneva 88 strain neonates. Leaf assays of transgenic

plants also killed 100% of OX4319L/Cry1-Ac hybrids, indicating high levels of *Bt* toxin expression.

3.4.2.3 Restocking of plant material

All cages started with 20 broccoli plants of their respective cultivar. Plants were replaced after 4 weeks or when defoliated due to larval feeding, by cutting at the base and placing on replacement plants to allow larvae to transfer. If moth populations grew beyond the capacity of the maximum food supply (estimated by exceeding their cage's plant material within one generation), the cage was terminated.

3.4.2.4 Establishment of cage populations

In all treatments, replicates were initiated by the release of diamondback moth adults from the founder strain into the cages. In treatments involving *Bt* broccoli, 200 randomly selected adults were released. In treatments involving wild-type plants, seven males and females (total 14 adults) were released into the cages. Due to *Bt* selection, this gave approximately equal starting population densities in Generation 1, in all cages.

3.4.2.5 fsRIDL treatments and modelling

Homozygous OX4319L males for release into the cages were produced by rearing egg collections from a stock colony in the absence of tetracycline in larval feed. Haphazard visual screening of eclosed adults revealed no females over the experimental period. OX4319L releases in this experiment were proportional: daily estimates of adult male recruitment for each cage were used to calculate a daily male release number for

that cage, dependent on the release ratio pre-determined for that treatment. A proportional release rate, rather than a constant number, was applied to reduce the likelihood of population extinction and thereby allow the exploration of the effect of fsRIDL transgene releases on population dynamics and resistance management. Release ratios were selected in advance based on the outcomes of a semi-continuous (discrete weeks) deterministic model, investigating the effects of each experimental treatment on population size. An explanation of the modelled OX4319L + *Bt* broccoli treatment is given below for demonstration.

Modelled moth populations on *Bt* broccoli treatments were started with 200 adults, wild-type broccoli modelled populations with 14. The sex ratio of these initial adults was assumed to be 0.5. The first two weeks of these modelled populations were conducted with no fsRIDL males. This was taken to be the establishment phase of the experiment as in the real cages the initial introductions of the founder line were allowed to mate and their progeny taken as the first generation. After two weeks, the number of pre-adults (PA) in this first generation was calculated (1).

$$PA = \frac{EA}{2} \times Y \qquad (1)$$

Where EA is the number of establishment phase adults introduced and Y is the reproductive rate of females (2R0) [taken here to be 16 as used in (Alphey et al., 2009)]. Using PA, a daily pupal recruitment rate (DPR) could be calculated for the establishment phase (2nd week) (2).

$$DPR = \left(PA \times \left(\frac{LP/TL}{LP}\right) \times LPS\right) \times FRG \qquad (2)$$

Where LP is the number of days spent as a larva, TL is the total pre-adult lifespan (i.e. egg-eclosing pupae) in days, LPS is the larva to pupa survival rate (here set as 0.8 – personal observation) and FRG is the frequency of *Bt* resistant genotypes in this generation (initially $0.25^2 = 0.0625$). From DPR the daily and weekly adult recruitment (DAR, WAR) and subsequently the weekly male and female adult recruitment (WMR, WFR) could be calculated (3).

$$DAR = DPR \times PAS \qquad (3)$$

Where PAS is the pupa to adult survival (set as 0.8 personal observation) and WAR = DAR × 7, and WMR,WFR = WAR/2. Once pupae began eclosing from this first generation (week 3) the PA calculation was modified to reflect their contribution to population growth (4).

$$PA(t+1) = PA(t) - (DPR(t) \times 7) + \left(\frac{Y}{ALE} \times 7\right) \times WFR(t) \qquad (4)$$

With ALE being the Average Life Expectancy of females and assuming a constant rate of oviposition over this time period. At this point, the chosen fsRIDL overflooding ratio could be integrated with a measure of OX4319L competitiveness to calculate an estimate of the progeny share (PS) won by released OX4319L insects in that week by equation 5.

$$PS = (1 - RSI)e^{\left(\frac{-3}{OR}\right)} + RSI \qquad (5)$$

Where RSI is the RSI of the OX4319L strain (here set as 0.37) and OR is the overflooding ratio for that week (constant if proportional release rates were chosen but variable between weeks if release rates were constant). An exponentially limited

function was chosen to represent progeny share as this is more realistic than simply multiply RSI by the overflooding ratio. Here, progeny share is limited at 1, and while between a 1:1 and 20:1 release ratio drastically increases progeny share, increases beyond this point begin to have reduced effectiveness.

Finally, the effects of fsRIDL introgression and *Bt*-induced lethality could be combined in a single DPR function (6).

$$DPR(t+1) = \left(\left(PA \times \left(\frac{\frac{LP}{TL}}{LP}\right) \times LPS\right) \times FRG\right) \times PS(t) \quad (6)$$

In generations other than the first, FRG was taken to be 1 as it was assumed strong selection will have reduced any initial susceptibility alleles in the cage to extinction.

These equations could subsequently be modified to model the effects of fsRIDL releases or *Bt* crops on their own. It should be noted, however that the point of creating these equations was not to explore the dynamics of fsRIDL releases and *Bt* or fsRIDL releases alone, both of which have been achieved comprehensively in previous publications (Schliekelman and Gould, 2000; Schliekelman et al., 2005; Alphey et al., 2007; Alphey et al., 2009). It was instead to devise a rough estimate of fsRIDL release rates which would demonstrate the combined action of fsRIDL and transgenic crops within the confines of this experimental protocol. As such, these equations are less complex, and their subsequent definition above less detailed, than would have been necessary if the former point was their goal.

In Treatments 2 and 3 (low-OX4319L release treatments), the intended over-flooding ratio was 5:1 (OX4319L: wild-type males). However, due to insect rearing limitations this was limited to 3:1 in Generation 1, and increased to 5:1 thereafter. These

low release rates, in combination with *Bt* broccoli plants, were predicted to maintain a relatively constant population size, but insufficient to cause population suppression when used alone. Similarly, the over-flooding rates in Treatment 4 (high-OX4319L release), initially 20:1 and increased to 40:1 in Generation 2 as production capacity increased, were predicted to be sufficient for population suppression when used alone.

Where small populations are expanding in the absence of limiting biotic or abiotic factors, stochastic effects make it difficult to accurately predict the rate of population increase. In response to higher-than-predicted population growth and limited plants available, Treatment 2 was reduced to two replicates in Generation 3 (closed-down cage selected at random). In Generation 4, the number of plants in each Treatment 1 cage was reduced to five (while maintaining per-plant population density levels) in response to limited plant availability. This was achieved by randomly harvesting 25% of the leaves on each plant within the cage, removing all insect and plant material from the cage, and then restocking the cage with five new plants and returning harvested leaves. This process took place between Generations 3 and 4 and as generations were discrete, and the vast majority of the population was present as larvae or pupae on plants, this allowed accurate culling of the population. In Generation 3, requirements for OX4319L male moths exceeded production capacity, so Treatment 4 was reduced to one replicate only.

3.4.2.4 Data collection

Population size estimation: The number of 2nd-4th instar larvae and pupae on each plant in each cage were counted once per week. Help was provided during periods

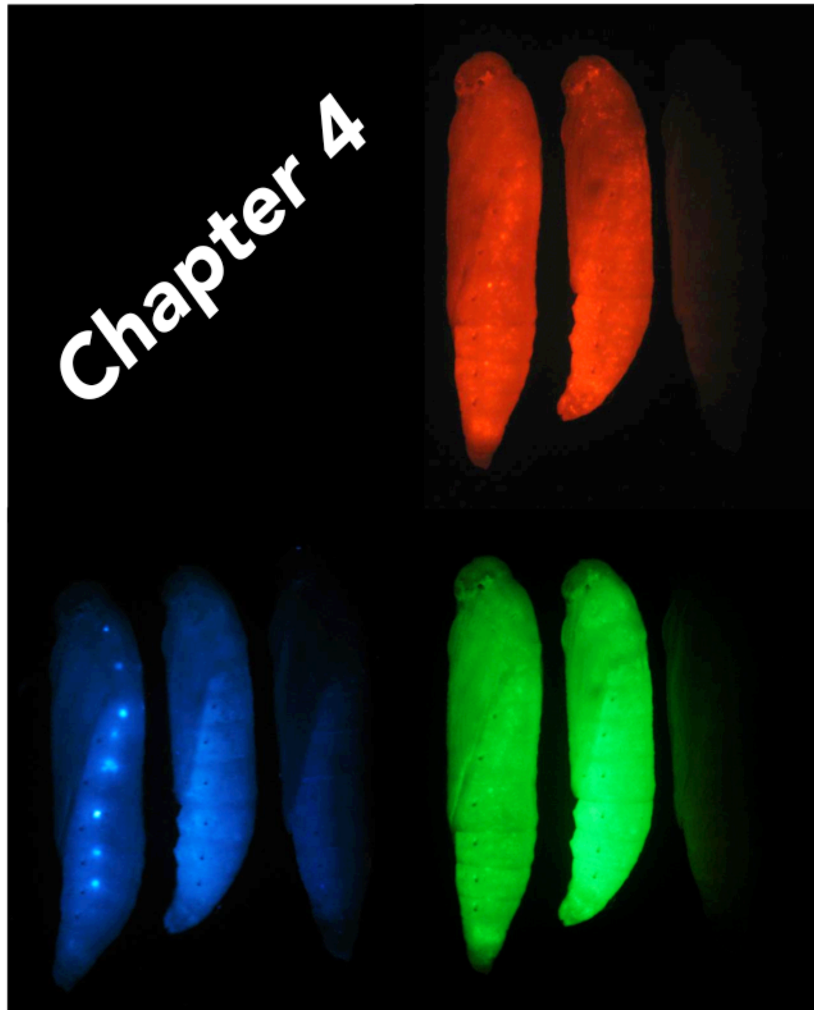
of intense counting by Ju Yao *Fluorescence assay*: Fluorescence data was collected from cages in which OX4319L males were released (treatments 2, 3 and 4). In the first generation, this was attempted by hanging cabbage juice soaked parafilm in cages to act as an oviposiiton source. However, this failed to attract ovipositing females, leading to a lack of data for treatment 3 and small sample sizes for treatments 2 and 4. Collected eggs were transferred to filter paper by paintbrush and resulting larvae were reared on tetracycline augmented artificial diet (100 μg/ml), a dose high enough to suppress female lethality. Larvae reaching pupation were screened for presence/absence of the DsRed transgene. From generation 2 onwards this technique was modified so that eggs were collected from 8-10 leaves selected at random as each cage reached maximum egg-laying potential (judged by female recruitment data in each population, collected from eclosion cages) and then reared and screened in the same way as previously. Due to the small size of the population in treatment 4 in its final generation, fluorescence data was not collected from this cage for this generation. *Bt resistance assays*: With the exception of Treatment 4, *Bt* resistance data were collected from all cages in the final generation they were run. Eggs were collected and reared using the same protocol as used from generation 2 for fluorescence assays. For each cage, two *Bt* survival assays and one control assay were performed. For *Bt* assays, tetracycline diet was poured into 30-ml plastic pots and 500 μl of 10 ppm *Bt* (Dipel®, Valent BioSciences Corp., Libertyville IL, USA) - shown previously to discriminate between homozygous-resistant and other genotypes (Shelton et al., 2000) - pipetted onto the dried surface. Non-*Bt* controls were prepared in the same way, with no added *Bt* solution. At 3rd instar, larvae were transferred onto the air-dried diet surface. Exact numbers of larvae per replicate differed between cages according to availability of eggs. A minimum of 33 larvae per pot was used for the first *Bt* assay; 11 per pot for the second *Bt* assay; and all

control replicates contained 20 larvae. Mortality was assessed 72 h later with surviving larvae taken to be homozygous for the resistant trait. For comparison, the founder strain was subjected to the same assay, in the generation prior to experimental initiation. *Male recruitment rate estimation*: For those cages requiring OX4319L male introductions, daily male recruitment rate was assessed by the removal of four randomly selected plants/cage/week. These were placed into smaller cages in an adjacent glasshouse maintained in the same environmental conditions. The number and sex ratio of the adults eclosing from these plants were recorded daily, and eclosed adults were then returned to their respective experimental cages. From these data an estimate of the eclosion rates in the main cage was made, from which the number of OX4319L males required to achieve the respective over-flooding ratio for each cage was calculated. This method is likely to have underestimated the recruitment rate in each of the main cages as insects pupating on the inside or floor of the main cage were not included in the recruitment rate estimation (as they had no chance of being moved into one of the smaller cages). As such, the overflooding ratios in this experiment (calculated from these recruitment rates) are likely to have been overestimated.

3.4.2.4 Statistics

For comparisons between treatment peak population densities in Generation 3, ANOVA was used, followed by Tukey's HSD for pair-wise comparisons. For Generation 4, a t-test was used as only two treatments were compared. For *Bt* assay results, results from the two *Bt* assays within each cage were summed to avoid pseudo-replication and then corrected for control mortality using a Henderson-Tilton correction. For comparisons between treatments, a Logit GLM for Categorical Data Analysis was

used followed by pair-wise comparisons using Generalized Linear Hypothesis Testing with each cage acting as a replicate. Data were analysed using R (Version 2.15.0).


Chapter 4

Chapter 4: Investigation of the Q inducible expression system for transgene control in the diamondback moth

4.1 Introduction

For a gene expression system to be compatible with an autocidal control strategy it must: be conditional with a low level of basal expression; be relatively compact in nucleotide sequence; show high levels of induction and repression; and, ideally, be compatible with an artificial diet. Additionally, the overall mechanism of regulation must be compatible with the biology of the species being transformed. For example, a light-inducible gene expression system (Wang et al., 2012; Ma et al., 2013) would be highly appropriate for aquatic species such as mosquitoes, but potentially less so for agricultural pests where larvae have an extensive burrowing phase. If such a system is to be used in conjunction with other gene expression systems within the same organism, their regulation (induction or repression) must be orthogonal. With these conditions in mind, the Q-system (Potter et al., 2010) was identified as a potential gene expression system to replace, or use in combination with, the tet-off system for genetic pest management strategies.

The Q gene cluster on which the Q system is based is an endogenous quinic acid (QA) catabolism circuit from the fungus *Neurospora crassa*. This pathway converts QA to protocatechuic acid and consists of seven tightly linked genes. Five of these encode structural proteins and two (qa-1F (QF) and qa-1S (QS)) act as regulators of the system. Under restrictive conditions (in the absence of QA) the transactivator QF is bound by

the suppressor QS, preventing the induction of the catabolism circuit. However, when QA is present, its binding to QS prevents the repression of QF (de-repression) and induction of structural genes is induced. Interestingly, as well as induction of target structural genes, QF also induces its own transcription (through binding to its upstream binding site QUAS) and QS, presumably to ensure a rapid switching off of the system once QA has been depleted (Giles et al., 1985; Giles et al., 1991). The Q-system is a binary, inducible transgene expression system utilising the QF, QS and QUAS sequences from the Q gene cluster (Potter et al., 2010) (Figure 4.1).

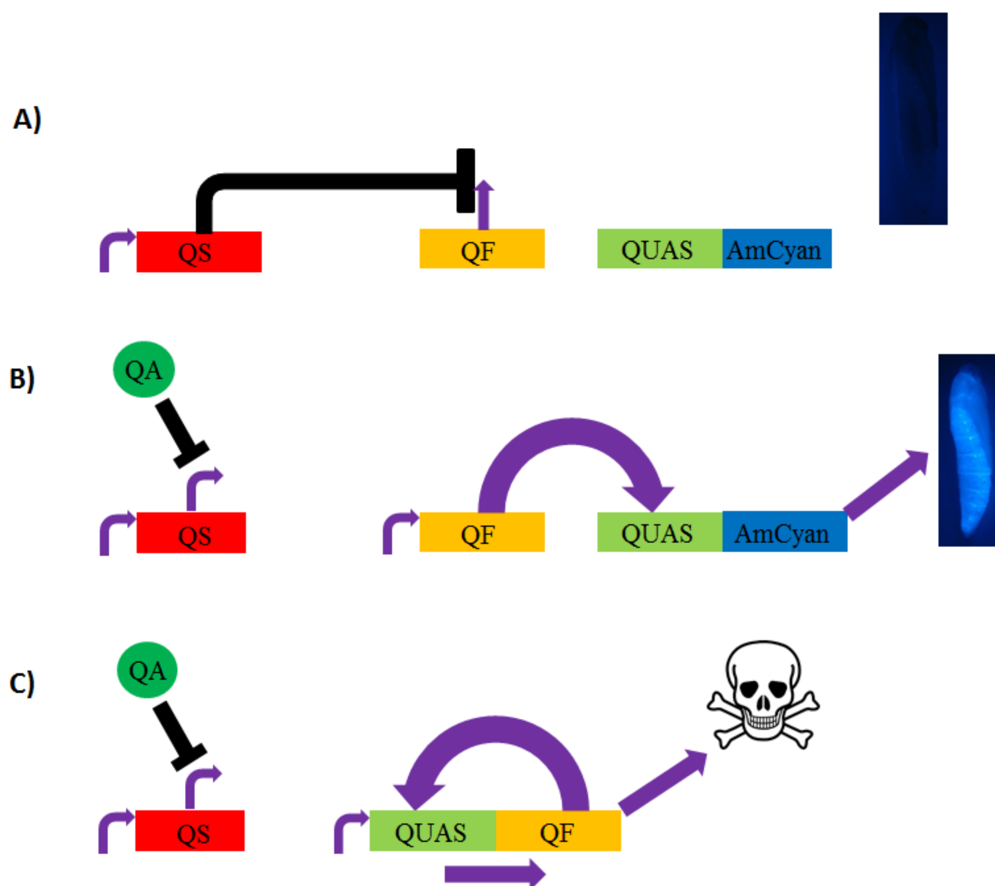


Figure 4.1: Schematic showing various potential uses of the Q-system. (A) and (B), traditional use of the Q-system as a conditional gene expression system, in this case driving the expression of the fluorescent protein AmCyan. (A) Shows the system in its constitutive, repressed state in the absence of quinic acid (QA). (B) Shows the system in its induced state, in the presence of QA, allowing the fluorescent phenotype. (C) Represents the proposed conversion of the Q system into a lethal cassette. By placing QUAS upstream of QF, a lethal positive feedback loop is created, in the presence of QA. An analogous phenotype could be engineered by replacement of AmCyan in (A) and (B) with a lethal gene.

By inserting a target gene downstream of the QUAS binding site, it has been shown in *D. melanogaster* that rapid, high-level expression can be induced in the presence of QA, while basal expression is minimal in its absence. If the Q-system could be modified such that auto-regulation of QF is restored and enhanced (i.e. QF induces its own high level transcription) this might represent an analogous, and orthogonal, alternative to tet-off RIDL (Figure 4.1).

Current RIDL/fsRIDL strategies rely on restrictive (lethal) conditions to be present in the field, made possible by the repressible tet-off system. As the Q-system is inducible it cannot be easily integrated in an analogous bi-sex strategy, that is, as a population suppression tool. Such a role would require the induction of the system (the presence of QA) in the field. The use of inducible lethal/sterile transgenes have been proposed as pest control tools whereby the engineered construct is allowed to spread within a target population prior to its induction (Davis et al., 2000; Thresher, 2007). However, the efficacy of such strategies is predicted to be highly dependent on transgene-associated fitness costs. Their successful use would require the generation of transgene insertions with extremely low fitness costs and/or the use of gene-drive systems to aid their persistence prior to the lethal effect being triggered (Schliekelman and Gould, 2000). In addition, some means of reliably exposing the wild population to the inducer in the field would be required. This may prove difficult with a dietary supplement such as quinic acid. Due to these potential complications, these strategies have remained largely theoretical. For the Q-system to be used in conjunction with tet-off RIDL it would thus need to be made sex-specific and used as a sexing system. In this scenario it would replace the tet-off system as the genetic sexing tool, while an orthogonal tet-off bi-sex transgene could be used as the means of population control in the field. The inducible nature of the Q-system - a disadvantage for use as a bi-sex tool

- would aid its employment as a sexing system. Specifically, as the system is constitutively repressed, any deleterious effect should only be present for the time period necessary to kill females. In comparison, fsRIDL under the control of the tet-off system is constitutively active. Thus, it is probable that tTAV continues to accumulate in adults after sexing and in the field, potentially contributing to the reduction in OX4319L male longevity and competitiveness observed relative to wild-type (Chapter 2, Sections 2.2.5.2 and 2.2.7).

The aim of this project was to investigate whether the Q-system can be employed to control gene expression within a genetic pest management framework, initially in the diamondback moth. Previous use of this system in *D. melanogaster* (Potter et al., 2010; Potter and Luo, 2011), *Caenorhabditis elegans* (Maupas) (Wei et al., 2012) and the zebrafish (*Danio rerio* Hamilton) (Subedi et al., 2014) suggests that this system is highly transferrable across large phylogenetic distances. However, the use of this system in non-model organisms and, specifically, its employment in a lethal context, remains unexplored. Also, there are concerns over the toxicity of components of the system (QF) when expressed ubiquitously (Potter et al., 2010). Preliminary experiments sought to investigate potential deleterious effects of QA when used as a dietary supplement for insects in which RIDL has been previously developed. Subsequently, constructs were built using the Q-system to drive a fluorescent reporter gene and transformation of this system was attempted in the diamondback moth. These test constructs divided the Q system into two parts, QS and QF-QUAS-reporter gene, to increase flexibility and problem-solving capacity. These experiments encountered difficulties in transforming components of the Q-system and thus its dynamics were further explored through transient transgene expression assays which do not require full transformation.

4.2 Results and Discussion

4.2.1 Quinic acid toxicity

QA is a naturally available chemical (for example, present at 1% in cranberry juice (Nollet, 2000)) and has been shown to have negligible effects when fed at high concentrations, over multiple generations, to *D. melanogaster* (Potter et al., 2010). However, due to biological differences of target RIDL species compared to *D. melanogaster*, the toxicity of QA was investigated. This potential toxicity was explored in three RIDL targets in which the Q-system could be employed (*Ae. aegypti, C. capitata* and diamondback moth). The impact of life history development parameters were investigated at different concentrations of QA: 0.98 mg/ml, 2.4 mg/ml, 7.5 mg/ml and 23.7 mg/ml, with 0.0 mg/ml as a control.

Results:

QA did not have a significant effect on proportional survival of medfly or diamondback moth for any of the life stages assessed (Figure 4.2A-F). For *Ae. aegypti*, however, high mortality occurred at the highest concentration of QA (100% mortality after 2 days), leading to a highly significant effect on larval survival overall ($Z = 14.01$, $df = 9$, $p < 2 \times 10^{-16}$) (Figure 4.2G). As no larvae reach pupation at this highest concentration, it was excluded from the analysis of pupae-adult mortality. In spite of these data being removed, the effect of QA on pupal-adult survival was still significant ($Z = 2.342$, $df = 7$, $p = 0.019$) (Figure 4.2H). Thus, QA had a highly significant effect on larval-adult (lifetime) mortality ($Z = 13.73$, $df = 9$, $p < 2 \times 10^{-16}$) (Figure 4.2I).

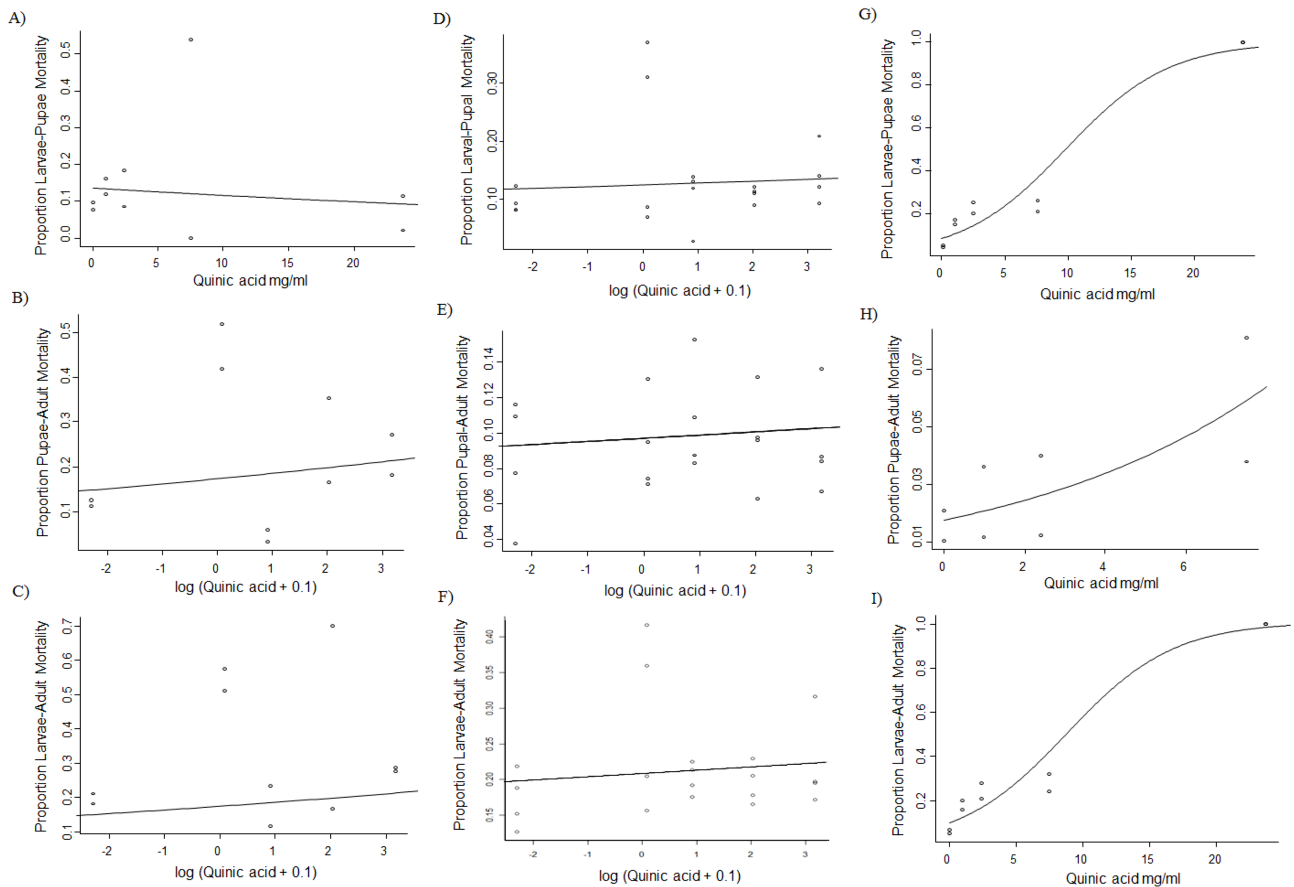


Figure 4.2: Graphs showing the proportional mortality recorded for three commercially important insect species, over three different time periods when reared at different QA concentrations. Columns from left to right refer to medfly (A-C), diamondback moth (D-F) and *Ae. aegypti* (G-I). Rows from top to bottom refer to proportion larvae-pupae mortality (A, D, G), proportion pupae-adult mortality (B, E, H) and proportion larvae-adult mortality (C, F, I).

Despite the significant toxic effect of high QA concentrations observed in *Ae. aegypti*, at lower concentrations mortality did not differ greatly relative to the no-QA control (including that previously used to induce the system, 7.5 mg/ml (Potter et al., 2010)). It is known that mosquitoes are more sensitive to the tet-off system than other species, potentially due to their higher intake of tetracycline from their aquatic environment (personal communication, Dr Luke Alphey). If this increased sensitivity to soluble molecules also applied to QA, it is conceivable that lower dosages than 7.5 mg/ml might be sufficient to induce the system, further reducing any concerns over QA

toxicity. These results indicate that QA is not significantly toxic at appropriate concentrations to induce the Q-system. Therefore research into this system was continued.

4.2.2 Design and transformation of modular constructs

Initially, the repressor (QS) and activator/effector (QF-QUAS-DsRed) were separated into two independent constructs. This allows the activities of the two components to be investigated individually and potential issues isolated. Lines transformed with these constructs are identifiable by their blue fluorescing transformation markers (AmCyan). However, only those carrying the QF construct should show red fluorescence (DsRed). Once lines have been generated for both constructs they can be made homozygous for their respective insertions and crossed together. Absence of red fluorescence will indicate repression of QF. These double heterozygotes will be reared on different concentrations of QA to investigate whether this leads to incremental de-repression of the system, indicated by DsRed fluorescence.

4.2.2.1 Repressor construct

The QS2 construct, OX4673, was designed with Dr Tarig Dafa'alla using Vector NTI Advance 11.5 (Figure 4.3). Construct map and individual components are detailed in the Appendix Figure A.3A and Table A.1.

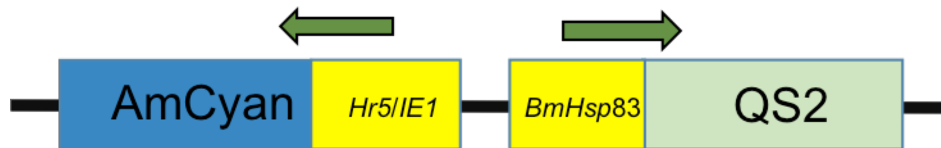


Figure 4.3: Schematic showing the components of the OX4673 construct relating to the Q system. Yellow boxes represent promoter sequences which control downstream open reading frames in the direction of the green arrows (direction of transcription). QS2 is the suppressor gene in the Q-system (suppresses QF2). AmCyan is a marker gene which fluoresces blue under the appropriate wavelength of light and which is used to isolate transformants.

After construction, the repressor construct plasmid was micro-injected into 1403 diamondback moth eggs. Of these, 466 G_0 injection survivors yielded one G_1 male transformant. This individual was out-crossed to wild-type females, founding the OX4673A line (Figure 4.4).

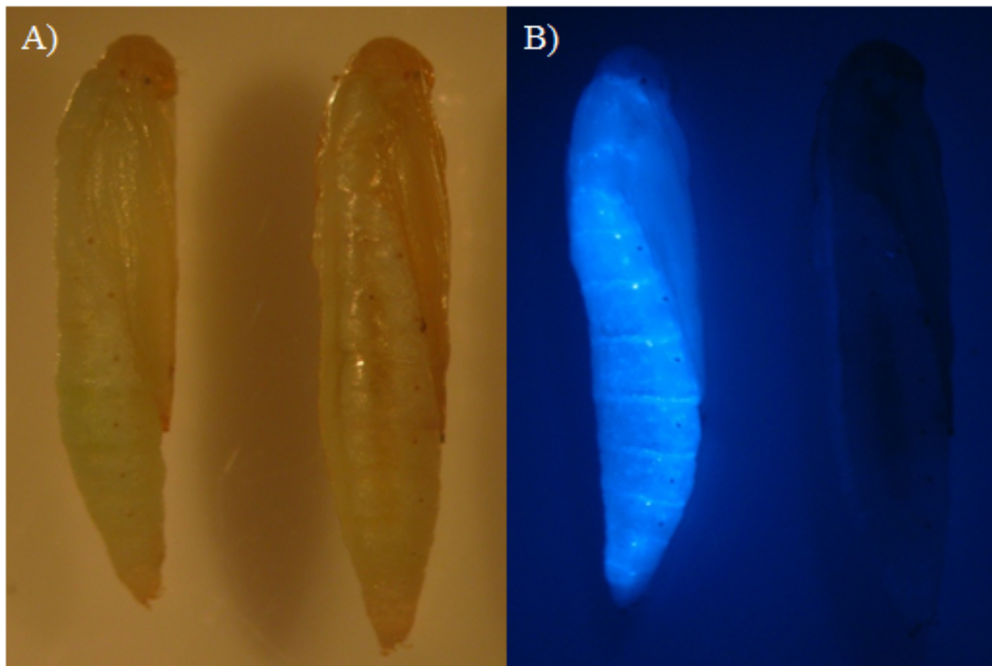


Figure 4.4: Photograph of DBM pupae taken under (A) bright field and (B) blue fluorescent protein excitation filters. The pupa on left is transgenic (OX4673A) while that on the right is wild-type. Under bright field (normal light), OX4673A is indistinguishable from wild type. However, when exposed to the correct wavelength of light, AmCyan proteins in the transgenics fluoresce blue, permitting easy identification.

For a suppressor transgene to function effectively it should be expressed at sufficiently high levels and in areas appropriate for repressing its target. In the case of OX4673A, the promoter chosen to control QS2 (*Bmhsp*83) is known to drive strong, early expression in lepidopterans (personal communication, Dr Neil Morrison). However, the magnitude and spatial/temporal characteristics of expression can be dramatically affected by positional effects (Chapter 2, section 2.2.5). Therefore, the expression profile of QS2 was characterised using one-step RT-PCR on 1st instar, 2nd instar and pupal OX4673A samples (Figure 4.5). These results show that QS2 expression is taking place throughout the life-stages tested, a positive indication for this line's use as a suppressor strain.

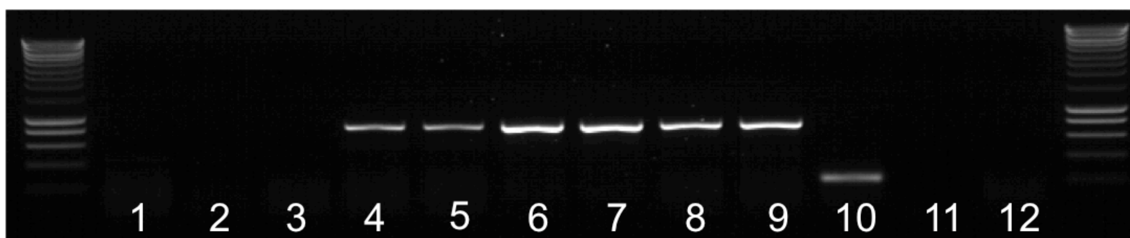


Figure 4.5: Results of one-step RT-PCR using primers specific to QS2. Lanes 1-3 represent wild-type 1st and 2nd instar, and pupae, respectively (wild-type control). Lanes 4-9 represent OX4673A 1st and 2nd instar, and pupae respectively (two replicates of each). Lane 10 is a positive control (endogenous *dsx* gene), lane 11 is a negative control (H_2O) and lane 12 is a no-RT control.

4.2.2.2 Activator/effector construct

The QF2 construct was designed with Dr Tarig Dafa'alla on Vector NTI Advance 11.5 and named OX4651 (Figure 4.6). Construct map and details of components are detailed further in the Appendix Figure A.3B and Table A.1.

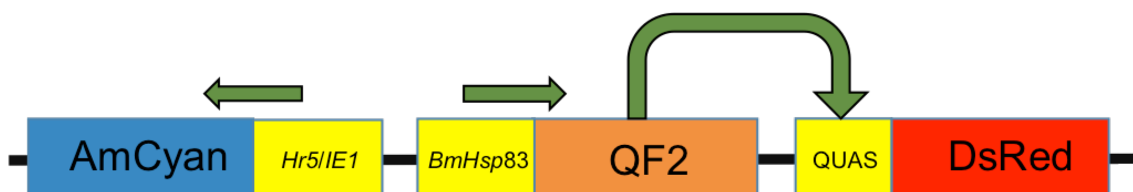


Figure 4.6: Schematic showing the components of the OX4651 construct relating to the Q system. Yellow boxes represent promoter and binding sequences which direct downstream open reading frames in the direction of the green arrows (direction of transcription). QF2 is the transactivator gene in the Q-system (and is suppressed by QS2). In its constitutive state, this construct functions when QF2 is expressed and binds to QUAS (curved green arrow), driving the downstream expression of DsRed. AmCyan is a marker gene which fluoresces blue under the appropriate wavelength of light and which is used to isolate transformants.

After construction, the activator/effector plasmid was micro-injected, along with a DNA helper plasmid carrying a copy of the *piggyBac* transposase gene, into 3096 diamondback moth eggs. Of these, 683 individuals survived to pupation (G_0) and 15 displayed signs of transient transgene expression (DsRed chimeras, Figure 4.7).

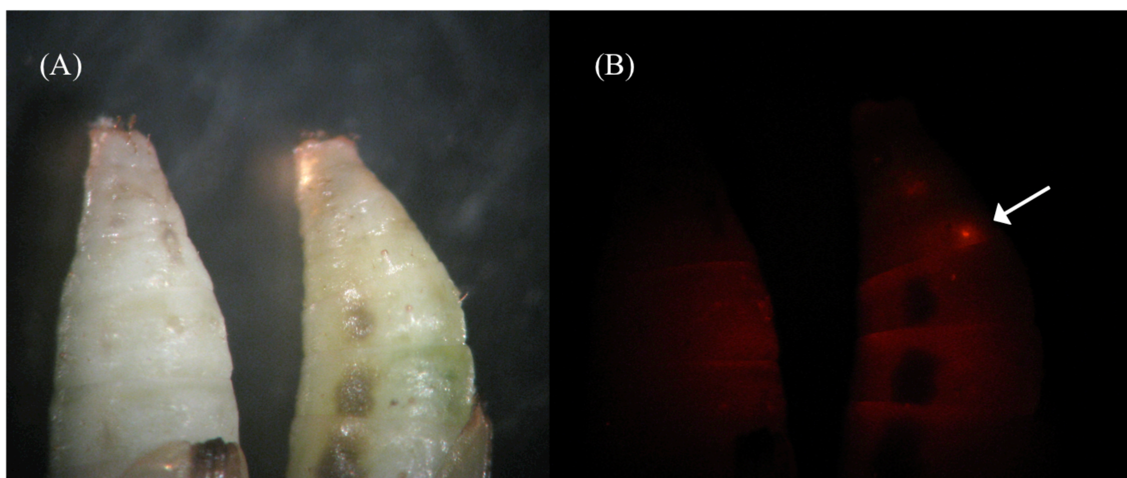


Figure 4.7: Photographs of diamondback moth pupae (posterior end) injected with construct OX4651 taken under bright field (A) and red fluorescent protein excitation filters (B). Under bright field (A), the pupae are indistinguishable. (B) From right to left the first pupae shows evidence of DsRed transient expression (signified by white arrow) while the left pupa does not.

However, no transformants were bred from these G_0s. For heritable transformation, the construct must be integrated into germ-line cells. Thus, transient

transgene expression does not necessarily lead to successful germ-line transformation, but is an indication that at least the transgene plasmid is present in the embryo. In order to increase the chances of transformation, a second round of injections took place, where the DNA transposase helper plasmid was substituted with *in vitro* synthesised mRNA encoding the *piggyBac* transposase. The use of mRNA transposase has been shown to significantly increase the number of insertion events in some insects (Kapetanaki et al., 2002; Pavlopoulos et al., 2004). OX4651 was injected along with transposase mRNA into 2500 eggs. To control for the possibility of researcher error being the cause for non-transformation, approximately 1,000 of these eggs were injected by a colleague (Adam Walker) who is highly experienced in micro-injection, having achieved 159 transformation events in the Lepidoptera. From these micro-injections, 750 G_0 individuals were collected and 30 showing transient expression of the marker were identified. Again, however, no transformants arose from these G_0s.

Micro-injection of this number of eggs would normally result in transformation, so this negative result was unexpected. Previously published experiments indicate similar issues in generating transformants ubiquitously expressing the QF protein in *D. melanogaster* (Potter and Luo, 2011). This research postulated that high-level expression of the QF protein may be toxic to transformed individuals, resulting in death. On one hand this is encouraging, as it implies that QF may be able to function in a similar way to tTAV, as a lethal effector in the RIDL positive feedback loop. However, this high toxicity also makes it difficult to generate live transformants, at least in the designed two-part system. A review of the literature was therefore conducted and three preliminary possibilities for attenuating the toxicity of QF identified. These were as follows:

- Replacement of the SV40 3′ UTR downstream of the QF2 sequence with the *hsp70* 3′ UTR. The 3′ UTR is involved in mRNA stability and replacement of a 'stable' 3′ UTR with a 'less stable' 3′ UTR may result in increased mRNA degradation prior to translation and thus decreased levels of protein.
- Placement of upstream Open Reading Frame (uORF) sequences within the *Bmhsp83* 5′ UTR, upstream of the QF2 sequence. uORF sequences are a form of post-transcriptional gene regulation and consist of start codons placed upstream of, and out of frame with the true ORF. Their ability to reduce protein expression levels are well-established (Calvo et al., 2009) and their use in insects has been investigated (Hayden and Bosco, 2008).
- Replacement of the *Bmhsp83* promoter with the *BmA3* or *Opie2* promoters. These promoters are thought to drive weaker fluorescent protein expression in pink bollworm than *Bmhsp83* (personal communication, Dr N. Morrison).

Of these, replacement of the 3′ UTR was selected as the most appropriate option. This was due to its successful employment in attenuating protein toxicity in previous research (Potter and Luo, 2011) and the ease with which it could be implemented. Inserting uORF sequences is potentially more sensitive, but requires a large degree of trial and error in engineering the placement and number of uORF sequences. To ensure agreement between suppressor and activator, changing the *Bmhsp83* promoter would also require a synchronous change in the QS2 construct for which a transgenic strain already exists. This revised construct (OX4765) was built and micro-injected into 2603 eggs, from which 1189 G_0 individuals were collected and 83 showing transient marker expression were identified. Again, no transformants were identified.

One possible reason (albeit unlikely due to the sheer number of individual repeats) for non-transformation is micro-injection error. Micro-injection aims to integrate the transgene construct into germ-line cells for heritable transformation to occur. To facilitate this, micro-injection occurs into the posterior of the pre-blastoderm embryo, from which the gonadal cells will form (Figure 4.8).

Figure 4.8: Diamondback moth egg being micro-injected on a glass microscope slide. Line reaching from top left corner of the photo down into the egg is the micro-injection needle. Small objects on the surface of the slide are scales lost from the wings of adults while ovipositing. Note the clear difference between the posterior end of the egg which is noticeably convex (and from which the lower portion of the insect will form), and the anterior part of the egg which is flattened. Micro-injection should aim for the posterior end of the egg to have the greatest chance of germ-line transformation.

In an attempt to negate factors which could be contributing towards failure of transformation, a novel microinjection protocol was devised. Normally, oviposition is induced on a microscope slide by painting the slide with cabbage extract and then placing it in an adult cage. This, however, leads to the random positioning of eggs across the slide making it difficult to accurately target the posterior end of the egg. Instead, a polyurethane strip was painted with cabbage extract and placed into the adult

cage. Oviposition occurred on the strip and eggs could then easily be lifted off of this surface and lined up on a microscope slide, in the correct orientation for micro-injection. This process was facilitated with the help of two colleagues, Matthew Gregory and Peter Elphick. Polyurethane was chosen as the substrate as it was found that eggs were more easily removed from this than from other materials such as glass or filter paper. This new technique also allowed individual eggs to be followed so that accurate development and hatch rate data could be collected. Simultaneously, the *piggyBac* ends of the construct were cloned and sequenced (performed by GATC Ltd and a colleague, Caroline Phillips) to check for irregularities that may have prevented genomic integration – none were found. Using the new microinjection technique, 2986 eggs were injected with a hatch rate of 31% (894 eggs hatched). Of these hatched larvae, 769 G_0s were collected giving a larva-to-pupa survival rate of 88.65%. Overall, this resulted in an egg-pupal survival rate of 25.8%. This figure falls within the margin of error estimated around the mean survival rate (egg-pupae) for all constructs injected into diamondback moth at Oxitec previously (24.4% ± 8.6 S.E., n = 34) as well as a subset of these constructs which gave rise to at least one transformant (31.1% ± 6.7 S.E., n = 25) – estimates provided by Matthew Gregory. Normally, the hatch rate in the wild-type strain used here (non-injected eggs) is approximately 90%. The larva-to-pupa survival rate is similarly high (c. 91%). Comparison of these data sets suggests that the mortality of pre-pupal individuals injected with OX4765 was not above what would be expected for eggs which had undergone micro-injection. If this were the case it may have been an indication of extreme toxicity of the construct to injected individuals (G_0s) (as has been observed previously for mosquitoes, personal communication, Dr Tarig Dafa'alla). Instead, the major cause of mortality in these OX4765 injected eggs was the trauma caused to the egg chorion and developing embryo by the injection needle or

plasmid mix, resulting in a large reduction in hatch rate relative to non-injected eggs. Again, from these G_0s no transformants were generated.

From these results, it was hypothesised that the QF2 protein is in some way toxic to lepidopterans when expressed more ubiquitously, i.e. at the G_1 stage (the progeny of injected individuals). This is because G_0 survival (injected individuals) did not appear to be affected post hatching, but full transformants could not be isolated from their offspring. It is plausible that throughout this work, germ-line transformation of injected individuals was occurring at a normal rate, but the transgenic progeny of these individuals (now expressing QF2 ubiquitously) never survived to a stage where they could be identified. As a transcriptional activator, it is possible that QF2 causes toxicity in a similar way to tTAV. In this case, toxicity occurs as a result of both 'squelching', where transcriptional machinery is recruited by the tTAV construct at high rates, starving other gene expression in the cell, and the induction of other genes at inappropriate times by tTAV protein. One potential way to reduce this toxicity is to suppress the system so that transformants can be isolated. For tTAV this can be achieved easily by rearing injected larvae on tetracycline diet. However, the dietary additive in the Q-system (QA) is an inducer rather than a repressor and as such the co-expression of the QS2 gene is required for suppression. With this in mind, a final attempt was made to transfer the Q-system to diamondback moth by creating a line that possessed copies of both the QF2 and QS2 transgenes.

A logical way to achieve this would have been to inject the QF2 construct directly into the already available suppressor line (OX4673A). However, two issues led to this strategy not being chosen. The first is the challenge posed by the interaction between QS2 and QF2. Suppression of the system occurs when QS2 protein binds to

QF2 protein, creating an inactive complex which is incapable of binding to QUAS (Giles et al., 1985; Giles et al., 1991). With the apparent toxicity of QF2 it is thus imperative that sufficient quantities of QS2 are expressed to complete this interaction. This would potentially be difficult to achieve with two independent insertions due to positional effects modifying expression patterns and strength. Secondly, due to the limited number of fluorescent protein markers available, the fluorescent marker gene (constitutively expressed and used to identify transformants) in both activator and suppressor constructs was AmCyan. This would not prove a problem if, as had been originally planned, homozygous lines of construct were generated independently, then crossed. However, if injected directly into OX4673A, dual QS2/QF2 transformants would only have been identifiable by the expression of DsRed (in the presence of QA). Furthermore, this would have been reliant on the components of the system interacting as hypothesised which may not have been likely, at least initially. For example, QS2 may not be repressed by QA, or QF2 may not have interacted correctly with QUAS. These and other issues could have been isolated if individual transformants had been generated but may have led to false negatives if the system was combined in this way. To circumvent this second issue, it would be possible to substitute the AmCyan marker in the activator construct with a phenotypically limited DsRed marker. Here, red fluorescence arising from the transformation marker could be discerned from that caused as a result of the binding of QUAS by QF2. Spatial control of gene expression in lepidopterans and other insects has been achieved through the use of the 3×P3 promoter which limits expression of transgene to the eyes and surrounding neural tissue (Thomas et al., 2002; Wimmer, 2003). However, the isolation of 3×P3 transformants in diamondback moth is made difficult at the pupal stage due to the sclerotization of the cuticle covering the eyes (Martins et al., 2012). Additionally, this solution does not

mitigate the possible differences in expression levels arising from independent transgene insertions. Thus, a revised construct, OX5046, in which the *ie1/Hr5*-AmCyan transformation marker cassette was replaced with *3×P3*-DsRed, was designed and built, but not injected. This construct was held in reserve and would be injected if time allowed and was necessary.

Based on the above issues, a revised activator construct was required where QS2 and QF2 were co-expressed, where the expression profiles of both transgenes were very similar, and where reporter and marker transgene fluorescence were not confounded. It was thought that the easiest way to satisfy these demands would be to combine QS2 and QF2 into one construct. Here only one transformation marker (AmCyan) would be required, and as both transgenes are at the same locus, their expression profiles should be identical. To further ensure that the expression characteristics were as similar as possible, the two transgene sequences (QF2 and QS2) were linked by a viral T2A bridge from the insect virus *Thosea assigna* (Gonzalez et al., 2011; Diao and White, 2012) and placed under the control of the same promoter. T2A belongs to a family of regulatory elements known as the CHYSELs (*cis*- acting hydrolase elements) which allow multiple sequences to be transcribed and translated within the same open reading frame – multicistronic mRNA (Ryan et al., 1991; de Felipe et al., 1999; Halpin et al., 1999). The characteristic of this family is a conserved 18-a.a. sequence which causes 'ribosomal-skipping'. Here, a peptide bond fails to form between the glycine and terminal proline amino acids within the T2A bridge, releasing the first polypeptide chain but allowing mRNA translation to continue on to the second coding sequence. Thus, if two sequences are joined by a T2a bridge they will be transcribed together but translated as two separate proteins. The advantage of this using this system here is that, theoretically, equimolar concentrations of each of the two conjoined sequence are produced. In

reality, the efficiency of this ribosomal skipping event vary between members of the CHYSEL family and the experimental conditions used. In this case, to ensure that, if any protein was expressed at a higher level it was the suppressor, QS2 was placed upstream of QF2 (at the 5′ end of the T2A bridge) in the construct. Finally, the *Opie2* promoter was used to replace *Bmhsp83* in driving dual Q-system component expression as this is anecdotally known to be a weaker promoter in lepidopterans.

This revised QS-T2A-QF2 construct was designed with the aid of Dr Tarig Dafa'alla on Vector NTI Advance 11.5 and named OX5042 (Figure 4.9). Due to time constraints, this construct was built by a colleague, Caroline Phillips. A construct map and details of components are given in Appendix Figure A.3C and Table A.1.

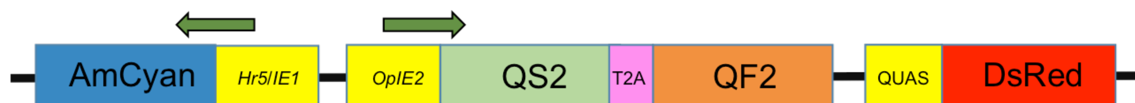


Figure 4.9: Schematic showing the components of the OX5042 construct relating to the Q system. Yellow boxes represent promoter and binding sequences which direct downstream open reading frames in the direction of the green arrows (direction of transcription). QF2 is the transactivator gene in the Q-system and is repressed by QS2. These two sequences are connected by a T2A bridge which causes ribosomal skipping during translation, allowing independent production of QS2 and QF2 polypeptides. In its constitutive state, this construct should be repressed as translated QF2 protein will be bound by QS2 protein preventing its binding to QUAS and the induction of downstream DsRed. Addition of QA should de-repress this process allowing DsRed expression. AmCyan is a marker gene which fluoresces blue under the appropriate wavelength of light and which is used to isolate transformants.

OX5042 was injected into 2,765 eggs, of which 1,463 survived to pupation (53% G_0 survival). An experiment conducted by a colleague (Matthew Gregory) prior to these injections, in which transient fluorescence was observed in G_0 individuals that had been injected with a fluorescent marker construct but no *piggyBac* helper, concluded that transient expression of a fluorescent marker does not require successful insertion of that transgene into the genome (i.e. that transcription from plasmid material alone is

sufficient to cause visible transient expression). The screening of pupal G_0 individuals for transience is a time-consuming event and in the light of these results, transient expression was only screened in embryos (Figure 4.10).

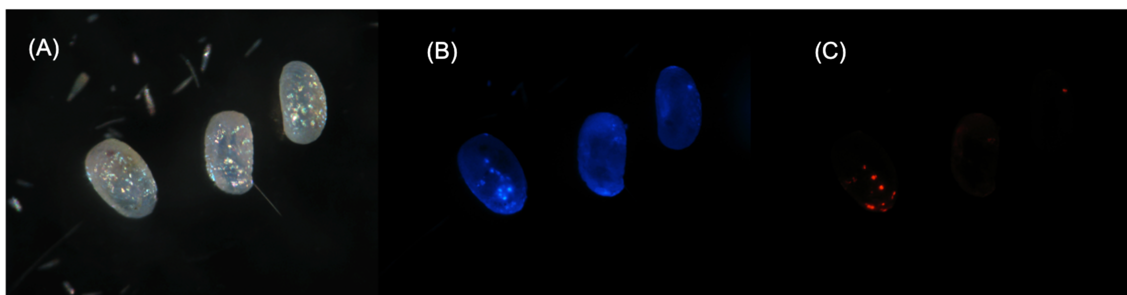


Figure 4.10: Photographs of diamondback moth eggs injected with construct OX5042 taken under bright field (A) and blue and red fluorescent protein excitation filters (B and C). This construct contains both AmCyan and DsRed transgenes. From left to right the first egg shows evidence of transience while the middle and right egg do not. Under bright field (normal light), the eggs are indistinguishable. However, when exposed to the correct wavelength of light, AmCyan and DsRed proteins within the transient egg fluoresce blue and red.

Interestingly, transient expression of both DsRed and AmCyan were observed, but red expression appeared to be c. 1 day delayed compared to blue. This may imply that, ultimately, QS2 is not able to suppress QF2 from activating the QUAS-DsRed reporter cassette but that, initially, some suppression may be taking place. However, the maturation time (period of time till protein becomes detectable) of the DsRed protein is known to be longer than AmCyan and this offers a potential alternative explanation of these results (Clontech, 2003). In any case, no transformants were isolated from these injections.

4.2.2.3 Transient expression assays

With no lines transformed with the QF2 transactivator, the behaviour of the Q-system can only be assessed via proxy. In the context of transgenesis, this proxy is transient fluorescence. Transient fluorescence occurs in the G_0 individual, that is, the individual which has undergone micro-injection. It is the expression of a transgene either from individual cells which have been transformed (and those derived from these cells) or from the non-integrated construct plasmid (essentially functioning as extra-chromosomal DNA; Matthew Gregory, unpublished data) (Figure 4.11)

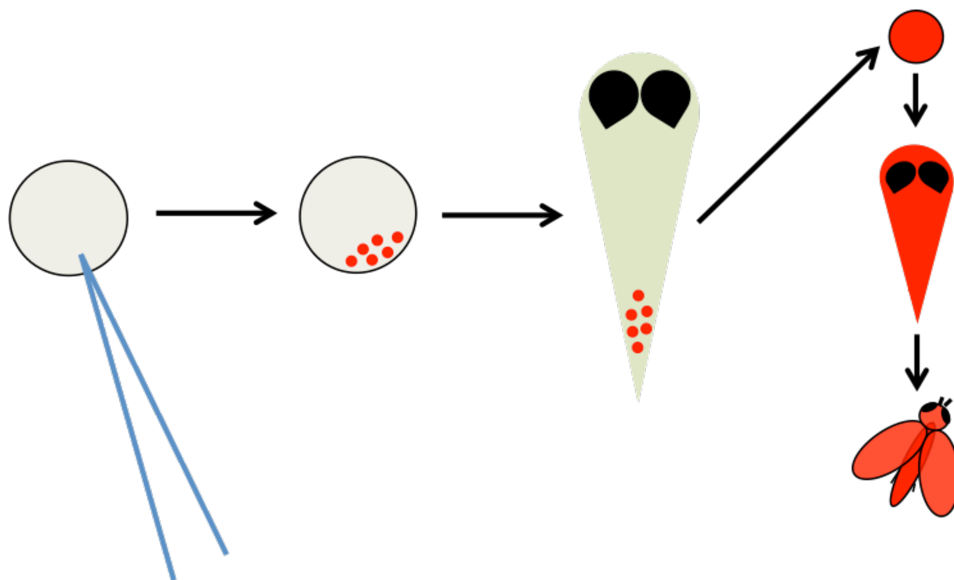


Figure 4.11: Schematic showing the process of transformation and the position of transient fluorescence in this process. From left to right, (1) eggs are microinjected. (2) Fluorescent transgenes may begin to express in the developing embryo. This could be a result of either chimeric expression (where the transgene has integrated into individual cells) or through extra-chromosomal expression (transcription and translation from injected construct plasmid without integration). (3) Transient expression, where present in embryos, may or may not be visible in developing pupae. If integration of the transgene has occurred into germ-line cell(s) gametes produced by this individual as an adult may contain a genomic copy of the transgene. If these gametes successfully contribute towards a zygote (F1 generation (4)), and this zygote is identified, a new transgenic line has been identified.

It is assumed that the protein-level interaction between QF2 and QS2 will be the same whether those proteins are expressed from chromosomal insertions or extra-chromosomal expression. Assessing the characteristics of transient expression of the DsRed effector in G_0 individuals thus provides an insight into what might be expected from this system if germline transformants could be generated. The observation of transient expression is a common form of preliminary analysis for transgene expression as it does not require germ-line transformation and has been used previously for the Q-system (Subedi et al., 2014).

Two transient expression assays were conducted. In the first, three constructs were each injected into wild-type pre-blastoderm eggs (1 construct in each egg cohort). These constructs were OX4765 (Figure 4.6 with modified 3'UTR), OX5042 (Figure 4.9) and OX4658. Construct OX4658 was a construct designed with the aid of Dr Tarig Dafa'alla on Vector NTI Advance 11.5. It is identical in structure to OX4651 (Figure 4.6) but with the *Bmhsp83*-QF2 cassette removed. Thus, this construct consisted of just a transformation marker (AmCyan), and QUAS-DsRed. Its purpose was to control for the DsRed basal expression rates in the other constructs, that is the degree to which QUAS-DsRed will produce fluorescent protein even in the absence of QF2. In addition to these constructs, a cohort of eggs was also injected with water to act as a control for possible fluorescence arising from damaged tissue around the injection site. All constructs contained AmCyan as a marker gene and DsRed as an effector. However, OX5042 contained both an effector activator and an activator suppressor (QF2 and QS2), OX4765 contained only the effector activator (QF2) and OX658 contained neither activator nor suppressor. Thus, comparing the proportion of transience events between constructs may indicate the level to which the Q-system is functional in this species/design. As these events are known to take place over a period of time (it may

take time for fluorescent protein concentrations to build up to visible levels) these cohorts of injected eggs were observed at a series of time points post injection (6hr, 12hr, 18hr, 36hr, 48hr).

In a second experiment, eggs from OX4673A (hereby the suppressor line) as well as wild-type eggs were used. These were both injected with the same QF2 activator construct used in the first transient expression assay (OX4765). Eggs were screened at 12 hr and 24 hr post-injection to look for potential reductions in DsRed transient expression which may be brought about by the constitutive expression of QS2 from genomic insertions of the integrated OX4673A transgene.

The levels of fluorescence expressed from the injected plasmid can be affected by factors such as egg age at injection and copy number/concentration of DNA mix transferred to the embryo. As these are likely to vary between assays, it is not the aim of this analysis to directly compare transient expression levels between the two experiments but rather to compare egg cohorts within assays and from these, draw general conclusions about the properties of the Q-system.

Result and Discussion

Assay 1: Encouragingly, eggs injected with water showed neither AmCyan nor DsRed fluorescence. As such, this cohort was not included in further analysis due to potential issues with large numbers of zero results in binary analysis. The proportions of each cohort of eggs which showed DsRed and/or AmCyan fluorescence at each of the time points is shown in Figure 4.12.

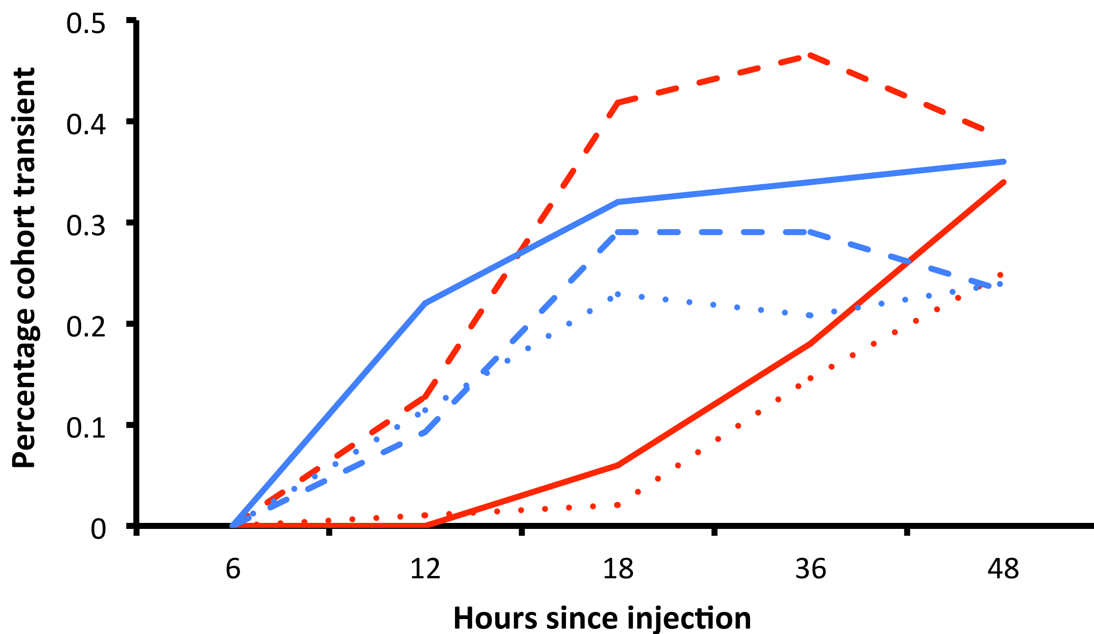


Figure 4.12: Proportion of egg cohorts injected with three transgene constructs each carrying AmCyan and DsRed fluorescent markers. Solid lines represent the cohort of eggs injected with OX4658, dashed lines the cohort injected with OX4765 and dotted lines the cohort injected with OX5042. Blue lines represent AmCyan fluorescence, red lines DsRed fluorescence.

AmCyan and DsRed data were analysed independently using the R package, Bild, which performs logistic regression on binary longitudinal (repeated measurements) data. Comparisons were made against OX4658 as this is the transient expression control construct. For AmCyan it was found that construct was not a significant predictor of transience (constructs did not differ significantly in their levels of transience) (4658-4765 $t = -0.431$, $p = 0.66$; 4658-5042, $t = -0.513$, $p = 0.60$). As was expected, time was a highly significant predictor (time, $t = 4.173$, $p < 0.001$), however constructs did not differ significantly over time (no significant interaction terms – 4765: time, $t = -0.535$, $p = 0.59$; 5042: time, $t = -0.658$, $p = 0.510$). Transience levels increased with time (as hypothesised due to the build-up of protein to visible levels). However, this behaviour did not differ between constructs. The AmCyan marker gene and its regulatory machinery are identical in all three constructs. Thus, these results act as a baseline to show that there appears to be agreement between transient expression levels

and the underlying genetic circuitry being investigated (an assumption of this experiment).

For DsRed data it was found that construct was a significant predictor of transient expression, with OX4765 but not OX5402 showing a significantly higher proportion transient expression than OX4658 (4658:4765, $t = 2.870$, $p = 0.004$; 4658:5042, $t = -0.073$, $p = 0.942$). Again, time was a highly significant predictor of transient expression (Time, $t = 4.526$, $p < 0.001$), however, constructs in this data set differed significantly in their interaction with time. OX4765 but not OX5042 showed a significantly different (lower) slope than OX4658 (4658:4765, $t = -2.455$, $p = 0.014$; 4658:5042, $t = -0.196$, $p = 0.84$). These results are interesting as they show (along with Figure 4.12) that there was substantial basal DsRed expression observed in eggs injected with the control construct OX4658. The "repressed" construct OX5402 showed similar characteristics to this baseline level and this may imply that QS2 protein in these eggs was able to suppress the transactivator from causing DsRed transcription to above basal levels. An alternative explanation, however, is that the QS2-T2A-QF2 cassette in this construct was not functioning properly and thus the only transient expression arose from basal DsRed expression. The delay of DsRed expression compared with AmCyan in the OX5042 construct observed previously (Section 4.2.2.2), was replicated in this experiment. In Figure 4.12 it can be seen that 12 h post-injection, eggs injected with OX5042 had begun to display signs of AmCyan transience while no DsRed transient eggs were identified. However, by 18-24 h post-injection DsRed expression in these eggs had begun to reach visible levels. Again, this is a potential indication that the co-expression of QS2 and QF2 in these constructs regulated the expression of DsRed. This behaviour is in contrast to the "unrepressed" QF2 construct (OX4765) which showed both AmCyan and DsRed expression (at similar levels) from 12 h post-injection and a

significantly higher construct effect. The significantly lower slope coefficient generated by the Bild package for this construct is difficult to interpret as it would appear from the results shown in Figure 4.12 that the rate of increase in transient expression was higher for this construct than the others. It may be that the decreased number of transient eggs observed in the final time-point reduced the slope estimate (and increased the intercept estimate) for this cohort.

Assay 2: The percentage of each cohort which showed transient expression for AmCyan and DsRed at each time-point is shown in Figure 4.13.

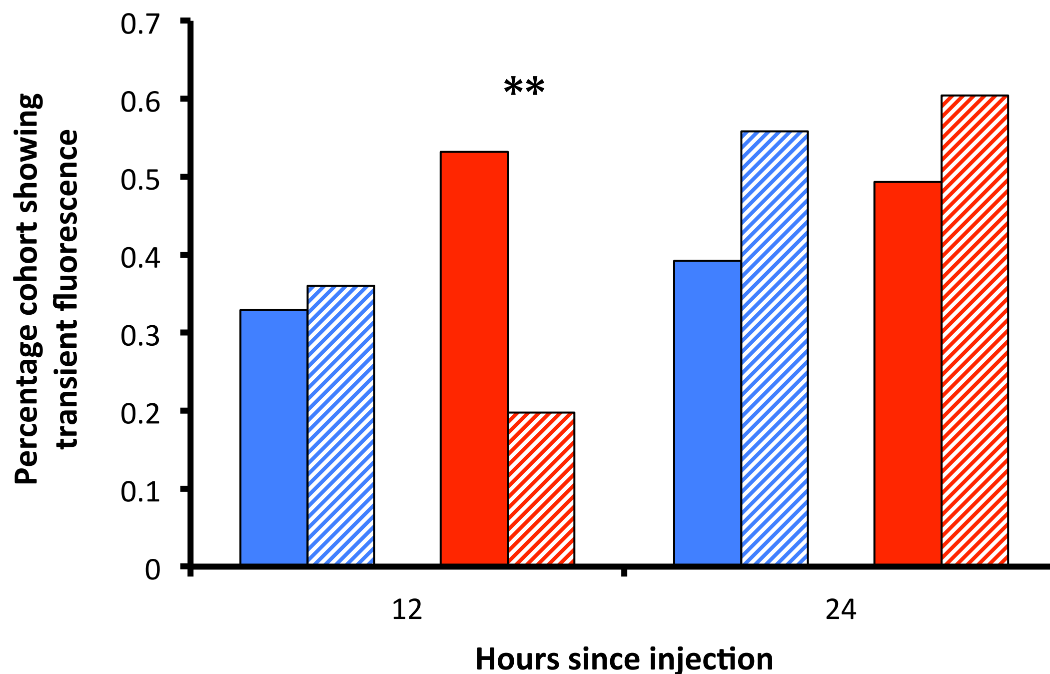


Figure 4.13: Percentage of each egg cohort displaying transient expression of two fluorescent proteins – DsRed (red bars) and AmCyan (blue bars) - at two time-points after microinjection with OX4675. Solid bars are those cohorts consisting of wild-type eggs. Hatched bars are eggs from the OX4673A line and carry genomic insertions of the QS2 suppressor transgene.

Overall, transient expression levels in the second assay were higher than those of the first. However, the same general trend was present. Throughout the experiment, AmCyan transient expression levels did not differ significantly between wild-type and suppressor line eggs (12 h: $X^2 = 0.067$, df = 1, p = 0.795; 24 h: $X^2 = 0.034$, df = 1, p = 0.853). This is interesting as it implies that expression of AmCyan from the genomic transgene insertions in the suppressor line do not contribute substantially to the visible levels of transient expression in this assay, at least on these timescales. On the other hand, DsRed transient expression levels, although faint and difficult to assess, were found to differ significantly between the two egg cohorts. At 12 h post-injection, significantly more eggs showed transient expression in the wild-type cohort than in the suppressor line cohort ($X^2 = 6.6546$, df = 1, p-value = 0.00989). However, by 24 h post-injection, this difference was no longer apparent ($X^2 = 1.62$, df = 1, p = 0.202). These results imply that, at least initially, the QS2 protein expressed by the suppressor line is able to suppress QF2 protein deriving from injected copies of the OX4675 construct. However, as time progresses, the level of QS2 expression is insufficient to maintain QF2 suppression, and by 24 h none was evident. These results corroborate those from the previous assay, in which eggs injected with the construct containing both activator and suppressor (OX5042) showed significantly less DsRed transient expression than those injected with construct containing just the activator (OX4765), at least initially. This loss of suppressive capacity occurred earlier in the second assay than the first. One possible reason for this is that each cell in the suppressor line contains, at most, two copies of the QS2 transgene, whereas when injected with OX5042, multiple copies of the transgene (containing both activator and suppressor) are likely to have been present.

In summary, these experiments provide some evidence that the interaction between QF and QS at the protein level, on which the Q-system is reliant, appears to be

maintained in the Lepidoptera. However, the suppressive capacity of QS seems to be limited, with each experiment showing a loss in this ability by the end time point. This suggests that multiple copies of QS may be required for full suppression of QF, a finding which may aid future construct design in this system. Unfortunately, with no time left to pursue this avenue in attempting transformation of the Q-system, this work must be left for future researchers.

4.3 Conclusion and Summary

The aim of this chapter was to explore the feasibility of utilising the Q-transgene expression system in the diamondback moth. The conclusions of this investigation are that this system is unsuitable, at least in its current form, for ubiquitous transgene control in this species.

As a binary transgene expression system, both an activator and suppressor are required to test the Q-system. Here, a total of 13,410 eggs were injected with various re-deigned QF2 constructs in an attempt to generate an activator line, with no success in recovering any transgenic G_1 individuals. Lepidopterans are known to have a relatively low rate of transformation success (Marec et al., 2005b). However, even taking this into account, the probability of generating no transformed lines after this many injection attempts is estimated to be less than 3×10^{-3} (Appendix Section A.1). The most probable cause for this failure is that this protein (QF2) is significantly toxic in this species and induces death in individuals showing ubiquitous expression prior to them being identified.

The toxicity of QF, specifically when used in conjunction with ubiquitous promoters, has been cited as a potential concern in the wider use of the Q-system. This system was originally built to study various components of *Drosophila* neural development and as such its expression was primarily limited to a small range of tissues during specific time periods (Potter et al., 2010). Even with such restricted expression, transformation with QF constructs was challenging and required modifications to QF regulatory regions (replacement of 3′ UTR with less stable *Dmhsp70* 3′ UTR (Potter et al., 2010)) and coding sequences (de-optimising codon usage for *Drosophila* (Potter and Luo, 2011)). Transformation of QF with ubiquitous promoters has been unsuccessful in the two species in which it has been attempted (*D. melanogaster* and the zebrafish (Potter et al., 2010; Subedi et al., 2014)), and diamondback moth can now be added to this list. In the zebrafish, even transient expression of QF constructs designed using ubiquitous promoters resulted in high levels of embryonic mortality (43%, although this was reduced by decreasing injected plasmid concentrations), while in both species successful transformation could only be achieved using tissue-specific promoters or enhancer traps. That mortality was caused across this large phylogenetic range attests to the basal nature of the toxicity involved. Thus, it is in retrospect no surprise that difficulty was experienced in generating transformants in lepidopterans designed to express QF2 more ubiquitously in this chapter.

In their most recent publication, Subedi *et al*. (2014) state that they have been successful in producing truncated forms of QF that maintain the ability to activate QUAS and be repressed by QS, but show lower toxicity (to *Drosophila*). However, these variants of QF are still at the testing stage and results have not yet been published, so they were unavailable during this research. In our attempt to reduce the toxicity of QF, this gene was combined with QS in a single construct connected by a T2A

ribosomal skipping bridge. However, this was also unsuccessful in producing transformants. Subsequent findings (Subedi et al., 2014) suggest that there is not in fact an equimolar relationship between QS and QF; i.e. a 1:1 ratio will not result in full QF suppression. With the apparent lethality of this protein to Lepidoptera and our design of OX5042 as an equimolar system, it is perhaps not surprising that this construct failed to generate transformants.

With the apparent high toxicity of ubiquitous QF2 expression to pre-pupal stages of the diamondback moth, the Q-system was investigated at the embryonic level via transient fluorescence, as has been achieved previously in the zebrafish. In a preliminary experiment it was found that, even in the absence of its activator, QUAS-DsRed showed high levels of basal transient expression. A construct containing both activator and suppressor (OX5042) showed similar levels of DsRed transient expression to this basal expression, while a construct containing just the activator (OX4765) showed significantly higher levels of transient expression. In a second experiment, these results were corroborated when the level of DsRed transient expression arising from injected OX4765 construct was significantly lower in eggs containing a genomic insertion of the suppressor transgene (QS) than in wild-type eggs. Both of these effects, however, decreased substantially with time. These results function as a proof of concept in demonstrating that QS repression of QF is functional in the Lepidoptera, but suggest that full repression of the system may be difficult to achieve.

Serious doubts remain as to whether the Q-system is suitable for transgene control within a genetic pest management framework. Significant further work is required to characterise this system, especially in terms of facilitating its ubiquitous expression. Additionally, the apparent challenge in ‘balancing’ the expression levels of QS and QF in order to effectively suppress the system may prove an obstacle in

generating effective RIDL lines. In *Drosophila* S2 cells, even five copies of the QS gene were unable to cause full repression of a single QF gene (in fact, expression levels remained c.10× that of baseline levels). In the zebrafish, QF transient expression remained at 200% of basal expression levels even in the presence of QS. In addition, the basal expression of the QUAS-reporter cassette was high in zebrafish, and here in transient assays. Combined with the toxicity of the activator and the difficulty in fully repressing the system, it is likely that this basal expression of a potential lethal effector would cause serious fitness costs to transformed individuals, potentially reducing their usefulness for autocidal release programmes.

As refinement of the Q-system continues, it is probable that many of these issues will be addressed. However, this is likely to take a considerable period of trial and error over potentially a number of years. For example, in the case of the X-chromosome shredding system, creation of a functional phenotype to prevent male-lethality required protein-level engineering to destabilise the *I-Ppo*I endonuclease. The timeframe between publication of the original system and its subsequent re-engineering was approximately 6 years (Windbichler et al., 2008; Galizi et al., 2014). The existence of multiple untested alternatives to the Q-system, such as expression systems controlled by plant hormones (Bierfreund et al., 2003) or light wavelength (Wang et al., 2012; Ma et al., 2013) make this timeframe unattractive, especially for a commercial product. As such, future research should focus on the potential of these alternative systems, at least until the challenges related to the ubiquitous expression and suppression of QF are addressed.

4.4 Materials and Methods

4.4.1 Micro-injection

Injections used a micro-manipulator 5171 (Eppendorf – Hamburg, Germany). Treatment of eggs, injection protocol and rearing of potential transformants followed the methods of Martins, Naish et al, (2012).

4.4.1 Quinic acid toxicity

In experiments conducted by Potter et al., (2010) a QA concentration of 7.5 mg/ml is used to induce the Q-System. In these experiments, 7.5 mg/ml was therefore used as a starting concentration to test the toxicity of QA against three significant and unrelated insect pests: medfly, diamondback moth and *Ae. aegypti*. A logarithmic scale was used to create a range of values around this concentration with the logged values of each experimental concentration separated by an interval of 1. Experimental concentrations of QA were 0.98 mg/ml, 2.4 mg/ml, 7.5 mg/ml and 23.7 mg/ml with 0.0 mg/ml added as a control. For medfly and diamondback moth, different amounts of QA stock solution (neutralised to pH 6.7 with NaOH) were mixed with artificial diet in rearing containers/cups to create the experimental concentrations required, with an equivalent amount of distilled water added to the control. For *Ae. aegypti,* different amounts of QA stock solution were pipetted into plastic pots and the volume in each pot made up to 300 ml using distilled water.

For the diamondback moth and medfly experiments, 100 eggs were placed into each container/cup with two replicates per treatment for medfly and four replicates per treatment for diamondback moth. Five days later, eggs were inspected and the number

of unhatched eggs counted. Upon pupation and eclosion, larvae-to-pupae, pupae-to-adult and larvae-to-adult proportional survival was calculated.

For *Ae. aegypti*, 100 first-instar larvae were transferred by hand into each deli pot (two replicates per treatment). Larvae were fed an appropriate quantity of fish food daily. Proportional survival to pupae and adults was calculated as above.

Proportional survival in each life stage was analysed using a logistic regression GLM and binomial errors. Where data showed signs of over-dispersion, the explanatory variable (+ 0.1) was logged and quasibinomial errors used.

4.4.2 Construction of OX4673 (repressor construct)

The QS2 sequence was taken from Potter et al., (2011) and codon-optimised for *Ae. aegypti*, diamondback moth and medfly. A *Bsa*I recognition site was added at the 5′ end of the start codon such that half the start codon would be removed upon digestion with *Bsa*I. An inverted *Bsa*I site was also added to the 3′ end of the QS2 sequence with nonsense sequence added upstream of this site to act as a linker region with the 3′ UTR. This artificial sequence was synthesised by Geneart.

1. *Bmhsp83*Pro + 5′ UTR was amplified from plasmid OX4430/1 using primer 2792 *Bmhsp*-bsa-f (designed to leave a *Not*I restriction site overhang when digested with *Bsa*I) and primer 1807 *Bmhsp83*-5utr-bsa-r (designed to leave a partial start codon overhang when digested with *Bsa*I). Amplified fragment digested with *Bsa*I and purified on an agarose gel and minElute column.
2. QS2 was amplified from its Geneart plasmid using primer 2793 QS2-bsa-f (designed to leave a partial start codon overhang when digested with *Bsa*I) and

primer 2794 QS2-bsa-r (designed to leave a nonsense linker region overhang when digested with *Bsa*I). Amplified fragment was digested with *Bsa*I and purified on an agarose gel and minElute column.

3. *BmHsp*83 3′ UTR + SV40 3′ UTR was amplified from plasmid #4430/1 with primer 2795 *BmHsp83*-3utr-bsa-f2 (designed to leave a nonsense linker region overhang when digested with *Bsa*I) and primer 2796 SV40-fse-r2 (designed to leave a *Fse*I overhang when digested with *Bsa*I). Amplified fragment was digested with *Bsa*I and purified on an agarose gel and minElute column.
4. Digested plasmid backbone #4221 with *Not*I and *Fse*I. Purified on an agarose gel and normal spin column.
5. Fragments and vector were ligated using Fermentas Rapid Ligation kit. Ligated vector was transformed into competent *E. coli* cells and colonies allowed to grow overnight. Individual colonies were PCR-screened using diagnostics primers Primer 2798 Diag-Qs2 and primer 224 Diag-pb3. Colonies showing bands of the correct size were selected and their DNA digested using *Age*I and *Sex*I. Colonies showing the correct digested fragment sizes were sent for sequencing (GATC Ltd). Of these colonies, two showed the correct sequence for OX4673 and were purified by maxipreps.

4.4.2.1 OX4673A expression profiling using RT-PCR

RNA was extracted from wild-type and OX4673A 1st and 2nd instar and pupae (Qiagen RNeasy mini kit). Fifty individuals were pooled for each 1st instar replicate, five individuals pooled for each 2nd instar replicate, and one individual per pupal replicate. One replicate was performed for each wild-type life-stage and two for each

OX4673A life stage. After quantification of RNA concentration in each sample (IMPLEN Nanophotometer P300), 0.5µg was taken for One-step RT-PCR was performed on these RNA samples (SuperScript Invitrogen) using primers specific to QS2 coding sequence Primer 2800 + Primer 2310. The positive control used RNA from the wild-type pupal replicate and primers specific to the diamondback moth endogenous *doublesex* gene Primer 3482 + Primer 3544. H_20 control used H_20 instead of RNA and NO-RT control was identical to the positive control but no reverse transcriptase was added. This controlled for the amplification of genomic DNA.

4.4.3 Construction of OX4651 (activator construct)

The QF2 sequence was taken from Potter and Luo (2011) and codon-optimised for *Ae. aegypti*, diamondback moth and medfly. A *Bsa*I recognition site was added at the 5′ end of the start codon such that half the start codon would be removed upon digestion with *Bsa*I. An inverted *Bsa*I site was also added to the 3′ end of the QF2 sequence with nonsense sequence added upstream of this site to act as a linker region with the 3′ UTR. This artificial sequence was synthesised by Geneart.

1. QF2 was amplified from its Geneart plasmid using primer 2807 QF2-bsa-f (designed to leave a partial start codon overhang when digested with *Bsa*I) and primer 2808 QF2-bsa-r (designed to leave a nonsense linker region overhang when digested with *Bsa*I). Amplified fragment was digested with *Bsa*I and purified on an agarose gel and minElute column.
2. *BmHsp*83 3′ UTR + SV40 3′ UTR was amplified from plasmid #4430/1 with primer 2795 *Bmhsp83*-3utr-bsa-f2 (designed to leave a nonsense linker region

overhang when digested with *Bsa*I) and primer 2809 SV40-bsa-r18 (designed to leave a nonsense linker region overhang when digested with *Bsa*I). Amplified fragment was digested with *Bsa*I and purified on an agarose gel and minElute column.

3. QUAS was amplified from its Geneart plasmid using primer 2810 QUAS-bsa-f (designed to leave a nonsense linker region overhang when digested with *Bsa*I) and primer 2811 QUAS-bsa-r (designed to leave a partial 3′ end of QUAS overhang when digested with *Bsa*I). Amplified fragment was digested with *Bsa*I and purified on an agarose gel and minElute column.
4. *Dmhsp70* minipromoter + *adh*-intron was amplified from OX513 using primer 2812 *Hsp*70-bsa-f (designed to give a partial 5′ end of QUAS overhang when digested with *Bsa*I) and primer 2813 *Adh*-int-bsa-r (designed to give a partial *Avr*II restriction site overhang when digested with *Bsa*I). Amplified fragment was digested with *Bsa*I and purified on an agarose gel and minElute column.
5. DsRed-SV40 was amplified from OX4583 using primer 2814 nls-bsa-f (designed to give a partial *Avr*II restriction site overhang when digested with *Bsmb*I) and primer 2815 SV40-bsa-r19 (designed to attach a *Fse*I restriction site to the 3′ end of the SV40 3′ UTR). Amplified fragment was digested with *Bsmb*I and *Fse*I and purified on an agarose gel and minElute column.
6. Digested fragments as well as the digested vector and *BmHsp*83 Promoter+5′UTR from OX4673 construction were ligated using Fermentas Rapid Ligation kit. Ligated vector was transformed into competent *E. coli* cells and colonies allowed to grow overnight. Individual colonies were PCR-screened using diagnostics primers primer 1127 Diag2-dmhsp and primer 224 Diag-pb3. Colonies showing bands of the correct size were selected and their DNA

digested using *Age*I and *Bgl*II. Colonies showing the correct digested fragment sizes were sent for sequencing (GATC Ltd.). Of these colonies, two showed the correct sequence for OX4651 and were purified using maxipreps.

4.4.4 Construction of basal expression control construct OX4658

1. Construct OX4651 was digested with *Not*I and *Asc*I. Klenow fragment was added in the presence of dNTPS and incubated at 37°C for 30min. This creates blunt ends for ligation. Digested vector was purified on an agarose gel and a normal spin column.
2. OX4651-QUAS-DsR was ligated using Fermentas Rapid Ligation Kit. Ligated vector was transformed into competent *E. coli* cells and colonies allowed to grow overnight. Individual colonies were PCR-screened using diagnostics primer 956) Diag5-ie1 + 825) Diag4-DsRed. Colonies showing bands of the correct size were selected and their DNA digested using *Nhe*I and *Avr*II. Colonies showing the correct digested fragment sizes were sent for sequencing (GATC Ltd.). Of these six colonies, all showed the correct sequence for OX4658 and two were purified using maxipreps.

4.4.5 Construction of modified activator construct OX4765

1. Construct OX4651 was digested with restriction enzymes *Spe*I and *Asc*I and purified on an agarose gel and normal spin column.Pro-QF2 was amplified from OX4651 using primer 2871) seq-Bmhsp83pro-6 + 3070) QF2-bsa-r3. PCR

products were quantified on an agarose gel and then purified on a spin column. After digesting purified fragments with the restriction enzyme *Bsa*I, fragment was purified again on an agarose gel and then minElute spin column.

2. Dmhsp70 3′UTR was amplified from drosophila gDNA using primers 3071) Dmhsp70 3′UTR- f + 3072) Dmhsp70 3′UTR-bsa-r. Fragment was purified and quantified as above.
3. OX4651-QF-Dmhsp70 3′UTR was ligated using a Fermentas Rapid ligation kit. Ligated vector was transformed into competent *E. coli* cells and colonies allowed to grow overnight. Individual colonies were PCR-screened using diagnostics primers, primer 2858) Diag2-QF2 + 954) Diag *Dmhsp*. Colonies showing bands of the correct size were selected and their DNA digested using *Xho*I. Colonies showing the correct digested fragment sizes were sent for sequencing (GATC Ltd). Of these colonies, three showed the correct sequence for OX4765 and were purified using maxipreps.

4.4.6 Construction of modified activator construct OX5046

1. Construct OX4765 was digested with the *Not*I and *Kpn*2I restriction enzymes and purified on an agarose gel and normal spin column.
2. *3×P3*-DsRed was amplified from OX4271 using primer 3422) *3×P3*-bsmb-f6 + 3423) DsR-bsmb-r3. These primers were designed to give a partial *Not*I and *Kpn2*I restriction site overhangs when digested with *Bsmb*I. Fragment was purified on an agarose gel and a minElute column.
3. OX4765-*3×P3*-DsRed was ligated using Fermentas Rapid Ligation kit. Ligated vector was transformed into competent *E. coli* cells and colonies allowed to

grow overnight. Individual colonies were PCR-screened using diagnostics primer 825) Diag4-DsRed and primer 1874) Diag-bmhsp83pro. Colonies showing bands of the correct size were selected and their DNA digested using *Not*I and *Kpn*2I. Colonies showing the correct digested fragment sizes were sent for sequencing (GATC Ltd.). Of these colonies, two showed the correct sequence for OX5046 and were purified using maxipreps.

4.4.7 Transient expression assays

Assay 1: 51, 86 and 96 pre-blastoderm wild-type eggs were injected with the OX4658, OX4765 and OX5042 constructs. 61 wild-type eggs were also injected with H_20 to act as a control. These were subsequently screened for both AmCyan (blue) and DsRed (red) fluorescence at 6, 12, 18, 36 and 48 hours post injection. Analysis was conducted using the R Package Bild for analysis of binary longitudinal (repeated measurement) data.

Assay 2: 79 wild-type and 86 OX4673A eggs were injected with the OX4765 construct. Screening followed the protocol listed for assay 1. However, after observing the results of the first assay, eggs were only screened at the 12 and 24 hour marks as these appeared to be the most informative. Analysis was conducted separately for each fluorescent protein at each time point using a binomial proportions test in R.

Chapter 5

Chapter 5: Conclusions

Lepidopterans rank amongst the most economically important orders of insect pests worldwide. Their control is primarily through the large-scale use of synthetic chemical insecticides. This reliance on insecticides has had significant ecological consequences on off-target species and caused widespread resistance to these compounds. In response to these concerns, more selective insecticides such as *Bt* transgenic crops have been developed to target the Lepidoptera. However, there is now growing concern over the potential for resistance to develop to these new control methods.

One lepidopteran pest that shows an almost unparalleled ability to develop insecticide resistance is the diamondback moth. The application of the SIT, which has been so successful in the area-wide control of some Tephritid fruitflies and other dipteran species, has so far not been applied against the diamondback moth. The reasons for this also apply to a range of other lepidopteran species for which SIT programmes would be highly desirable, but which are absent. That is, the lack of economically feasible means of sorting males from females prior to release, and the high costs of sterilising radiation doses to released male fitness. This thesis sought to address these issues through the development and testing of a female-specific autocidal genetic control system for the diamondback moth.

A line of diamondback moth carrying a transgene conferring female-specific, tetracycline-repressible lethality based on the sex alternate splicing of the pink bollworm *doublesex* gene was developed: OX4319L. The results of Chapter 2 show that this line displays an appropriate lethal phenotype to function effectively both as a

genetic sexing system, and as a means of autocidal population management. Further investigation demonstrated that the transgene has not dramatically reduced the mating competitiveness of homozygous OX4319L males relative to their wild-type counterparts (including factors such as sperm competition and temporal changes in fecundity), at least in the laboratory. Estimates of OX4319L relative mating competitiveness (RSI) from these experiments (0.41 and 0.37) were significantly lower than those of wild-type individuals and indeed, lower than RSI values calculated for other fsRIDL strains such as the OX3097D olive fly and OX3864A medfly (RSI = 0.56 and 0.46, respectively) (Ant et al., 2012; Leftwich et al., 2014). However, they are higher than that generated for OX513A *Ae. aegypti* (RSI = 0.31, although higher estimates were generated at different densities (Massonnet-Bruneel et al., 2013)), which has been successfully deployed in field suppression trials. These estimates are also higher than the minimum recommended value for irradiated medfly in SIT (RSI= 0.2).

Comparison of these estimates to RSI values of strains conventionally used in SIT (such as the medfly Vienna-8 *tsl* strain) is complicated by the wide range of estimates generated from different studies. For Vienna-8 these vary from extremely low (c. 0.2) (Shelly et al., 2005; Paranhos et al., 2006) to relatively high (0.48) (Morrison et al., 2009). The effects of irradiation also appear to vary considerably, with no change in mating competitiveness in some cases (Wong et al., 1983; Shelly et al., 2005) and in others up to two-fold decreases in sexual performance and negative effects on a range of complex mating behaviours (Calcagno et al., 2002; Lux et al., 2002). Comparison with the relatively sparse literature on moth strains used in SIT is also difficult as these few studies have tended to concentrate on aspects of competitiveness such as pheromone response in wind tunnels and trap response/recapture rates in the field. In both cases, irradiation has been shown to cause significant decreases in competitiveness (Suckling

et al., 2002; Stringer et al., 2013). Due to the regulatory issues surrounding the field testing of genetically modified organisms, field experiments with OX4319L were not possible in the scope of this thesis. Wind tunnel pheromone response experiments, however, are currently underway in Cornell University, USA, with initial results indicating good performance by OX4319L (personal communication, Michael Bolton). Nonetheless, it is believed that the experiments described in this thesis provide a suitably robust preliminary examination of the mating competitiveness of OX4319L.

Experiments investigating the population genetics of the OX4319L transgene insertion were able to expand on these results. Estimates of relative fitness derived from the evolutionary trajectories of transgenic genotypes imply that this line is less susceptible to wild-type allele stock-colony contamination than traditional chromosomal-translocation-based GSS methods (*tsl* medfly strains). Nonetheless, the fitness costs estimated in these experiments were high enough for population models to conclude that persistence of this transgene in the wild is extremely unlikely, even if fully permissive conditions - sufficiently high concentrations of tetracycline in host plants to disengage engineered lethality - are found. Linked to these experiments, analysis of transgene heterozygotes concluded that rearing larvae on host plant material did not affect the penetrance of the larval female-lethal phenotype in OX4319L, while sufficient tetracycline to repress the system is unlikely to be taken up by host plants in the field. Together, these results provide a robust demonstration of the non-persistence of this transgene in the wild, a concern often voiced over the release of transgenic organisms (Wallace, 2014).

Future research into the competitiveness of this line should have two goals; to examine more complex behaviours such as predator avoidance, pheromone response and flight ability which could, at least initially, be tested in wind tunnels/flight mills or

mesocosm assays; and to explore the extent to which transgene-associated fitness costs to these and other behaviours may be exacerbated under adverse field conditions. This would require preliminary studies to be conducted on the mating biology of the diamondback moth in the field, focusing on characteristics such as male and female remating propensity, as surprisingly little about this is known. Data gathered from such experiments would further inform the debate over the utility of co-releasing males and females in autocidal release programmes as, if males mate relatively few times, this supports the idea that co-released females would act as "sperm sinks" (Hight et al., 2005). This work should be supplemented by research into maximising the competitiveness of released OX4319L males. In the medfly, both bacterial dietary supplements (Gavriel et al., 2011) and exposure of pre-release adults to attractants such as ginger root oil (methyl eugenol) (Shelly et al., 2007) has been shown to significantly increase sexual performance. It is highly probable that the competitiveness of OX4319L males estimated here could be increased by similar means (Robinson and Hendrichs, 2005).

Additionally, a prudent and interesting direction of research would be to investigate the degree to which penetrance of the lethal phenotype in this line exhibits plasticity, perhaps in response to altered environmental conditions or diets. In disease-refractoriness strategies employing transinfections of mosquito strains with the bacterium Wolbachia, variation in temperature in particular has been cited as a potential issue limiting field efficacy, and this may also apply to the expression of lethal effectors in transgenic insects (Murdock et al., 2012). This work would combine well with research into resistance against the fsRIDL phenotype, whether that be behavioural - as has been seen previously for the SIT in medfly (McInnis et al., 1996) - or molecular/genetic (such as mutation of the insertion locus or some form of metabolic

defence against tTAV lethality). As with all pest management strategies, resistance formation against fsRIDL insects is possible, and genetic systems designed to pre-empt this, such as the system of redundant lethals proposed in Figure 5.1, should be investigated.

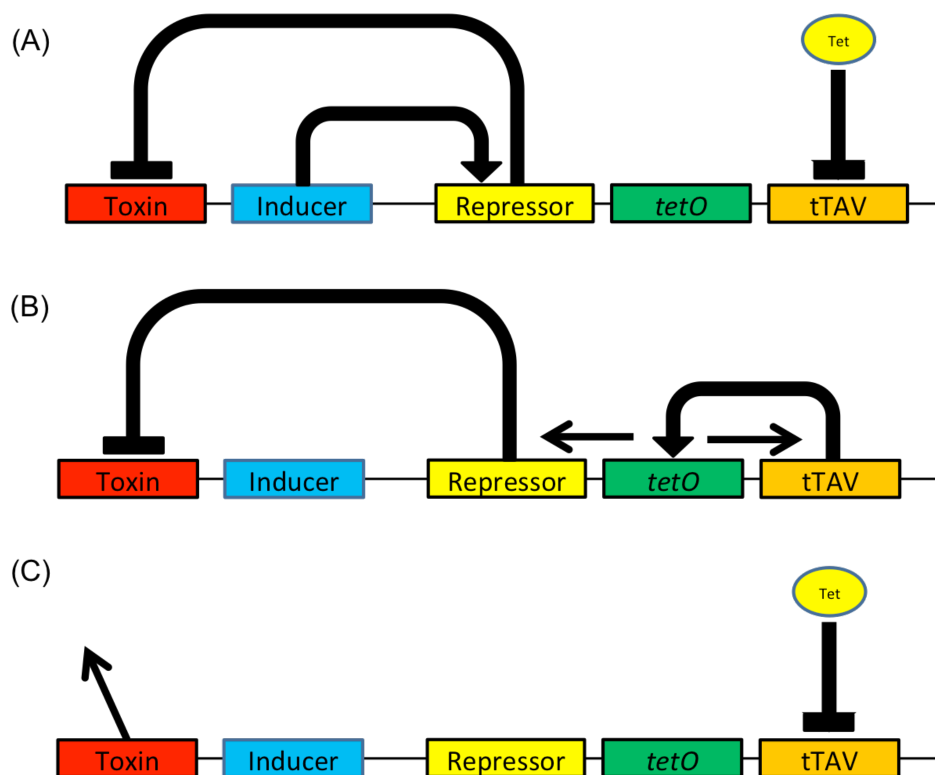


Figure 5.1: Proposed design for a safeguard system based on a series of redundant lethal effectors. The system is identical to the tetracycline-repressible RIDL lethal system but takes advantage of the bidirectional nature of the *tetO* enhancer to repress a second lethal system (here generically represented as 'Repressor' and 'Toxin') as well as a second inducible expression system (here generically represented as 'inducer'). During **Mass-Rearing** (A), tTAV lethality is repressed as in the RIDL system by dietary tetracycline supplements. In addition, the inducible system is activated (by dietary or climatic conditions) to repress the Toxin. In the **Field** (B), tTAV lethality is engaged by the absence of tetracycline, which simultaneously drives the expression of a repressor, suppressing the redundant lethal system. But still resulting in the death of the insect. (C) **Resistance:** If a breakdown in the tTAV positive feedback loop occurs in the field (in this case simplified as tetracycline being present, but could also be a result of mutation within the tet-off locus), tTAV lethality is disengaged but expression of the secondary toxin is simultaneously de-repressed, leading to the death of the 'resistant' individual prior to mating.

Chapter 3 sought to test the utility of OX4319L as an Integrated Pest Management tool. This was conducted using two proof-of-principle cage experiments. The first demonstrated that the sustained release of OX4319L males into a closed population can cause dramatic reductions in its reproductive capacity, in this case resulting in extinction. This suppression occurred at a rate that was comparable with previous experiments investigating genetic control strategies in the olive fly, medfly, and two mosquito species (de Valdez et al., 2011; Ant et al., 2012; Galizi et al., 2014; Leftwich et al., 2014).

A second experiment sought to investigate the proposed synergies between autocidal strategies which target females, and insecticides that have a dominant mode of action (that is, where insecticide resistance is recessive). Modelling has suggested that the survival of male transgenic progeny when an fsRIDL system is used in the field will result in the introgression of susceptible genetic backgrounds into wild populations, effectively diluting the frequency of homozygous resistance (Alphey et al., 2007; Alphey et al., 2009). This mechanism of resistance management is analogous to the high-dose/refugia strategy currently employed in *Bt* crops. The results of this experiment demonstrated that treatments which combined *Bt* broccoli plants and low-level releases of OX4319L males limited diamondback moth population growth to a significantly higher extent than when either strategy was used in isolation. These findings were corroborated by *Bt* susceptibility assays which showed that, although the frequency of resistance had risen significantly from founder populations in the combined *Bt* broccoli and OX4319L treatments, it was still significantly lower than in cages where *Bt* plants were used in isolation.

Results from these experiments strongly support the IPM potential of insects carrying female-lethal transgenes. The first demonstrates the efficacy of OX4319L as a

control measure in its own right, while the second lends weight to the argument that female-lethal transgenes can function as effective resistance management tools for many conventional insecticides, as well as *Bt* cotton and maize, which currently are grown on more than 75 million ha (James, 2013). Similarly, area-wide SIT programmes against pink bollworm in Arizona have provided an effective addition to IPM in delaying development of resistance to *Bt*-expressing cotton (Tabashnik et al., 2010). The findings described here, however, indicate that the release of insects carrying a female-lethal transgene can, additionally, actively counteract the spread of insecticide resistance in a pest population. This is a benefit that conventional SIT does not provide: in SIT, all offspring die so there is no introgression of susceptible alleles from the released insects into the wild population. The primary advantage of this difference is that fsRIDL releases can actively spread susceptibility alleles into pest populations even under relatively low release rates (assuming they are not immediately selected against). Thus, if combined with a periodic control measure (such as insecticidal sprays), or a constitutively acting pesticide (such as *Bt*) where there are gaps between growing seasons, fsRIDL releases will result in highly susceptible pest populations when and if these other control measures are eventually employed.

The efficiency of a transgenic insect strain in providing these effects will likely depend on performance and survival of male moths in the field. Previous studies on OX4319L indicate that this transgene insertion is associated with a small but significant fitness cost in the laboratory. In these cage experiments there was evidence that this cost may be exacerbated in more realistic environments. As previously stated, the true pest management potential of this strain will only be known when it is tested in the field, which would be reliant on regulatory approval. Beyond this work on competitiveness, further empirical research is required on how the population dynamics of target

populations respond to the suppressive forces of female-lethal alleles. In particular, the effects of immigration and density-dependence have been extensively modelled in the context of bisex and female-specific autocidal releases, and investigated empirically during radiation-based SIT programmes. However, no field releases of fsRIDL strains have taken place to date, and those of bisex RIDL strains have focused on demonstrating the suppression potential of this technology, rather than investigating more nuanced applied ecological questions. This is to be expected of a novel strategy being deployed for the first time. It is hoped that as regulatory approval (and thus field releases) of these strains occurs, there will be further opportunities to explore these avenues.

One of the main arguments for the use of mating-based autocidal strategies is their high compatibility with other pest management methods in IPM programmes. This provides a wide range of potential further research should OX4319L be deployed in the field. For example, if used as an alternative to broad-spectrum insecticides (or in combination with more selective compounds) the species-specificity of fsRIDL would potentially reduce off-target effects on predator and parasitoids, therefore allowing them to establish and offer valuable biocontrol services, as is the case in traditional SIT programmes (Robinson and Hendrichs, 2005). The importance of conserving these natural enemy communities in IPM programmes has often been cited (Furlong et al., 2013). Indeed, they may be more effective in limiting diamondback moth populations than are insecticidal sprays, as well as acting against a wider range of pests (Bommarco et al., 2011). Studies demonstrating the retention of natural enemy complexes in *Bt* cotton fields with reduced insecticidal sprays have been conducted (Lu et al., 2012), and would provide a framework on which to design investigations of fsRIDL insects' ability to provide similar effects. It is likely that these benefits would also extend to the

conservation of other beneficials such as pollinators, as well as birds which may be threatened by the employment of some insecticides in agricultural areas (Hallmann et al., 2014; IUCN Task Force on Systemic Pesticides, 2014).

Finally, if fsRIDL lines are to be commercially applicable they must be financially competitive against current pest management options, such as pesticides. This will require economic analysis of both their respective costs and effectiveness in the field, similar to that performed previously for lepidopteran SIT programmes (Yang et al., 1998). The results of Chapter 3 indicate that combining fsRIDL insects with *Bt* crops can dramatically reduce (here by 75 – 88%) the number of released insects required to maintain a given level of control (Chapter 3, Figure 3.4A - similar pest population densities for low level OX4319L + *Bt* broccoli treatment and high level OX4319L treatment). Therefore, it may prove that combining fsRIDL insects with other pest management options provides not only a more sustainable pest management approach, but also the most economically attractive option. In the case of OX4319L, the exploration of these issues will first require the development of large-scale commercially applicable mass-rearing systems. These are currently unavailable for the diamondback moth. However, the existence of related systems used to rear diamondback moth for biocontrol efforts (parasitoid production), and other lepidopteran species such as the pink bollworm and codling moth for SIT programmes, implies that this will be feasible.

Chapter 4 set out to investigate the suitability of the 'Q' binary transgene expression system for application to RIDL type control in the diamondback moth. In most current RIDL constructs, lethality is caused by the accumulation of large quantities of tTAV via a positive feedback mechanism. The simplest forms of these systems (and

thus the easiest to engineer) use ubiquitous promoters causing this accumulation to take place across the entire organism. This allows a conditional lethal phenotype to be generated without prior knowledge of sensitive tissues/life stages and means with which to target them (i.e. tissue specific promoter/enhancers). A major disadvantage of using a ubiquitous design, however, is that global (untargeted) expression of the system is likely to result in increased toxicity, fitness costs and difficulty in generating transformants, relative to a system where expression is spatially or temporally restricted. The results of this chapter demonstrate that transformation of the QF activator gene in diamondback moth is difficult/impossible using ubiquitous promoters and/or current designs of this protein. The most likely explanation for this is toxicity caused by QF to an as-yet-unknown tissue type in pre-pupal stages. The potential for transcriptional activators in transgenes to cause toxicity is well-established (Natesan et al., 1999; Rajeswaran et al., 2007) and this effect has been observed in attempts to transform *D. melanogaster* and the zebrafish with QF. Despite this toxicity, however, assays measuring the rate of DsRed expression in plasmid-injected embryos (transient expression) provided evidence to suggest that the underlying Q-system mechanism will function in the Lepidoptera. The proportion of embryos showing DsRed transient expression was higher when the QF transactivator formed part of the injected construct, implying that QUAS binding by QF results in expression of downstream genes. This expression was reduced, either when the repressor gene QS formed part of the injected construct, or where genomic copies of QS were present. This implies that QS was capable of repressing QF to some degree.

These results provide a glimmer of hope for the Q-system in this context. If research into this system is to continue, however, it will require investigation of how to minimise the toxicity of the QF protein. If ubiquitous expression were required, this

would necessitate the use of redesigned QF proteins engineered to show reduced toxicity. These sequences are in the initial stages of being released (Flystock, 2014). A second, potentially more promising avenue of research, would be to transform enhancer trap lines of QF and then combine them with the existing ubiquitously expressing QS line. Enhancer trap constructs are designed without enhancer sequences. Thus, the expression characteristics of an integrated enhancer trap transgene will be controlled by endogenous enhancer sequences present in the DNA adjacent to the locus of insertion (O'Kane and Gehring, 1987). As the integration of a *piggyBac* transgene is essentially a random process, each enhancer trap line should therefore show its own unique expression profile, often limited to very specific tissues. This restricted expression (and therefore toxicity) may allow the successful transformation of QF lines. The dynamics of the system could then be observed solely in those tissues in which DsRed expression was taking place (Potter et al., 2010). If enhancer sequences that allowed QF transformation could be identified, and these combined with effectors able to cause lethality when expressed in those tissues, this could facilitate the use of the Q-system in a RIDL context without the need to express components ubiquitously (as well as identifying promising promoter/enhancer targets for future RIDL systems in the Lepidoptera). This strategy has been used in transgenic strains of *Ae. aegypti* mosquitoes, in which a (functionally lethal) flightless phenotype is limited to females through a sex–specific indirect flight muscle promoter (*AeAct-4*).

In conclusion, this thesis provides evidence that the release of insects carrying female-lethal transgenes could form part of effective and sustainable future pest management strategies, and describes the development of such an fsRIDL system in the diamondback moth. Looking forward, there is great scope for further work, especially if approval for the field use of this line is granted. The modular nature of transgene design

and the universality of DNA imply that the OX4319 system should also be highly transferrable to other economically important lepidopteran pest species, for example the fall armyworm (*Spodoptera frugiperda* Smith) and corn earworm (*Helicoverpa zea* Boddie) both of which exhibit high rates of insecticide resistance. Furthermore, it could provide a reliable means of pre-release sex-sorting for existing codling moth or pink bollworm SIT programmes, which would be highly valuable but is currently not possible. It is hoped that the research conducted here contributes to the advancement of the field of genetic pest management and the improvement of agricultural sustainability.

Appendix

4319L Flanking 5'
GACGACAGAAAGGGCGTGGTGCGGAGGGCGGTGTGATGATGACAGAAAGG
GCGTGGTGCGGAGGGCGGTGTGATCAGACTTGGAGCACCTGCCATATTATT
TTCTATTCTATTTGGTGACACTAAGGCCACTTTTAATGGCTTTTGCTTTGGTT
CATGAACGGTGAAAAGTAAATCATACATCTATTATTGATTATCTATGTACAG
TCAGCAAACAAGCTACATTGGCCAGCCCAGTACAAAAACATTAATTTTGTG
ATCCACTGACAGTTGACACATGACATGAACAATACTTAACTAACAAAATGA
ACTTGTACAGATCGAGCTTGTCCAGCATGGTGGACTAGGCCTAAACCCATCC
TTCATTGGAAGGAGACCCATGAAATGGTCATGTAAAATTGCAAATTATTTCT
CGGTGTACAAATAAATTTTACTCTATCTATCTACAGTTTTATACAACTCATG
TAGGCTCCTTTATTGCAGCCATCCACATGTGAAGGGTGTTAATATATGGGAA
ACTCTGAACCTATCTGAGATGGCCACACTCAGGCATAGAATAACTGATGCA
ATCTCTGTGCAGCAGCGATGCCTCAAGGCTATGTCACACCTGATAATACTCC
CCATACTTTGCAAGTATCTGCTGAATGACACAGATATCACCAGAGCATTGG
AAAAATTATGTTAGTGCATTGAATGTTTCTTTTGTAATTCTCCTATGAAATA
ACAAACATGTACATTGTACCGTGCAATGAAGTTCCAACGTGACCCAAGCCA
AGTTCAAAGCCTTGATTGTGAAGAGCTCATAGTTTTACTTCAAAGGGTCGT
TGGCTCATTTAACCCTAGAAAGATAGTCTGCGTAAAATTGACGCATGCATTC
TTGAAATATTGCTCTCTCTTTCTAAGTAGCGCGAATCCGTCGCTGTGCATTTA
GGACATCTCAGTCGCC (5'PB)

4319L Flanking 3'
(3'PB)CAGACCGATAAAACACATGCGTCAATTTTACGCATGATTATCTTTAAC
GTACGTCACAATATGATTATCTTTCTAGGGTTAAATCTTTGAAACGCATTTT
GATTATAAGTACCTATTGATGTACCTAGAACACATATTATAATAATTATCCA
CTTACCAAGAAACCGATTGAATATCTTTAAAACAACCTATCAGTTAGATAA
AGAGTTCTCTTCACTGTTCAGTTGTTTAATGTAGGCTCCTACTGGGTAGTGT
ACAACGGTCCGCCGGCGTCCTATCAAATTATTTTGAAGTAGGTACCTACTTA
CGTAGATAACACATAGATAACATAACTGGAAGCACTTACATTAGAAAACGG
TAATCAAATCACCTTCGATGTCACTGAGTGCGCTACTATCCTATCGGCTATC
GTACAATGAAATGAAATAAACAATAAACATTAACTCACCAATAAAGTAATG
AAATAAAATATCCAGCTCTCTGACAACACGCACGAAACAACCAGTGTTGCC
GCAACGGAGTTCTCAAAACGCACATATCCAGCGCTGAAAACGTACATTTAG
GGTTAAAACCGTCCATGAGCTGTTTTTTACTAAAATCACTCAATTTAGCTTT
TCCAGCTCTTTCGCACCTGCTCGTCAGCAGAAGTACTATATTTTAATAGATT
TTAGTCGTGAGAAACGTACCTATATTTATTATAGCATTTTAAAAAGTGTTAC
AATGTCCTAAAACCTTCAGAGTAGGTACCCCGCAGTTTTATGTATGATATAC
TTATACTCATTGTAATCATGTAATAATCATATTGGTTGAGAAAATAATCATA
CAAGAAAATCAAAATCGTAACTAATTGTGGTTAATAATTTTTTTGCTGCGAT
TCCAAAAAAAAATACGTAAAATAGTCCATAAACAAAGATTTTACACATGATA
ATTATGATATTGGAAGTTAG

Figure A.1: Genomic sequence flanking the OX4319L transgene insertion. Red sequence represents the 5' and 3' *piggybac* ends. Black arrows show the position of the transgene. Underlined sequences show the positions of the flanking primers 4319LF1, 4319LF2, 4319LR1 and 4319LR2.

Female comparison

```
                     1                                                50
» DsxPBWF      (1)        AGCTAAACAATCTGCCCTAGGCCTCGACGAGGCTTCTGGAAAAAT
» DsxDBMF      (2)   GAACAAGCTAAACAATCTGCCCTAGGCCTCGACGAGGCTTCTGGAAAAAT

                     51                                              100
» DsxPBWF     (47)   TGGTGAAGGGAAGACGATCATCAACGAGTACGCGAGGAAGCACATCTGAA
» DsxDBMF     (52)   TGGTGAAGGGAAGACGATCATCAACGAGTACGCGAGGAAGCACATCTGAA

                     101                                             150
» DsxPBWF     (97)   CATCTTCGGTGGCCACGAGCTGAGGAACTCGACTCGCTAACGGACCGAAA
» DsxDBMF    (102)   CATCTTCGGTGGCCACGAGCTGAGGAACTCGACTCGCTAACGGACCGAAA

                     151                                             200
» DsxPBWF    (147)   CCACCATGGGCAGCCGCCTGGATAAGTCCAAAGTCATCAACTCCGCGTTG
» DsxDBMF    (152)   CCACCATGGGCAGCCGCCTGGATAAGTCCAAAGTCATCAACTCCGCGTTG

                     201                                             250
» DsxPBWF    (197)   GAGCTGTTGAACGAAGTTGGCATTGAGGGACTGACGACCCGCAAGTTGGC
» DsxDBMF    (202)   GAGCTGTTGAACGAAGTTGGCATTGAGGGACTGACGACCCGCAAGTTGGC

                     251                                             300
» DsxPBWF    (247)   GCAGAAGCTGGGCGTGGAGCAGCCCACCCTCTACTGGCACGTGAAGAATA
» DsxDBMF    (252)   GCAGAAGCTGGGCGTGGAGCAGCCCACCCTCTACTGGCACGTGAAGAATA

                     301                                             350
» DsxPBWF    (297)   AGCGGGCGCTGCTGGATGCCCTGGCCATCGAGATGCTCGACCGCCACCAC
» DsxDBMF    (302)   AGCGGGCGCTGCTGGATGCCCTGGCCATCGAGATGCTCGACCGCCACCAC

                     351                                             400
» DsxPBWF    (347)   ACGCATTTTTGCCCGTTGGAAGGCGAGTCCTGGCAGGACTTCCTCCGCAA
» DsxDBMF    (352)   ACGCATTTTTGCCCGTTGGAAGGCGAGTCCTGGCAGGACTTCCTCCGCAA

                     401                                             450
» DsxPBWF    (397)   TAACGCCAAGTCGTTCCGCTGCGCTCTGCTGTCCCACCGAGACGGTGCCA
» DsxDBMF    (402)   TAACGCCAAGTCGTTCCGCTGCGCTCTGCTGTCCCACCGAGACGGTGCCA

                     451                                             500
» DsxPBWF    (447)   AAGTCCATCTCGGCACGCGCCCGACCGAAAAGCAATACGAGACACTGGAG
» DsxDBMF    (452)   AAGTCCATCTCGGCACGCGCCCGACCGAAAAGCAATACGAGACACTGGAG

                     501                                             550
» DsxPBWF    (497)   AACCAGCTCGCGTTCCTGTGCCAGCAAGGCTTCAGCCTGGAAAATGCTCT
» DsxDBMF    (502)   AACCAGCTCGCGTTCCTGTGCCAGCAAGGCTTCAGCCTGGAAAATGCTCT

                     551                                             600
» DsxPBWF    (547)   CTACGCTCTGAGCGCCGTCGGTCACTTTACCCTGGGCTGCGTGCTGGAGG
» DsxDBMF    (552)   CTACGCTCTGAGCGCCGTCGGTCACTTTACCCTGGGCTGCGTGCTGGAGG

                     601                                             650
» DsxPBWF    (597)   ACCAAGAGCATCAAGTCGCAAAAGAGGAGCGCGAGACCCCAACAACCGAT
» DsxDBMF    (602)   ACCAAGAGCATCAAGTCGCAAAAGAGGAGCGCGAGACCCCAACAACCGAT

                     651                                             700
» DsxPBWF    (647)   TCGATGCCCCCACTGCTGCGTCAGGCAATCGAGCTGTTCGATCATCAAGG
» DsxDBMF    (652)   TCGATGCCCCCACTGCTGCGTCAGGCAATCGAGCTGTTCGATCATCAAGG
```

```
                      701                                               750
» DsxPBWF    (697)  AGCCGAGCCGGCATTCCTGTTCGGCTTGGAGCTGATTATCTGCGGATTGG
» DsxDBMF    (702)  AGCCGAGCCGGCATTCCTGTTCGGCTTGGAGCTGATTATCTGCGGATTGG

                      751                                               800
» DsxPBWF    (747)  AAAAGCAACTGAAATGCGAGTCGGGCTCGGGCCCCGCCTACAGCCGCGCC
» DsxDBMF    (752)  AAAAGCAACTGAAATGCGAGTCGGGCTCGGGCCCCGCCTACAGCCGTGCC

                      801                                               850
» DsxPBWF    (797)  CGCACCAAGAACAACTACGGCAGCACCATCGAGGGCCTGCTGGATCTGCC
» DsxDBMF    (802)  CGCACCAAGAACAACTACGGCAGCACCATCGAGGGCCTGATGGATCTGAC

                      851                                               900
» DsxPBWF    (847)  GGATGATGATGCCCCGGAGGAGGCGGGCCTGGCCGCCCCGCGCCTGAGCT
» DsxDBMF    (852)  GGATGATGATGCCCCGGAGGAGGCGGGCCTGGCCGCCCCGCGCCTGAGCT

                      901                                               950
» DsxPBWF    (897)  TCCTGCCGGCCGGACACACCCGCCGCCTGTCGACCGCCCCGCCGACCGAC
» DsxDBMF    (902)  TCCTGCCGGCCGGACACACCCGCCGCCTGTCGACCGCCCCGCCGACCGAC

                      951                                              1000
» DsxPBWF    (947)  GTGAGCCTGGGCGATGAGCTGCACCTGGATGGCGAGGATGTGGCGATGGC
» DsxDBMF    (952)  GTGAGCCTGGGCGATGAGCTGCACCTGGATGGCGAGGATGTGGCGATGGC

                      1001                                             1050
» DsxPBWF    (997)  CCACGCCGATGCCCTGGACGACTTCGACCTGGACATGCTGGGCGATGGCG
» DsxDBMF   (1002)  CCACGCCGATGCCCTGGACGACTTCGACCTGGACATGCTGGGCGATGGCG

                      1051                                             1100
» DsxPBWF   (1047)  ATAGCCCGGGACCGGGATTCACCCCGCACGATAGCGCCCCCTACGGCGCC
» DsxDBMF   (1052)  ATAGCCCGGGACCGGGATTCACCCCGCACGATAGCGCCCCCTACGGCGCC

                      1101                                             1150
» DsxPBWF   (1097)  CTGGATATGGCCGATTTCGAGTTCGAGCAGATGTTCACCGACGCCCTGGG
» DsxDBMF   (1102)  CTGGATATGGCCGATTTCGAGTTCGAGCAGATGTTCACCGACGCCCTGGG

                      1151                                             1200
» DsxPBWF   (1147)  CATCGATGAATACGGCGGCTAACACCGGTGGCCACGAGCTGAGGAACTCG
» DsxDBMF   (1152)  CATCGATGAATACGGCGGCTAACACCGGTGGCCACGAGCTGAGGAACTCG

                      1201                                             1250
» DsxPBWF   (1197)  ACTCGCCAGAAAATGCTGAGCGAAATTAATAATATAAGTGGTGTACTATC
» DsxDBMF   (1202)  ACTCGCCAGAAAATGCTGAGCGAAATTAATAATATAAGTGGTGTACTATC

                      1251                                             1300
» DsxPBWF   (1247)  GTCGTCCATGAAGTTATTTTGCGAATGATACTTTGTTTTGTATGTGCTGT
» DsxDBMF   (1252)  GTCGTCCATGAAGTTATTTTGCGAATGATACTTTGTTTTGTATGTGCTGT

                      1301                                             1350
» DsxPBWF   (1297)  GTGTTGTGTGGACTTTTGCTGTGCGTTGCTGTTTGCGATGGAAGGACTAT
» DsxDBMF   (1302)  GTGTTGTGTGGACTTTTGCTGTGCGTTGCTGTTTGCGATGGAAGGACTAT

                      1351                                             1400
» DsxPBWF   (1347)  TGTGTCGTCGCCACGCCGGACTATTCGCACATTGGGTGGTCCACCAGTGG
» DsxDBMF   (1352)  TGTGTCGTCGCCACGCCGGACTATTCGCACATTGGGTGGTCCACCAGTGG


                      1401                                             1450
» DsxPBWF   (1397)  CGGATGTACGAGCGGTCGCTGTGCTCGCTCCTGGAGCTGCAAGCGCGCAA
» DsxDBMF   (1402)  CGGATGTACGAGCGGTCGCTGTGCTCGCTCCTGGAGCTGCAAGCGCGCAA
```

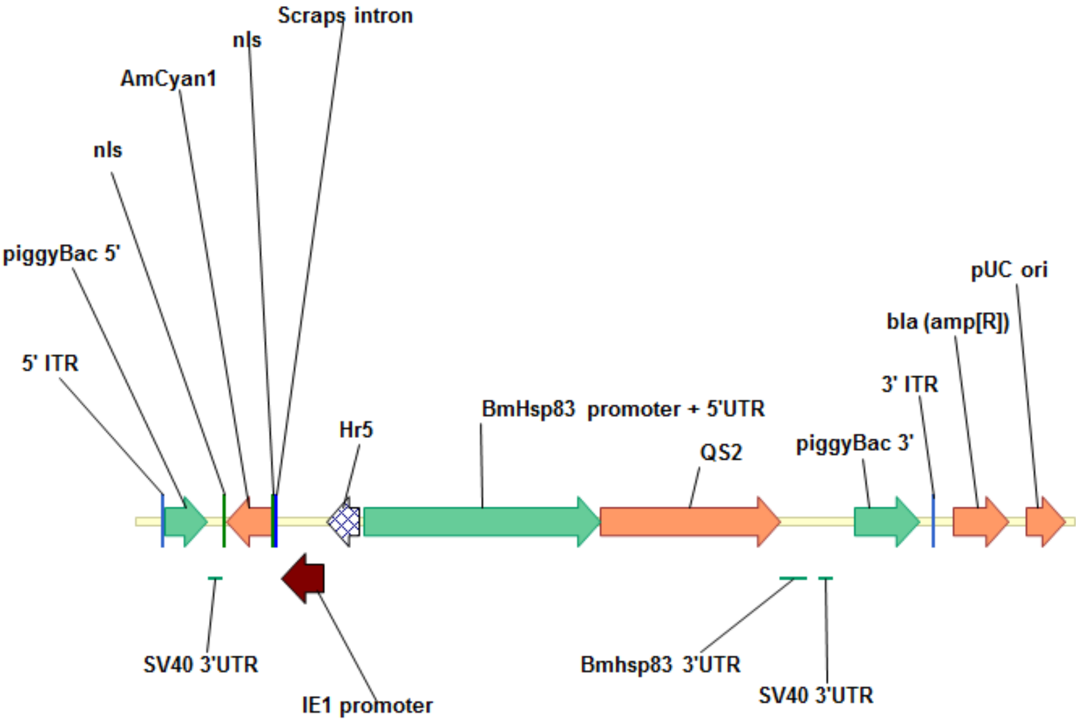
```
                   1451                                              1500
» DsxPBWF  (1447) AGGGACGTACTCGGTGTGCTGCTCACCCCGCTACGTCATCGCGCCCGAGT
» DsxDBMF  (1452) AGGGACGTACTCGGTGTGCTGCTCACCCCGCTACGTCATCGCGCCCGAGT

                   1501                                              1550
» DsxPBWF  (1497) ACGCGTCACACCTGTTGCCTCTGCCGCTTACCACGCAGAGATCATCCCCG
» DsxDBMF  (1502) ACGCGTCACACCTGTTGCCTCTGCCGCTTACCACGCAGAGATCATCCCCG

                   1551
» DsxPBWF (•1546) C
» DsxDBMF (•1551) C
```

<u>Male comparison</u>

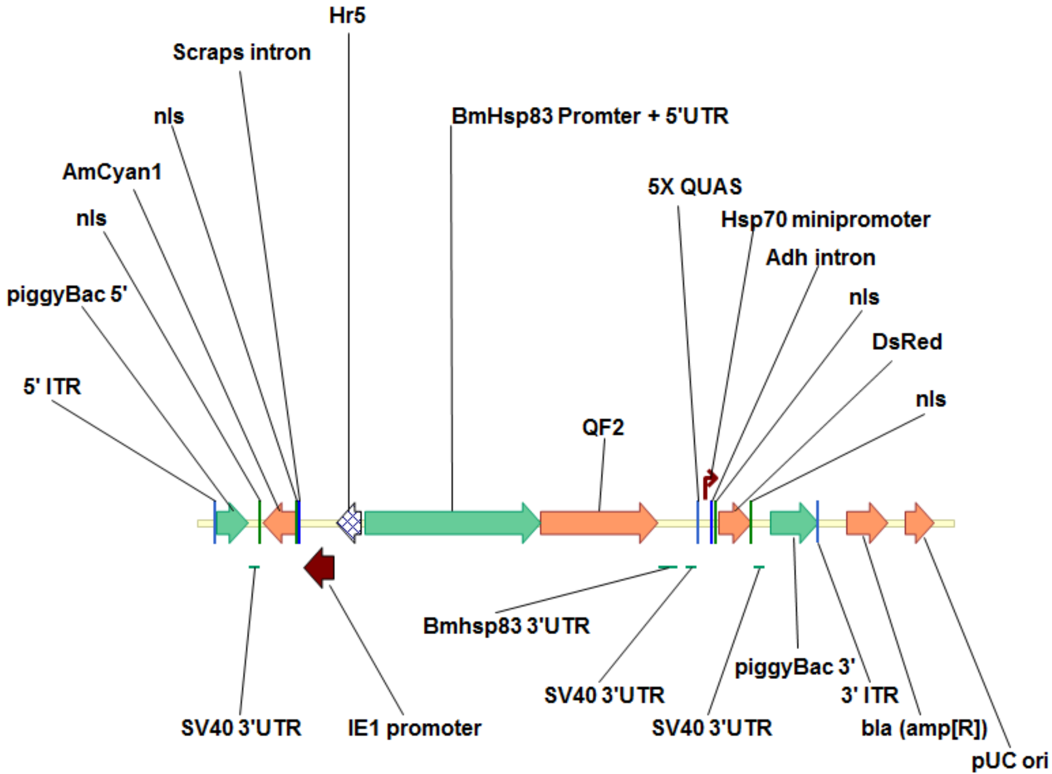
```
                  1                                                  50
» DsxPBWM     (2) GCTGAACAAGCTAAACAATCTGCCCTAGGCCTCGACGAGGCTTCTGGAAA
» DsxDBMM     (2) GCTGAACAAGCTAAACAATCTGCCCTAGGCCTCGACGAGGCTTCTGGAAA

                  51                                                100
» DsxPBWM    (52) AATTGGTGAAGCACATTGGGTGGTCCACCAGTGGCGGATGTACGAGCGGT
» DsxDBMM    (52) AATTGGTGAAGCACATTGGGTGGTCCACCAGTGGCGGATGTACGAGCGGT

                  101                                               150
» DsxPBWM   (102) CGCTGTGCTCGCTCCTGGAGCTGCAAGCGCGCAAAGGGACGTACTCGGTG
» DsxDBMM   (102) CGCTGTGCTCGCTCCTGGAGCTGCAAGCGCGCAAAGGGACGTACTCGGTG

                  151                                               200
» DsxPBWM   (152) TGCTGCTCACCCCGCTACGTCATCGCGCCCGAGTACGCGTCACACCTGTT
» DsxDBMM   (152) TGCTGCTCACCCCGCTACGTCATCGCGCCCGAGTACGCGTCACACCTGTT

                  201                             235
» DsxPBWM   (202) GCCTCTGCCGCTTACCACGCAGAGATCATCCCCGC
» DsxDBMM   (202) GCCTCTGCCGCTTACCACGCAGAGATCATCCCCGC
```

Figure A.2: Sequence alignment of primary PCR bands amplified from diamondback moth and pink bollworm OX4319 transgenic females (first sequence) and males (second sequence) using primers specific to the shared *Pgdsx* exons. Female specific band showed >99% sequence agreement between the two species with only 2bp divergence (in yellow) within the coding sequence. It is likely that this is a result of sequencing error, rather than differences in splicing. A small divergence also occurred at the beginning of the alignment as a result of partial transcription of the 3' end of the hsp70 minimal promoter in diamondback moth. Primary male transcripts were identical.

A)



B)



C)

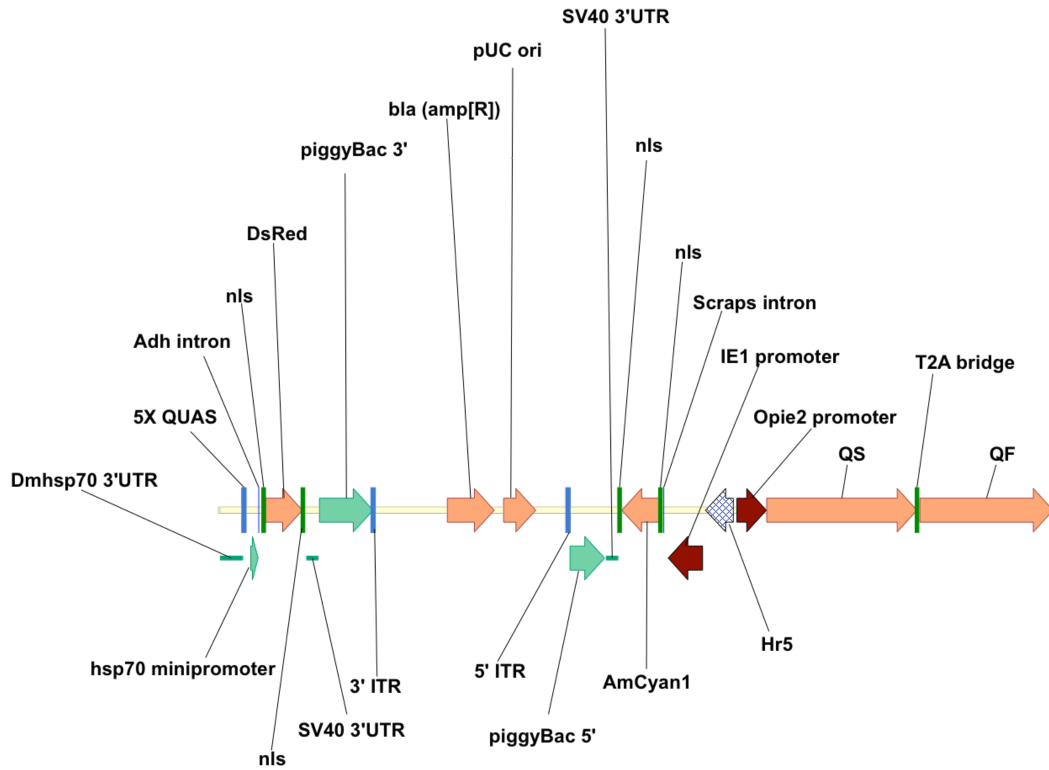


Figure A.3: Schematic maps of the primary constructs (OX46858 (A), OX4651 (B) and OX5042 (C)) used in this thesis. Maps produced by Vector NTI version 5.1. Detailed explanations of construct components are given in table A.1

Table A.1: Details of genetic components of constructs shown in figure A.3

Name on plasmid map	Construct component	Component function	Donor	Detailed description
5 X QUAS	Regulatory sequence	Target site for QF binding	*N. crassa*	Part of the QA catabolism circuit from *N. crassa*. In the endogenous system is the upstream binding site for QF self-induction. When placed upstream of a target gene allows expression of that gene when bound by QF. Five copies
adh intron	Diagnostics sequence	Is spliced out of pre-mRNA	*D. melanogaster*	An intron which is spliced out of pre-mRNA allowing the distinction between cDNA and gDNA in RT-PCR analysis.
AmCyan	Fluorescent protein coding sequence	Express AmCyan (blue)	*Anemonia majano* (coral)	A fluorescent protein which, when visualised under the correct wavelength fluoresces blue (458 nm). No fluorescence occurs under white light and this protein confers no known fitness changes. Here used as a central transformation marker under the control of a constitutive promoter.
bla (amp[R])	Antibiotic resistance marker	Confers resistance to ampicillin	*Salmonella paratyphi*	Allows the selection of transformed bacterial cells
Bmhsp83 3'UTR	Regulatory sequence	Stabilises mRNA	*B. mori*	Regulatory sequence aiding the stabilisation of mRNA
BmHsp83 Promoter +5'UTR	Promoter + 5' regulatory sequence	Controls expression of QS2	*B. mori*	Heat shock protein 83 promoter and 5' UTR regulatory sequence. Allows strong early expression of downstream gene.
DsRed	Fluorescent protein coding sequence	Express DsRed (red)	*Discosoma* Sp (coral)	A fluorescent protein which, when visualised under the correct wavelength fluoresces red (583nm). No fluorescence occurs under white light and this protein confers no known fitness changes.
Hsp70 minipromoter	Regulatory sequence	Controls expression of DsRed	*D. melanogaster*	When QUAS is bound by QF this brings the transactivator into contact with this minimal promoter allowing the expression of DsRed
IE1/Hr5	Promoter/	Controls	*Autographa*	Promoter from *immediate-*

	enhaner sequences	expression of AmCyan	*californica* nuclear polyhedrosis virus (AcMNPV)	*early-1* gene and *hr5* enhancer region. Allows strong early expression of target protein.
nls	Nuclear localisation sequence	Localises DsRed protein into the nuclei of cells	synthetic	NLS marks proteins for transport into the nuclei of cells. This allows for spatial accumulation of protein expression increasing visualisation of fluorescence patterns.
Opie2 promoter	Regulatory sequence	Controls expression of QS2-T2A-QF2	*Orgyia pseudotsgata* nucleopolyhed rosis virus	Promoter sequence causing early ubiquitous expression of downstream sequences
piggyBac 5' *piggyBac* 3' 5' ITR 3' ITR	Vector sequence	Germline transformation	*piggyBac* from *Trichoplusia ni* (moth)	Elements from the *piggyBac* DNA transposon. A transposon is an autonomous mobile genetic element capable of inducing its own replication within a host genome. Replication of the transposon is dependent on a transposase encoded within the open reading frame (ORF) of the transposon, and inverted terminal repeats (ITR) occurring at the 5' and 3' ends of the transposon DNA sequence. In this case, the *piggyBac* 5' and 3' sequences as well as their relevant ITR sequences have been added to the relevant ends of the sequence to be inserted. The transposase ORF under the control of the *D. melanogaster hsp70* promoter was cloned into a helper plasmid and simultaneously injected along with the construct allowing integration (Handler and Beeman, 2003).
pUC ori	Bacterial origin of replication	Plasmid replication point	*E. coli*	Allows high rate replication of construct within transformed bacterial cells.
QF2	Transcriptional activator	Binds to QUAS inducing downstream expression	*N. crassa*	Part of the QA catabolism circuit from *N. crassa*. Binds to QUAS sequence inducing expression of downstream genes. Is itself bound and repressed by QS.
QS2	Regulatory gene	Represses the activity of	*N. crassa*	Part of the quinic acid catabolism circuit from *N. crassa*. Binds to the QF

		QF2		transactivator protein preventing its induction of QUAS- target gene. Is itself bound and repressed by QA.
Scraps intron	Regulatory sequence for 5'UTR	Requirement for translation	*D. melanogaster*	Stabilises mRNA and required for translation of mRNA
SV40 3' UTR	Regulatory sequence 3' UTR	Stabilises mRNA	Simian virus 40	Regulatory sequence aiding the stabilisation of mRNA
T2A viral bridge	Ribosomal skipping sequence	Allows multicistronic translation of mRNA	*Thosea asigna* insect virus	Is placed in frame between two coding sequences. During translation, when a ribosome encounters this sequence a peptide bond between two amino acids is not formed (skipped) allowing the release of the primary polypeptide while translation of the second sequence continues. (Gonzalez et al., 2011; Diao and White, 2012).

Table A.2: Sequences of primers used in this thesis.

Primer name/number	Sequence
1127 Diag2-dmhsp	TAAACAAGCGCAGCTGAACAAGC
1377 RTPBWMEXONR	GCGGGGATGATCTCTGCGTGGT
1807 *Bmhsp83*-5utr-bsa-r	ACGCGGTCTCACATTTTCTTTACTGCTTTTTGTTTAATTCAC
1855 3816RTEX1	GCTGAACAAGCTAAACAATCTGCCCTA
1874 Diag-bmhsp83pro	AACGAATAAATAGCATTAGAATCTGCTC
218 PB3	GGTGACCGGTATGGCCCTGTCCAACAAGTTCATCG
224 Diag-pb3	TTCCGTACAATAATGCCATAGGCCAC
250) MI3F	ATAAGAATGCGGCCGCAAAGTGCAATTGGCTAAAAACCG
2792 *Bmhsp*-bsa-f	GCGCGGTCTCAGGCCGCTACGGATAGCTGTCTACAATACAGTGG
2793 QS2-bsa-f	ACGCGGTCTCTAATGAACACCATCCCAGCCC
2794 QS2-bsa-r	ACGCGGTCTCTGAGGATCCTTAGCTGATCTGGGTGGCGA
2795 *BmHsp83*-3utr-bsa-f2	ACGCGGTCTCACCTCGATCTCTACTTTAACTGTAGGAACCATGTC
2796 SV40-fse-r2	CTATGGCCGGCCTGTTCCTGTGATGATCATAATCAGC
2798 Diag-Qs2 224 Diag-pb3	GCCATGCTGCAGGACTTCAG
2807 QF2-bsa-f	ACGCGGTCTCTAATGCCGCCGAAGCGCAAG
2808 QF2-bsa-r	ACGCGGTCTCTGAGGTTACTGCTCGTAGGTGTTGATGTCG
2809 SV40-bsa-r18	ACGCGGTCTCATACGTGTTCCTGTGATGATCATAATCAGC
2810 QUAS-bsa-f	ACGCGGTCTCACGTAGGCGCGCCATAGAGCTCGGGTAATCGCTTATCC
2811 QUAS-bsa-r	GGTACCGGTCTCACTGCAGCAATTC
2812 *Hsp*70-bsa-f	ACGCGGTCTCAGCAGAAACGAGCGCCGGAGTATAAATAG

2813 *Adh*-int-bsa-r	ACGCGGTCTCACTAGGTTTCTAAAGGTGTTATAAATCAAATTAG
2814 nls-bsa-f	AGCGCGTCTCACTAGGCCACCATGGGAGATCCCAC
2815 SV40-bsa-r19	TAGTGGCCGGCCCGGGTAAGATACATTGATGAGTTTGGACA
2858 Diag2-QF2	CGCAGTTCATGACCAACCTGG
2871 seq-Bmhsp83pro-6	GCGAAACACTGAACAGCTTAGG
3070 QF2-bsa-r3	GCTAGGTCTCGATCCTTACTGCTCGTAGGTGTTGATGTCG
3071 Dmhsp70 3'UTR- f	CGGTGGTCTCAGGATCCAGCCAAATAGAAAATTATTCAGTTCCTG
3072 Dmhsp70 3'UTR-bsa-r	ACGTGGTCTCTCGCGCCGGTTCCATTTTGTAATCCGTA
3422 *3×P3*-bsmb-f6	AGCGCGTCTCAGGCCGCTCGCCCGGGGATCTAATTC
3423 DsR-bsmb-r3	GACGCGTCTCTCCGGATCACAGGAACAGGTGGTGGCG
4319LF1	CCCATACTTTGCAAGTATCTGCTG
4319LF2	CACAGATATCACCAGAGCATTGGA
4319LR1	CATCGAAGGTGATTTGATTACCGT
4319LR2	GTGTTATCTACGTAAGTAGGTACCTACTTC
825 Diag4-DsRed	CTCGATCTCGAACTCGTGGC
954 Diag Dmhsp	TTTCGCTTAGCGACGTGTTCAC
956 Diag5-ie1	GCGCATCACAAAGACATCG
PB5-out	CTCTGGACGTCATCTTCACTTACGTG
2310 Diag-Bmhsp83-3utr	GTCTTTGGCTGTACACCGATG
2800 QS2-seq-3	GCGAGAACACCGACTGGATC
3482 DBM-dsx-e2-f1	CGGTGAACATCGAGAACCTGGT
3544 DBMdsxMR4	GCAGCACAGCGAGTACGTGTCC

Summary of injection success model

The model used to estimate the probability of success (transformation of at least 1 line) given a certain number of injections was generously provided by a colleague Matthew Gregory. As this model and its definitions will constitute part of Matt's thesis they will not be explained in great detail here. However, to summarise briefly, Matt collected data on transformation success from a decade of injections at Oxitec, and also from the insect transformation literature. He was able to divide this data up by order and species and from it, build a predictive model of transformation success, given a certain number of injections, or G_0 survivors. The Q-system was used in this data-set, however was removed when the predictions in this thesis were made. The probability of non-transformation given in Chapter 4 was based on the total number of injections made using constructs which contained QF. However, even when the number injected was broken down by construct, the probability of failure was still extremely low (OX4651 – 0.0913%; OX4765 – 0.0376%; OX5042 – 0.0903%), based on all known data on previous injection success in the diamondback moth.

Papers

The following papers arose from work conducted during this thesis.

Jin, L.,* Walker, A.S.,* Fu, G.,* **Harvey-Samuel, T.D.**,* Dafa'alla, T., Miles, A., Marubbi, T., Granville, D., Humphrey-Jones, N., O'Connell, S., Morrison, N.I., Luke Alphey, L., (2013) 'Engineered Female-Specific Lethality for Control of Pest Lepidoptera', *ACS Synthetic Biology*, **3**, 160-166 (* = first author)

Harvey-Samuel, T.D., Ant, T., Gong, H., Morrison, N.I., Alphey, L., (2014) 'Population-level Effects of Fitness Costs associated with Female-Lethal Transgene Insertions in Two Pest Insects' *Evolutionary Applications*, **7**, 597-606

Harvey-Samuel, T.D., Morrison, N.I, Walker, A.S., Marubbi, T., Yao, J., Collins, H.L., Gorman, K., Davies, T.G.E., Alphey, N., Warner, S., Shelton, A.M., Alphey, L., (2015) 'Pest control and resistance management through releases of insects carrying a male-selecting transgene' *BMC Biology*, **13**:49

Chapter cover page photos

1. Sunrise over a cabbage field in Lompoc, California – credit: Kevin Cole (www.kevinlcole.com)

2. Female OX4319L adult photographed under red fluorescence filter. DsRed fluorescing throughout but especially in the thin membranous wing veins - credit: Tim Harvey-Samuel

3. Cage of *Bt* transgenic broccoli plants used in experiments in this chapter - credit: Tim Harvey-Samuel

4. Diamondback moth pupae co-transformed with DsRed, AmCyan and ZsGreen fluorescent protein markers - credit: Adam Walker, Oxitec

5. First instar tomato leaf miner moth larva (*Tuta absoluta* Meyrick) mining in tomato leaf - credit: Derric Nimmo, Oxitec

References

Abbott, W.S. (1925). 'A method of computing the effectiveness of an insecticide' *J. Econ. Entomol* **18***,* 265-267.

Acharya, L., and McNeil, J.N. (1998) 'Predation risk and mating behavior: the responses of moths to bat-like ultrasound' *Behav. Ecol*. **9***,* 552-558.

Ahmad, M. (1999) *Initial frequencies of alleles for resistance to Bacillus thuringiensis toxins in field populations of Plutella xylostella and Helicoverpa armigera,*Thesis (Ph.D.)—University of Adelaide.

Ahrens, R.N.M., and Devlin, R.H. (2011) 'Standing genetic variation and compensatory evolution in transgenic organisms: a growth-enhanced salmon simulation' *Transgenic Res* **20***,* 583-597.

Allinghi, A., Calcagno, G., Petit-Marty, N., Cendra, P.G., Segura, D., Vera, T., Cladera, J., Gramajo, C., Willink, E., and Vilardi, J.C. (2007) 'Compatibility and competitiveness of a laboratory strain of *Anastrepha fraterculus* (Diptera : Tephritidae) after irradiation treatment' *Fla. Entomol*. **90***,* 27-32.

Alphey, N., Bonsall, M.B., and Alphey, L. (2009) 'Combining Pest Control and Resistance Management: Synergy of Engineered Insects With *Bt* Crops' *J. Econ. Entomol*. **102***,* 717-732.

Alphey, N., Coleman, P.G., Donnelly, C.A., and Alphey, L. (2007) 'Managing insecticide resistance by mass release of engineered insects' *J. Econ. Entomol*. **100***,* 1642-1649.

Andow, D. (1991) 'Vegetational Diversity and Arthropod Population Response' *Annu. Rev. Entomol*. **36***,* 561-586.

Ankersmit, G.W. (1953) 'DDT Resistance in *Plutella maculipennis* (Curt) (Lep) in Java' *Bull. Entomol. Res*. **44***,* 421-425.

Ant, T., Koukidou, M., Rempoulakis, P., Gong, H., Economopoulos, A., Vontas, J., and Alphey, L. (2012) 'Control of the olive fruit fly using genetics-enhanced sterile insect technique' *BMC Biol.* **10***,* 51.

Awmack, C.S., and Leather, S.R. (2002) 'Host plant quality and fecundity in herbivorous insects' *Annu. Rev. Entomol* **47***,* 817-844.

Bailey, C.G. (1976) 'A quantitative study of consumption and utilization of various diets in the bertha armyworm, *Mamestra configurata* (Lepidoptera: Noctuidae)' *Can. Entomol.* **108***,* 1319-1326.

Barclay, H.J. (1982) 'The Sterile Release Method with Unequal Male Competitive Ability' *Ecol. Model.*. **15***,* 251-263.

Bariola, L.A., Keller, J.C., Turley, D.L., and Farris, J.R. (1973) 'Migration and Population Studies of the Pink Bollworm in the Arid West' *Environ. Entomol. 2,* 205-208.

Barry, J.D., Opp, S.B., Dragolovich, J., and Morse, J.G. (2007) 'Effect of adult diet on longevity of sterile Mediterranean fruit flies (Diptera : Tephritidae)' *Fla. Entomol.* **90***,* 650-655.

Bateman, A.J. (1948) 'Intra-Sexual Selection in Drosophila' *Heredity* **2***,* 349-368.

Bates, S.L., Zhao, J.Z., Roush, R.T., and Shelton, A.M. (2005) 'Insect resistance management in GM crops: past, present and future' *Nat. Biotechnol.* **23***,* 57-62.

Bax, N.J., and Thresher, R.E. (2009) 'Ecological, behavioral, and genetic factors influencing the recombinant control of invasive pests' *Ecol. Appl.* **19***,* 873-888.

Beketov, M.A., Kefford, B.J., Schäfer, R.B., and Liess, M. (2013) 'Pesticides reduce regional biodiversity of stream invertebrates' *Proc. Natl. Acad. Sci. U. S. A.* **110**, 11039-11043.

Bellen, H.J., O'Kane, C.J., Wilson, C., Grossniklaus, U., Pearson, R.K., and Gehring, W.J. (1989) 'P-Element-Mediated Enhancer Detection - a Versatile Method to Study Development in Drosophila' *Genes. Dev.* **3**, 1288-1300.

Bergelson, J., and Purrington, C.B. (1996) 'Surveying patterns in the cost of resistance in plants' *Am. Nat.* **148***,* 536-558.

Bierfreund, N.M., Reski, R., and Decker, E.L. (2003) 'Use of an inducible reporter gene system for the analysis of auxin distribution in the moss *Physcomitrella patens*' *Plant Cell Rep.* **21***,* 1143-1152.

Binbahari, I. (1994) 'Radiation-Induced Changes in Reproductive Ability of Diamondback Moth (Lepidoptera, Plutellidae)' *J. Econ. Entomol.* **87***,* 1190-1197.

Bloem, K.A., Bloem, S., and Carpenter, S. (2005) *Impact of Moth Suppression/Eradication Programmes using the Sterile Insect Technique or Inherited Sterility*, In Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management, Dyck, V. A., Hendrichs, J. and Robinson, A. S. eds., (Springer) pp. 677-700.

Bloem, S., and Carpenter, J.E. (2001) 'Evaluation of population suppression by irradiated Lepidoptera and their progeny' *Fla. Entomol.* **84***,* 165-171.

Bommarco, R., Miranda, F., Bylund, H., and Bjorkman, C. (2011) 'Insecticides Suppress Natural Enemies and Increase Pest Damage in Cabbage' *J. Econ. Entomol.* **104***,* 782-791.

Brinton, F.E., Proverbs, M.D., and Carty, B.E. (1969) 'Artificial diet for mass production of the codling moth, *Carpocapsa pomonella* (Lepidoptera: Olethreutidae)' *Can. Entomol.* **101***,* 577-584.

Bushland, R.C., Lindquist, A.W., and Knipling, E.F. (1955) 'Eradication of Screw-Worms through Release of Sterilized Males' *Science* **122***,* 287-288.

Calcagno, G.E., Manso, F., and Vilardi, J.C. (2002) 'Comparison of mating performance of medfly (Diptera : Tephritidae) genetic sexing and wild type strains: Field cage and video recording experiments' *Fla. Entomol.* **85***,* 41-50.

Calkins, C.O., and Parker, A.G. (2005) *Sterile Insect Quality*, In Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management, Dyck, V. A., Hendichs, J. and Robinson, A. S. eds., (Springer) pp. 269-296.

Calvo, S.E., Pagliarini, D.J., and Mootha, V.K. (2009) 'Upstream open reading frames cause widespread reduction of protein expression and are polymorphic among humans' *Proc. Natl. Acad. Sci. U. S. A.* **106***,* 7507-7512.

Cameron, P.J., Walker, G.P., Penny, G.M., and Wigley, P.J. (2002) 'Movement of potato tuberworm (Lepidoptera : Gelechiidae) within and between crops, and some comparisons with diamondback moth (Lepidoptera : Plutellidae)' *Environ. Entomol.* **31***,* 65-75.

Cao, J., Zhao, J.Z., Tang, J.D., Shelton, A.M., and Earle, E.D. (2002) 'Broccoli plants with pyramided Cry1Ac and Cry1C *Bt* genes control diamondback moths resistant to Cry1A and Cry1C proteins' *Theor. Appl. Genet.* **105***,* 258-264.

Carpenter, J.E., Bloem, S., and Marec, F. (2005) *Inherited Sterility in Insects*, In Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management, Dyck, V. A., Hendichs, J. and Robinson, A. S. eds., (Springer) pp. 115-146.

Carriere, Y., and Tabashnik, B.E. (2001) 'Reversing insect adaptation to transgenic insecticidal plants' *Proc. R. Soc. B* **268***,* 1475-1480.

Carriere, Y., Crowder, D.W., and Tabashnik, B.E. (2010) 'Evolutionary ecology of insect adaptation to *Bt* crops' *Evol. Appl.* **3***,* 561-573.

Carson, R. (1962). *Silent Spring* (Boston: Houghton Mifflin).

Cattaneo, M.G., Yafuso, C., Schmidt, C., Huang, C.Y., Rahman, M., Olson, C., Ellers-Kirk, C., Orr, B.J., Marsh, S.E., Antilla, L., Dutilleu, P., and Carriere, Y. (2006), Farm-scale evaluation of the impacts of transgenic cotton on biodiversity, pesticide use, and yield' *Proc. Natl. Acad. Sci. U. S. A*. **103***,* 7571-7576.

Catteruccia, F., Godfray, H.C.J., and Crisanti, A. (2003) 'Impact of genetic manipulation on the fitness of *Anopheles stephensi* mosquitoes' *Science* **299***,* 1225-1227.

Chapman, J.W., Reynolds, D.R., Smith, A.D., Riley, J.R., Pedgley, D.E., and Woiwod, I.P. (2002), 'High-altitude migration of the diamondback moth *Plutella xylostella* to the UK: a study using radar, aerial netting, and ground trapping' *Ecol. Entomol*. **27***,* 641-650.

Chen, C., Huang, H., Ward, C.M., Su, J.T., Schaeffer, L.V., Guo, M., and Hay, B.A. (2007) 'A synthetic maternal-effect selfish genetic element drives population replacement in Drosophila' *Science* **316***,* 597-600.

Christin, M.S., Menard, L., Giroux, I., Marcogliese, D.J., Ruby, S., Cyr, D., Fournier, M., and Brousseau, P. (2013) 'Effects of agricultural pesticides on the health of *Rana pipiens* frogs sampled from the field' *Environ. Sci. Pollut. R.*, **20***,* 601-611.

Cirio, U., and Murtas, I.d. (1974) *Status of Mediterranean fruit fly control by the sterile-male technique on the island of Procida*. in Food and Agriculture Organization; International Atomic Energy Agency: The sterile-insect technique and its field applications : Proceedings of a Panel on the Practical Use of the Sterile-Male Technique on Insect Control organized by the Joint FAO/IAEA Division of Atomic Energy in Food and Agriculture (Vienna, Austria).

Clontech. (2003) *Living Colors Reef Coral Fluorescent Protein Vectors* https://www.uam.es/personal_pdi/ciencias/pbonay/protocolos/LivingColors.pdf Accessed 2014.

Cooley, L., Kelley, R., and Spradling, A. (1988) 'Insertional Mutagenesis of the Drosophila Genome with Single P-Elements' *Science* **239***,* 1121-1128.

Corby-Harris, V., Drexler, A., de Jong, L.W., Antonova, Y., Pakpour, N., Ziegler, R., Ramberg, F., Lewis, E.E., Brown, J.M., Luckhart, S., and Riehle, M.A. (2010) 'Activation of Akt Signaling Reduces the Prevalence and Intensity of Malaria Parasite Infection and Lifespan in *Anopheles stephensi* Mosquitoes' *PloS Path*. **6***,* e1001003.

Courchamp, F., Clutton-Brock, T., and Grenfell, B. (1999) 'Inverse density dependence and the Allee effect' *TREE*. **14***,* 405-410.

Davis, S.A., Catchpole, E.A., and Fulford, G.R. (2000) 'Periodic triggering of an inducible gene for control of a wild population' *Theor. Popul. Biol.* **58***,* 95-106.

de Felipe, P., Martin, V., Cortes, M.L., Ryan, M., and Izquierdo, M. (1999) 'Use of the 2A sequence from foot-and-mouth disease virus in the generation of retroviral vectors for gene therapy' *Gene Ther.* **6***,* 198-208.

de la Torre, A., Iglesias, I., Carballo, M., Ramirez, P., and Jesus Munoz, M. (2012) 'An approach for mapping the vulnerability of European Union soils to antibiotic contamination' *Sci. Total Environ.* **414***,* 672-679.

de Valdez, M.R.W., Nimmo, D., Betz, J., Gong, H., James, A.A., Alphey, L., and Black, W.C. (2011) 'Genetic elimination of dengue vector mosquitoes' *Proc. Natl. Acad. Sci. U. S. A.* **108***,* 4772-4775.

Diao, F., and White, B.H. (2012) 'A Novel Approach for Directing Transgene Expression in Drosophila: T2A-Gal4 In-Frame Fusion' *Genetics* ***190***, 1139-U356.

Dunier, M., and Siwicki, A.K. (1993) 'Effects of pesticides and other organic pollutants in the aquatic environment on immunity of fish: a review' *Fish. Shellfish Immunol*. **3**, 423-438.

Evenson, R.E., and Gollin, D. (2003) 'Assessing the impact of the Green Revolution, 1960 to 2000' *Science* ***300***, 758-762.

Facchinelli, L., Valerio, L., Ramsey, J.M., Gould, F., Walsh, R.K., Bond, G., Robert, M.A., Lloyd, A.L., James, A.A., Alphey, L., and Scott, T.W. (2013) 'Field Cage Studies and Progressive Evaluation of Genetically-Engineered Mosquitoes' *Plos Negl. Trop. Dis.* **7**, e2001.

FAO. (2014). *FAOSTAT*. http://faostat.fao.org/site/348/default.aspx. Accessed 2014.

FAO/IAEA/USDA. (2003) *Manual for product quality control and shipping procedures for sterile mass-reared Tephritid fruit flies,* International Atomic Energy Agency (Vienna, Austria).

Feder, A.F., Kryazhimskiy, S., and Plotkin, J.B. (2014)' Identifying Signatures of Selection in Genetic Time Series' *Genetics* **196**, 509-522.

Fisher, K. (2000) 'Genetic sexing strains of mediterranean fruit fly (Diptera : Tephritidae): Quality in mass-reared temperature-sensitive lethal strains treated at high temperatures' *J. Econ. Entomol.* **93**, 394-402.

Flystock. (2014) *QF lines*, http://flystocks.bio.indiana.edu/Browse/Qsystem/qf.php Accessed 2014.

Foley, J., DeFries, R., Asner, G., Barford, C., Bonan, G., Carpenter, S., Chapin, F., Coe, M., Daily, G., Gibbs, H., *et al.* (2005) 'Global consequences of land use' *Science* **309**, 570-574.

Franz, G. (2005) *Genetic Sexing Strains* in Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management, Dyck, V. A., Hendichs, J. and Robinson, A. S. eds., (Springer) pp. 407-451.

Fried, M. (1971) 'Determination of Sterile-Insect Competitiveness' *J. Econ. Entomol.* **64**, 869-872.

Fu, G., Condon, K.C., Epton, M.J., Gong, P., Jin, L., Condon, G.C., Morrison, N.I., Dafa'alla, T.H., and Alphey, L. (2007) 'Female-specific insect lethality engineered using alternative splicing' *Nat. Biotechnol.* **25**, 353-357.

Furlong, M.J., Pell, J.K., Pek Choo, O., and Abdul Rahman, S. (1995) ‘Field and laboratory evaluation of a sex pheromone trap for the autodissemination of the fungal entomopathogen *Zoophthora radicans* (Entomophthorales) by the diamondback moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae)’ *Bull. Entomol. Res*. **85***,* 331-337.

Furlong, M.J., Wright, D.J., and Dosdall, L.M. (2013) ‘Diamondback Moth Ecology and Management: Problems, Progress, and Prospects’ *Annu. Rev. Entomol.* **58***,* 517-541.

Galizi, R., Doyle, L.A., Menichelli, M., Bernardini, F., Deredec, A., Burt, A., Stoddard, B.L., Windbichler, N., and Crisanti, A. (2014) ‘A synthetic sex ratio distortion system for the control of the human malaria mosquito’ *Nat Commun* **5***,* 3977-3977.

Gato, R., Lees, R.S., Bruzon, R.Y., Companioni, A., Menendez, Z., González, A., and Rodríguez, M. (2014) ‘Large indoor cage study of the suppression of stable *Aedes aegypti* populations by the release of thiotepa-sterilised males’ *Memórias do Instituto Oswaldo Cruz* **109***,* 365-370.

Gavriel, S., Jurkevitch, E., Gazit, Y., and Yuval, B. (2011) ‘Bacterially enriched diet improves sexual performance of sterile male Mediterranean fruit flies’ *J. Appl. Entomol.* **135***,* 564-573.

Gavriel, S., Gazit, Y., and Yuval, B. (2010) ‘Effect of diet on survival, in the laboratory and the field, of sterile male Mediterranean fruit flies’ *Entomol. Exp. Appl.* **135***,* 96-104.

Geiger, F., Bengtsson, J., Berendse, F., Weisser, W.W., Emmerson, M., Morales, M.B., Ceryngier, P., Liira, J., Tscharntke, T., Winqvist, C*., et al.* (2011) ‘Persistent negative effects of pesticides on biodiversity and biological control potential on European farmland’ *Basic Appl. Ecol.* **12***,* 386-387.

Ghimire, M.N., Huang, F., Leonard, R., Head, G.P., and Yang, Y. (2011), ‘Susceptibility of Cry1Ab-susceptible and -resistant sugarcane borer to transgenic corn plants containing single or pyramided *Bacillus thuringiensis* genes’ *Crop Prot.* ***30****,* 74-81.

Giles, N.H., Case, M.E., Baum, J., Geever, R., Huiet, L., Patel, V., and Tyler, B. (1985) ‘Gene Organization and Regulation in the Qa (Quinic Acid) Gene-Cluster of *Neurospora crassa*’ *Microbiol. Rev*. ***49****,* 338-358.

Giles, N.H., Geever, R.F., Asch, D.K., Avalos, J., and Case, M.E. (1991) ‘Organization and Regulation of the Qa (Quinic, Acid) Genes in *Neurospora crassa* and Other Fungi’ *Heredity* **82***,* 1-7.

Goldewijk, K.K. (2001) ‘Estimating global land use change over the past 300 years: The HYDE Database’ *Global Biogeochem. Cycles* **15***,* 417-433.

Gong, P., Epton, M.J., Fu, G.L., Scaife, S., Hiscox, A., Condon, K.C., Condon, G.C., Morrison, N.I., Kelly, D.W., Dafa'alla, T., Coleman, P.G., and Alphey, L. (2005) ‘A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly’ *Nat. Biotechnol.* **23***,* 453-456.

Gonzalez, M., Martin-Ruiz, I., Jimenez, S., Pirone, L., Barrio, R., and Sutherland, J.D. (2011) 'Generation of stable Drosophila cell lines using multicistronic vectors' *Scientific Reports* **1***,* 75.

Gossen, M., and Bujard, H. (1992) 'Tight Control of Gene-Expression in Mammalian-Cells by Tetracycline-Responsive Promoters' *Proc. Natl. Acad. Sci. U. S. A*. **89***,* 5547-5551.

Gould, F., Anderson, A., Jones, A., Sumerford, D., Heckel, D.G., Lopez, J., Micinski, S., Leonard, R., and Laster, M. (1997) 'Initial frequency of alleles for resistance to *Bacillus thuringiensis* toxins in field populations of *Heliothis virescens*' *Proc. Natl. Acad. Sci. U. S. A.* **94**, 3519-3523.

Gould, F., Huang, Y., Legros, M., and Lloyd, A.L. (2008) 'A Killer-Rescue system for self-limiting gene drive of anti-pathogen constructs' *Proc. R Soc. B*. **275***,* 2823-2829.

Greene, G.L., Leppla, N.C., and Dickerson, W.A. (1976) 'Velvetbean Caterpillar - (Leipidoptera, Noctuidae) Rearing Procedure and Artificial Medium' *J. Econ. Entomol.* **69***,* 487-488.

Hajek, A. (2004) *Natural enemies: an introduction to biological control* (Cambridge: Cambridge University Press).

Haldane, J.B.S. (1931) 'Mathematical Darwinism: A Discussion of the Genetical Theory of Natural Selection' *Eugen. Rev*. **23***,* 115-117.

Hallmann, C.A., Foppen, R.P.B., van Turnhout, C., de Kroon, H., and Jongejans, E. (2014) 'Declines in insectivorous birds are associated with high neonicotinoid concentrations' *Nature*, **511**, 341-343.

Halpin, C., Cooke, S.E., Barakate, A., El Amrani, A., and Ryan, M.D. (1999) 'Self-processing 2A-polyproteins - a system for co-ordinate expression of multiple proteins in transgenic plants' *Plant Journal* **17***,* 453-459.

Hamilton, M.B. (2009) *Population genetics* (Oxford: Wiley-Blackwell).

Handler, A.M., and Beeman, R.W. (2003) 'United States Department of Agriculture - Agricultural Research Service: advances in the molecular genetic analysis of insects and their application to pest management' *Pest Manag. Sci.* **59***,* 728-735.

Harcourt, D.G. (1954) *The biology and ecology of the diamondback moth (Plutella maculipennis Curtis) in Eastern Ontario.* Thesis (Ph.D.)--Cornell University.

Harcourt, D.G. (1986) *Population dynamics of the diamondback moth in southern Ontario*. In Diamondback moth management: Proceedings of the First International Workshop at The Asian Vegetable Research and Development Centre. (Tainan, Taiwan).

Harris, A.F., McKemey, A.R., Nimmo, D., Curtis, Z., Black, I., Morgan, S.A., Oviedo, M.N., Lacroix, R., Naish, N., Morrison, N.I., *et al.* (2012) 'Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes' *Nat. Biotechnol.* **30**, 828-830.

Harris, A.F., Nimmo, D., McKemey, A.R., Kelly, N., Scaife, S., Donnelly, C.A., Beech, C., Petrie, W.D., and Alphey, L. (2011) 'Field performance of engineered male mosquitoes' *Nat. Biotechnol.* **29**, 1034-U109.

Hasan, F., and Ansari, M.S. (2011) 'Effects of different brassicaceous host plants on the fitness of *Pieris brassicae* (L.)' *Crop Prot*. **30**, 854-862.

Hayden, C.A., and Bosco, G. (2008) 'Comparative genomic analysis of novel conserved peptide upstream open reading frames in Drosophila melanogaster and other dipteran species' *BMC Genom.* **9**, 61.

Hendrichs, J., Franz, G., and Rendon, P. (1995) 'Increased Effectiveness and Applicability of the Sterile Insect Technique through Male-Only Releases for Control of Mediterranean Fruit-Flies during Fruiting Seasons' *J. Appl Entomol.* **119**, 371-377.

Hight, S.D., Carpenter, J.E., Bloem, S., and Bloem, K.A. (2005) 'Developing a sterile insect release program for *Cactoblastis cactorum* (Berg) (Lepidoptera : Pyralidae): Effective overflooding ratios and release-recapture field studies' *Environ. Entomol.* **34**, 850-856.

Hoa, N.T.Q., and Tien, N.T.T. (2001) 'Radiation Induced F1 Sterility in *Plutella xylostella* (Lepidoptera: Plutellidae): Potential for Population Suppression in the Field' *Fla. Entomol.* **84**, 199-208.

Honda, K., Miyahara, Y., and Kegasawa, K. (1992) 'Seasonal Abundance and the Possibility of Spring Immigration of the Diamondback Moth, *Plutella xylostella* (Linnaeus) (Lepidoptera, Yponomeutidae), in Morioka-City, Northern Japan' *Appl. Entomol. Zool.* **27**, 517-525.

Horn, C., Schmid, B.G.M., Pogoda, F.S., and Wimmer, E.A. (2002) 'Fluorescent transformation markers for insect transgenesis' *Insect Biochem. Mol. Biol.* **32**, 1221-1235.

Hu, X., Zhou, Q., and Luo, Y. (2010) 'Occurrence and source analysis of typical veterinary antibiotics in manure, soil, vegetables and groundwater from organic vegetable bases, northern China' *Environ. Pollut.* **158**, 2992-2998.

Huberson, J., Nemoto, H., and Hirose, Y. (1998) *Pesticides and Conservation of Natural Enemies in Pest Management* In Conservation Biological Control, Pedro A. Barbosa ed., (California: Academic Press) pp. 207-220.

Illingworth, C.J.R., Parts, L., Schiffels, S., Liti, G., and Mustonen, V. (2012) 'Quantifying Selection Acting on a Complex Trait Using Allele Frequency Time Series Data' *Mol. Biol. Evol.* ***29***, 1187-1197.

IRAC. (2013) *The Diamondback Moth, Plutella xylostella: Resistance Management is Key for Sustainable Control,* http://www.irac-online.org/content/uploads/plutella_poster_v3.1_15Feb13.pdf. Accessed 2014.

Irvin, N., Hoddle, M.S., O'Brochta, D.A., Carey, B., and Atkinson, P.W. (2004) 'Assessing fitness costs for transgenic *Aedes aegypti* expressing the GFP marker and transposase genes' *Proc. Natl. Acad. Sci. U. S. A.* **101***,* 891-896.

Ito, Y., and Yamamura, K. (2005) *Role of population and behavioural ecology in the sterile insect technique* in Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management., Dyck, V. A., Hendrichs, J. and Robinson, A. S. eds., (Springer) pp 177-208.

IUCN Task Force on Systemic Pesticides. (2014) *Worldwide integrated assessment on systemic pesticides* https://www.iucn.org/about/union/commissions/cem/cem_work/task_force_systemic_pesticides.cfm. Accessed 2014

James, C. (2013) *Global status of commercialized biotech/GM crops: 2013. ISAAA Briefs* http://www.isaaa.org/resources/publications/briefs/46/executivesummary/ Accessed 2014.

Jin, L., Walker, A.S., Fu, G., Harvey-Samuel, T., Dafa'alla, T., Miles, A., Marubbi, T., Granville, D., Humphrey-Jones, N., O'Connell, S., Morrison, N.I., and Alphey, L. (2013) 'Engineered Female-Specific Lethality for Control of Pest Lepidoptera'. *ACS Syn. Biol.* **2***,* 160-166.

Kapetanaki, M.G., Loukeris, T.G., Livadaras, I., and Savakis, C. (2002) 'High frequencies of Minos transposon mobilization are obtained in insects by using in vitro synthesized mRNA as a source of transposase' *Nucleic Acids Res*. **30***,* 3333-3340.

Karimzadeh, J., Bonsall, M.B., and Wright, D.J. (2004) 'Bottom-up and top-down effects in a tritrophic system: the population dynamics of *Plutella xylostella* (L.) and *Cotesia plutellae* (Kurdjumov) on different host plants' *Ecol. Entomol.* **29***,* 285-293.

Kenis, M., and Branco, M. (2010) 'Impact of alien terrestrial arthropods in Europe. Chapter 5' *BioRisk* **4***,* 51-71.

Kirsch, K., and Schmutterer, H. (1988) 'Low Efficacy of a *Bacillus thuringiensis* (Berl) Formulation in Controlling the Diamondback Moth, *Plutella xylostella* (L), in the Philippines' *J. Appl. Entomol.* **105***,* 249-255.

Kiuchi, T., Koga, H., Kawamoto, M., Shoji, K., Sakai, H., Arai, Y., Ishihara, G., Kawaoka, S., Sugano, S., Shimada, T*., et al.* (2014) 'A single female-specific piRNA is the primary determiner of sex in the silkworm' *Nature*. **509***,* 633-636.

Klassen, W. (2005). *Area-Wide Integrated Pest Management and SIT* in Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management, Dyck, V. A., Hendrichs, J. and Robinson, A. S. eds., (Springer) pp. 39-68.

Klassen, W., and Curtis, C.F. (2005) *History of the Sterile Insect Technique* in Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management, Dyck, V. A., Hendrichs, J. and Robinson, A. S. eds., (Springer) pp. 3-36.

Knipling, E.F. (1985) 'Sterile Insect Technique as a Screwworm *Cochliomyia hominivorax* Control Measure, the Concept and its Development' *Misc. Publ. Entomol. Soc. Am.* **62**, 4-7.

Knipling, E.F. (1979) *The basic principles of insect population suppression and management* in The Basic Principles of Insect Population Suppression and Management. (Washington, D.C.: US Dept. of Agriculture).

Knipling, E.F. (1955) 'Possibilities of Insect Control Or Eradication through the use of Sexually Sterile Males' *J. Econ. Entomol.* **48**, 459-462.

Knipple, D.C. (2013) 'Prospects for the use of transgenic approaches to improve the efficacy of the Sterile Insect Technique (SIT) for control of the codling moth Cydia pomonella Linnaeus (Lepidoptera: Tortricidae)' *Crop Prot.* **44**, 142-146.

Koudelova, J., and Cook, P.A. (2001) 'Effect of gamma radiation and sex-linked recessive lethal mutations on sperm transfer in *Ephestia kuehniella* (Lepidoptera : Pyralidae)' *Fla. Entomol*. **84**, 172-182.

Koval, T.M. (1996) 'Moths: Myths and mysteries of stress resistance' *Bioessays* **18**, 149-156.

Koyama, J., Teruya, T., and Tanaka, K. (1984) 'Eradication of the Oriental Fruit-Fly (Diptera, Tephritidae) from the Okinawa Islands by a Male Annihilation Method' *J. Econ. Entomol.* **77**, 468-472.

Krebs, J.R., Wilson, J.D., Bradbury, R.B., and Siriwardena, G.M. (1999) 'The second Silent Spring?' *Science* **400**, 612.

Kumar, K., Gupta, S.C., Baidoo, S.K., Chander, Y., and Rosen, C.J. (2005) 'Antibiotic uptake by plants from soil fertilized with animal manure' *J. Environ. Qual*. **34**, 2082-2085.

Kuriwada, T., Kumano, N., Shiromoto, K., Haraguchi, D., and Kohama, T. (2012) 'Suppressing effect of gamma-irradiated weevils on progeny production in the West Indian sweetpotato weevil *Euscepes postfasciatus* (Coleoptera: Curculionidae)' *Appl. Entomol. Zool.* **47**, 437-442.

Lachance, L.E., and Graham, C.K. (1984) 'Insect Radiosensitivity - Dose Curves and Dose-Fractionation Studies of Dominant Lethal Mutations in the Mature Sperm of 4 Insect Species' *Mutat. Res.* **127**, 49-59.

Lambert, M.R.K. (1997) 'Effects of pesticides on amphibians and reptiles in sub-Saharan Africa' *Rev. Environ. Contam. Toxicol.* **150**, 31-73.

Lambrechts, L., Koella, J.C., and Boete, C. (2008) ‘Can transgenic mosquitoes afford the fitness cost?’ *Trends Parasitol.* **24***,* 4-7.

Lance, D.R., and McInnis, D.O. (2005) *Biological basis of the sterile insect technique* in Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management. Dyck, V. A., Hendrichs, J. and Robinson, A. S. eds., (Springer) pp 69-94.

Landis, D., Wratten, S., and Gurr, G. (2000) ‘Habitat management to conserve natural enemies of arthropod pests in agriculture’ *Annu. Rev. Entomol*. **45***,* 175-201.

Lee, S., Chu, Y., and Talekar, N.S. (1995) ‘The mating behavior of diamondback moth (Lepidoptera: Plutellidae)’ *Chin. J. Entomol.* **15***,* 81-89.

Leftwich, P.T., Koukidou, M., Rempoulakis, P., Gong, H., Zacharopoulou, A., Fu, G., Chapman, T., Economopoulos, A., Vontas, J., and Alphey, L. (2014) ‘Genetic elimination of field-cage populations of Mediterranean fruit flies’ *Proc. R. Soc. B.* **281**.

Liebman, M. (1995). *Polyculture Cropping Cystems in Agroecology: The Science of Sustainable Agriculture.* M Altieri., ed. (Boulder CO: Westview Press), pp 205-218

Lipsitcht, M., Singer, R.S., and Levin, B.R. (2002) ‘Antibiotics in agriculture: When is it time to close the barn door?’ *Proc. Natl. Acad. Sci. U. S. A*. **99***,* 5752-5754.

Lu, Y., Wu, K., Jiang, Y., Guo, Y., and Desneux, N. (2012) ‘Widespread adoption of *Bt* cotton and insecticide decrease promotes biocontrol services’ *Nature* **487***,* 362-365.

Lux, S.A., Vilardi, J.C., Liedo, P., Gaggl, K., Calcagno, G.E., Munyiri, F.N., Vera, M.T., and Manso, F. (2002) ‘Effects of irradiation on the courtship behavior of medfly (Diptera, Tephritidae) mass reared for the sterile insect technique’ *Fla. Entomol.* **85***,* 102-112.

Lyman, R.F., Lawrence, F., Nuzhdin, S.V., and Mackay, T.F.C. (1996) ‘Effects of single P-element insertions on bristle number and viability in Drosophila melanogaster’ *Genetics* **143***,* 277-292.

Ma, Z., Du, Z., Chen, X., Wang, X., and Yang, Y. (2013) ‘Fine tuning the LightOn light-switchable transgene expression system’ *Biochem. Biophys. Res. Commun*. **440***,* 419-423.

Maa, C.J.W. (1986) *Ecological approach to male diamondback moth response to sex pheromone*, http://web.entomology.cornell.edu/shelton/diamondback-moth/pdf/85papers/1985DBM12.pdf. Accessed 2014.

Mackay, T.F.C., Lyman, R.F., and Jackson, M.S. (1992) ‘Effects of P-Element Insertions on Quantitative Traits in Drosophila-Melanogaster’ *Genetics* **130***,* 315-332.
Maimun, T., and Mahani, M.C. *Using radiation dose of 175Gy for sterile insect technique in diamondback moth* (1996) Proceedings of the Third Internation Worshop: The Management of Diamondback Moth and other Crucifer Pests. (Kuala Lumpur: Malaysia). pp 339-342

Mani, E., and Wildbolz, T. (1977) ‘Dispersal of Male Codling Moths (Laspeyresia Pomonella L) in Upper Rhine Valley’ *J. Appl. Entomol*. **83***,* 161-168.

Marec, F., Neven, L.G., and Fukova, I. (2007) *Developing transgenic sexing strains for the release of non-transgenic sterile male codling moths Cydia pomonella* in Area-Wide control of Insect Pests eds. Vreysen, M.J.B., Robinson. A.S., Hendrichs. G. (Springer), pp 103-111

Marec, F., Neven, L.G., Robinson, A.S., Vreysen, M., Goldsmith, M.R., Nagaraju, J., and Franz, G. (2005) ‘Development of genetic sexing strains in lepidoptera: from traditional to transgenic approaches’ *J. Econ. Entomol.* **98***,* 248-259.

Marec, F., Neven, L.G., Robinson, A.S., Vreysen, M., Goldsmith, M.R., Nagaraju, J., and Franz, G. (2005) ‘Development of genetic sexing strains in lepidoptera: from traditional to transgenic approaches’ *J. Econ. Entomol.* **98***,* 248-259.

Marrelli, M.T., Moreira, C.K., Kelly, D., Alphey, L., and Jacobs-Lorena, M. (2006) ‘Mosquito transgenesis: what is the fitness cost?’ *Trends Parasitol.* **22***,* 197-202.

Marrelli, M.T., Li, C., Rasgon, J.L., and Jacobs-Lorena, M. (2007) ‘Transgenic malaria-resistant mosquitoes have a fitness advantage when feeding on Plasmodium-infected blood’ *Proc. Natl. Acad. Sci. U. S. A.* **104***,* 5580-5583.

Martins, S., Naish, N., Walker, A.S., Morrison, N.I., Scaife, S., Fu, G., Dafa'alla, T., and Alphey, L. (2012) ‘Germline transformation of the diamondback moth, *Plutella xylostella* L., using the piggyBac transposable element’ *Insect Mol. Biol.* **21***,* 414-421.

Massonnet-Bruneel, B., Corre-Catelin, N., Lacroix, R., Lees, R.S., Kim Phuc Hoang, Nimmo, D., Alphey, L., and Reiter, P. (2013) ‘Fitness of Transgenic Mosquito *Aedes aegypti* Males Carrying a Dominant Lethal Genetic System’ *PLoS ONE,* **8***,* 1-7.

Matson, P.A., Parton, W.J., Power, A.G., and Swift, M.J. (1997) ‘Agricultural Intensification and Ecosystem Properties’ *Science* **277***,* 504-509.

Maung, N., (1998) *Control of Cabbage Diamondback Moth by using Sterile Insect Technique in Myanmar (1992-1998)* in Proceedings of FAO/IAEA International Conference on Area-Wide Control of Insect Pests integrating the Sterile Insect and related nuclear and other techniques (Penang, Malaysia).

Maxmen, A. (2013) ‘Crop pests under attack’ *Nature* **501***,* S15-S17.

Mazlan, N., and Mumford, J. (2005) ‘Insecticide use in cabbage pest management in the Cameron Highlands, Malaysia’ *Crop Pro*. **24***,* 31-39

Mckenzie, J.A. (1990) ‘Selection at the Dieldrin Resistance Locus in Overwintering Populations of Lucilia-Cuprina (Wiedemann)’ *Aust. J. Zool*. **38***,* 493-501.

McLaughlin, A., and Mineau, P. (1995) ‘The impact of agricultural practices on biodiversity’ *Agric. Ecosyst. Environ.* **55***,* 201-212.

Metz, T.D., Roush, R.T., Tang, J.D., Shelton, A.M., and Earle, E.D. (1995) 'Transgenic Broccoli Expressing a *Bacillus thuringiensis* Insecticidal Crystal Protein - Implications for Pest Resistance Management Strategies' *Mol. Breed*. **1***,* 309-317.

Mineau, P., and Whiteside, M. (2013) 'Pesticide Acute Toxicity Is a Better Correlate of U.S. Grassland Bird Declines than Agricultural Intensification' *PLoS ONE*, e57457.

Mo, J.H., Baker, G., Keller, M., and Roush, R. (2003) 'Local dispersal of the diamondback moth (*Plutella xylostella* (L.)) (Lepidoptera : Plutellidae)' *Environ. Entomol*. **32***,* 71-79.

Moreira, L.A., Wang, J., Collins, F.H., and Jacobs-Lorena, M. (2004) 'Fitness of anopheline mosquitoes expressing transgenes that inhibit plasmodium development' *Genetics* **166***,* 1337-1341.

Morrison, N.I., Franz, G., Koukidou, M., Miller, T.A., Saccone, G., Alphey, L.S., Beech, C.J., Nagaraju, J., Simmons, G., and Polito, L.C. (2010) 'Genetic improvements to the sterile insect technique for agricultural pests' *APJMBB*. **18***,* 275-295.

Morrison, N.I., Segura, D.F., Stainton, K.C., Fu, G., Donnelly, C.A., and Alphey, L.S. (2009), 'Sexual competitiveness of a transgenic sexing strain of the Mediterranean fruit fly, *Ceratitis capitata*.' *Entomol. Exp. Appl.* **133***,* 146-153.

Morrison, N.I., Simmons, G.S., Fu, G., O'Connell, S., Walker, A.S., Dafa'alla, T., Walters, M., Claus, J., Tang, G., Jin, L.*, et al.* (2012) 'Engineered Repressible Lethality for Controlling the Pink Bollworm, a Lepidopteran Pest of Cotton' *PLoS ONE,* **7***,* e50922.

Murdock, C.C., Paaijmans, K.P., Cox-Foster, D., Read, A.F., and Thomas, M.B. (2012) 'Rethinking vector immunology: the role of environmental temperature in shaping resistance' *Nat. Rev. Microbiol.* **10***,* 869-876.

Nagaraju, J., and Goldsmith, M.R. (2002) 'Silkworm genomics - progress and prospects' *Curr. Sci.* **83***,* 415-425.

Nagaraju, J., Gopinath, G., Sharma, V., and Shukla, J.N. (2014) 'Lepidopteran Sex Determination: A Cascade of Surprises' *Sex. Dev.* **8***,* 104-112.

Nagel, P., and Peveling, R. (2005) *Environment and the SIT* in Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management, Dyck, V. A., Hendrichs, J. and Robinson, A. S. eds., (Springer) pp. 499-524.

Natesan, S., Molinari, E., Rivera, V.M., Rickles, R.J., and Gilman, M. (1999) 'A general strategy to enhance the potency of chimeric transcriptional activators' *Proc. Natl. Acad. Sci. U. S. A.* **96***,* 13898-13903.

Nollet, L.M.L. (2000) *Food Analysis by HPLC* (New York: CRC Press).

Oerke, E.C. (2006) 'Crop losses to pests' *J. Agric. Sci*. **144***,* 31-43.

Ohbayashi, F., Suzuki, M.G., Mita, K., Okano, K., and Shimada, T. (2001) 'A homologue of the Drosophila doublesex gene is transcribed into sex-specific mRNA isoforms in the silkworm, *Bombyx mori*.' *Comp. Biochem. Physiol. B.* **128***,* 145-158.

O'Kane, C.J., and Gehring, W.J. (1987) 'Detection Insitu of Genomic Regulatory Elements in Drosophila' *Proc. Natl. Acad. Sci. U. S. A.* **84***,* 9123-9127.
Omar, D., and Mansor, M. (1993) *Effect of Substerilization Doses of Radiation on the Biology of Diamondback Moth* in Proceedings of Radiation Induced F1 Sterility in Lepidoptera for Area-Wide Control (Phoenix: USA) pp 3-9.

Orozco-Davila, D., Hernandez, R., Meza, S., and Dominguez, J. (2007) 'Sexual competitiveness and compatibility between mass-reared sterile flies and wild populations of *Anastrepha ludens* (Diptera : Tephritidae) from different regions in Mexico' *Fla. Entomol.* **90***,* 19-26.

Pane, A., Salvemini, M., Bovi, P.D., Polito, C., and Saccone, G. (2002) 'The transformer gene in *Ceratitis capitata* provides a genetic basis for selecting and remembering the sexual fate' *Development* **129***,* 3715-3725.

Paranhos, B.J., McInnis, D., Uramoto, K., Damasceno, I., Gonçalves, N., Alves, M.A., Costa, M., Walder, J., Malavasi, A., and Nascimento, A. (2006) *Sterile medfly males of the tsl Vienna 8 genetic sexing strain display improved mating performance with ginger root oil* in Fruit Flies of Economic Importance: From Basic to Applied Knowledge Proceedings of the 7th International Symposium on Fruit Flies of Economic Importance. (Salvador, Brazil).

Pardo-López, L., Soberón, M., and Bravo, A. (2013) '*Bacillus thuringiensis* insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection' *FEMS Microbiol. Rev*. **37***,* 3-22.

Pasyukova, E.G., Nuzhdin, S.V., Morozova, T.V., and Mackay, T.F.C. (2004) 'Accumulation of transposable elements in the genome of Drosophila melanogaster is associated with a decrease in fitness' *Heredity*. **95***,* 284-290.

Paton, D., Underhill, A., Meredith, J., Eggleston, P., and Tripet, F. (2013) 'Contrasted Fitness Costs of Docking and Antibacterial Constructs in the EE and EVida3 Strains Validates Two-Phase *Anopheles gambiae* Genetic Transformation System' *PLoS ONE* **8***,* e67364.

Pavlopoulos, A., Berghammer, A.J., Averof, M., and Klingler, M. (2004) 'Efficient transformation of the beetle *Tribolium castaneum* using the Minos transposable element: Quantitative and qualitative analysis of genomic integration events' *Genetics* **167***,* 737-746.

Pérez-Staples, D., Shelly, T.E., and Yuval, B. (2013) 'Female mating failure and the failure of mating in sterile insect programs' *Entomol. Exp. Appl.* **146***,* 66-78.

Phuc, H.K., Andreasen, M.H., Burton, R.S., Vass, C., Epton, M.J., Pape, G., Fu, G., Condon, K.C., Scaife, S., Donnelly, C.A., *et al.* (2007) 'Late-acting dominant lethal genetic systems and mosquito control' *BMC Biol.* **5***,* 11.

Pimentel, D. (2009) *Environmental and Economic Costs of the Application of Pesticides Primarily in the United States* in Integrated Pest Management: Innovation-development Process. Peshin, R., Dhawan A.K., eds., (Springer) pp 89-111.

Pimentel, D. (2005) Environmental and Economic Costs of the Application of Pesticides Primarily in the United States. Environment, *JSD* **7***,* 229-252.

Pimentel, D., and Pimentel, M. (1990) 'Comment: Adverse environmental consequences of the Green Revolution' *Popul. Dev. Rev.* **16***,* 329-332.

Pivnick, K.A., Jarvis, B.J., Gillott, C., Slater, G.P., and Underhill, E.W. (1990) 'Daily Patterns of Reproductive Activity and the Influence of Adult Density and Exposure to Host Plants on Reproduction in the Diamondback Moth (Lepidoptera, Plutellidae)' *Environ. Entomol.* **19***,* 587-593.

Potter, C.J., and Luo, L. (2011) 'Using the Q system in *Drosophila melanogaster*' *Nat. Protoc.* **6***,* 1105-1120.

Potter, C.J., Tasic, B., Russler, E.V., Liang, L., and Luo, L. (2010) 'The Q System: A Repressible Binary System for Transgene Expression, Lineage Tracing, and Mosaic Analysis' *Cell* **141***,* 536-548.

Prout, T. (1978) 'The joint effects of the release of sterile males and immigration of fertilized females on a density regulated population' *Theor. Popul. Biol.* **13***,* 40-71.

Proverbs, M.D., Newton, J.R., and Campbell, C.J. (1982) 'Codling moth: a pilot program of control by sterile insect release in British Columbia' *Can. Entomol*. **114***,* 363-376.

Rajeswaran, R., Sunitha, S., Shivaprasad, P.V., Pooggin, M.M., Hohn, T., and Veluthambi, K. (2007) 'The Mungbean yellow mosaic Begomovirus transcriptional activator protein transactivates the viral promoter-driven transgene and causes toxicity in transgenic tobacco plants' *Mol. Plant-Microbe Interact.* **20***,* 1545-1554.

Rasgon, J.L. (2009) 'Multi-Locus Assortment (MLA) for Transgene Dispersal and Elimination in Mosquito Populations' *PLoS ONE,* **4***,* e5833.

Rauh, V., Arunajadai, S., Horton, M., Perera, F., Hoepner, L., Barr, D.B., and Whyatt, R. (2011) 'Seven-Year Neurodevelopmental Scores and Prenatal Exposure to Chlorpyrifos, a Common Agricultural Pesticide' *Environ. Health Perspect*. **119***,* 1196-1201.

Raymond, B., Wright, D.J., and Bonsall, M.B. (2011) 'Effects of host plant and genetic background on the fitness costs of resistance to *Bacillus thuringiensis*' *Heredity* **106***,* 281-288.

Regier, J.C., Mitter, C., Zwick, A., Bazinet, A.L., Cummings, M.P., Kawahara, A.Y., Sohn, J., Zwickl, D.J., Cho, S., Davis, D.R., *et al.* (2013) 'A Large-Scale, Higher-Level, Molecular Phylogenetic Study of the Insect Order Lepidoptera (Moths and Butterflies)' *PLoS ONE,* **8***,* e58568.

Relyea, R.A., Schoeppner, N.M., and Hoverman, J.T. (2005) 'Pesticides and amphibians: The importance of community context' *Ecol. Appl.* **15***,* 1125-1134.

Rendon, P., McInnis, D., Lance, D., and Stewart, J. (2004) 'Medfly (Diptera : Tephritidae) genetic sexing: Large-scale field comparison of males-only and bisexual sterile fly releases in Guatemala' *J. Econ. Entomol.* **97***,* 1547-1553.

Rhode, R.H., Simon, J., Perdomo, A., Gutierre.J, Dowling, C.F., and Lindquis.Da. (1971) 'Application of Sterile-Insect-Release Technique in Mediterranean Fruit Fly Diptera-Tephritidae Suppression' *J. Econ. Entomol.* **64***,* 708-713.

Robert, M.A., Legros, M., Facchinelli, L., Valerio, L., Ramsey, J.M., Scott, T.W., Gould, F., and Lloyd, A.L. (2012) 'Mathematical Models as Aids for Design and Development of Experiments: The Case of Transgenic Mosquitoes' *J. Med. Entomol.* **49***,* 1177-1188.

Robinson, A.S. (2005) *Genetic Basis of the Sterile Insect Technique* in Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management, Dyck, V. A., Hendichs, J. and Robinson, A. S. eds., (Springer) pp. 95-114.

Robinson, A.S., and Hendrichs, J. (2005) *Future Development and Application* in Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management, Dyck, V. A., Hendichs, J. and Robinson, A. S. eds., (Springer) pp. 115-146.

Ruttan, V. (2002) 'Productivity growth in world agriculture: Sources and constraints'. *J. Econ. Persp.* **16***,* 161-184.

Ryan, M.D., King, A.M.Q., and Thomas, G.P. (1991) 'Cleavage of Foot-And-Mouth-Disease Virus Polyprotein is Mediated by Residues Located within a 19 Amino-Acid-Sequence' *J. Gen. Virol.* **72***,* 2727-2732.

Sabatier, R., Meyer, K., Wiegand, K., and Clough, Y. (2013) 'Non-linear effects of pesticide application on biodiversity-driven ecosystem services and disservices in a cacao agroecosystem: A modeling study' *Basic Appl. Ecol.* **14***,* 115-125.
Saeed, S., Sayyed, A.H., and Ahmad, I. (2010) 'Effect of host plants on life-history traits of Spodoptera exigua (Lepidoptera: Noctuidae)' *J. Pest Sci.* **83***,* 165-172.

San Andres, V., Ortego, F., and Castanera, P. (2007) 'Effects of gamma-irradiation on midgut proteolytic activity of the Mediterranean fruit fly, *Ceratitis capitata* (Diptera : Tephritidae)' *Arch. Insect Biochem. Physiol.* **65***,* 11-19.

Schäfer, R.B., Bundschuh, M., Rouch, D.A., Szöcs, E., von der Ohe, P.C., Pettigrove, V., Schulz, R., Nugegoda, D., and Kefford, B.J. (2012) 'Effects of pesticide toxicity, salinity and other environmental variables on selected ecosystem functions in streams and the relevance for ecosystem services' *Sci. Total Environ.* **415***,* 69-78.

Schäfer, R.B., Caquet, T., Siimes, K., Mueller, R., Lagadic, L., and Liess, M. (2007) 'Effects of pesticides on community structure and ecosystem functions in agricultural streams of three biogeographical regions in Europe' *Sci. Total Environ*. **382***,* 272-285.

Schliekelman, P., Ellner, S., and Gould, F. (2005) 'Pest control by genetic manipulation of sex ratio' *J. Econ. Entomol.* **98***,* 18-34.

Schliekelman, P., and Gould, F. (2000) 'Pest control by the release of insects carrying a female-killing allele on multiple loci' *J. Econ. Entomol.* **93***,* 1566-1579.

Schmitt, H., Stoob, K., Hamscher, G., Smit, E., and Seinen, W. (2006) 'Tetracyclines and tetracycline resistance in agricultural soils: Microcosm and field studies' *Microb. Ecol.* **51***,* 267-276.

Scolari, F., Schetelig, M.F., Bertin, S., Malacrida, A.R., Gasperi, G., and Wimmer, E.A. (2008) 'Fluorescent sperm marking to improve the fight against the pest insect *Ceratitis capitata* (Wiedemann; Diptera : Tephritidae)' *New Biotechnol.* **25***,* 76-84.

Scott, T.W., Rasgon, J.L., Black, W.C., and Gould, F. (2006) *Fitness studies: developing a consensus methodology* in Bridging laboratory and field research for genetic control of disease vectors. Scott, T.W., Rasgon, J.L., Black, W.C., Gould, F. eds., (Springer) 171-181.

Seo, Y.H., Cho, B.O., Kang, A.S., Jeong, B.C., and Jung, Y. (2010) 'Antibiotic uptake by plants from soil applied with antibiotic-treated animal manure' *Kor. J Soil Sci. Fert.* **43***,* 466-470.

Shelly, T.E., Edu, J., and Pahio, E. (2005) 'Lack of an irradiation effect on the mating performance of mass-reared males of the Mediterranean fruit fly' *Fla. Entomol.* **88***,* 547-548.

Shelly, T.E., McInnis, D.O., Rodd, C., Edu, J., and Pahio, E. (2007) 'Sterile insect technique and Mediterranean fruit fly (Diptera : tephritidae): Assessing the utility of aromatherapy in a Hawaiian coffee field' *J. Econ. Entomol.* **100***,* 273-282.

Shelton, A., Zhao, J., and Roush, R. (2002) 'Economic, ecological, food safety,and social consequences of the deployment of *Bt* transgenic plants' *Annu. Rev. Entomol*. **47***,* 856-881.

Shelton, A.M., Tang, J.D., Roush, R.T., Metz, T.D., and Earle, E.D. (2000) 'Field tests on managing resistance to *Bt*-engineered plants' *Nat. Biotechnol.* **18***,* 339-342.

Shukla, J.N., and Nagaraju, J. (2010) 'Two female-specific DSX proteins are encoded by the sex-specific transcripts of dsx, and are required for female sexual differentiation in two wild silkmoth species, *Antheraea assama* and *Antheraea mylitta* (Lepidoptera, Saturniidae)' *Insect Biochem. Mol. Biol*. **40***,* 672-682.

Simmons, G.S., McKemey, A.R., Morrison, N.I., O'Connell, S., Tabashnik, B.E., Claus, J., Fu, G., Tang, G., Sledge, M., Walker, A.S.*, et al.* (2011 'Field Performance of a Genetically Engineered Strain of Pink Bollworm' *PLoS ONE* **6***,* e24110.

Sinkins, S.P., and Gould, F. (2006) 'Gene drive systems for insect disease vectors' *Nature Rev. Genet.* **7***,* 427-435.

Smith, D.B., and Sears, M.K. (1982) 'Evidence for Dispersal of Diamondback Moth, *Plutella xylostella* (Lepidoptera, Plutellidae), into Southern Ontario' *Proc. Entomol. Soc. Ont.* **113***,* 21-27.

Stebbins, M.J., Urlinger, S., Byrne, G., Bello, B., Hillen, W., and Yin, J.C.P. (2001) 'Tetracycline-inducible systems for Drosophila' *Proc. Natl. Acad. Sci. U. S. A.* **98***,* 10775-10780.

Stiling, P. (1988) 'Density-Dependent Processes and Key Factors in Insect Populations' *J. Anim. Ecol*. **57***,* 581-593.

Stodola, T.J., Andow, D.A., Hyden, A.R., Hinton, J.L., Roark, J.J., Buschman, L.L., Porter, P., and Cronholm, G.B. (2006) 'Frequency of resistance to *Bacillus thuringiensis* toxin Cry1Ab in southern United States corn belt population of European corn borer (Lepidoptera : Crambidae)' *J. Econ. Entomol.* **99***,* 502-507.

Stringer, L.D., Sullivan, N.J., Sullivan, T.E.S., Mitchell, V.J., Manning, L.M., Mas, F., Hood-Nowotny, R.C., and Suckling, D.M. (2013) 'Attractiveness and competitiveness of irradiated light brown apple moths' *Entomol. Exp. Appl.* **148***,* 203-212.

Strunnikov, V. (1975) 'Sex Control in Silkworms' *Nature* **255***,* 111-113.

Subedi, A., Macurak, M., Gee, S.T., Monge, E., Goll, M.G., Potter, C.J., Parsons, M.J., and Halpern, M.E. (2014) 'Adoption of the Q transcriptional regulatory system for zebrafish transgenesis' *Methods* **66***,* 433-440.

Suckling, D.M., Hackett, J.K., Barrington, A.M., and Daly, J.M. (2002) 'Sterilisation of painted apple moth *Teia anartoides* (Lepidoptera: Lymantriidae) by irradiation' *NZPP* **55** 7-11.

Sugimoto, T.N., Fujii, T., Kayukawa, T., Sakamoto, H., and Ishikawa, Y. (2010) 'Expression of a *doublesex* homologue is altered in sexual mosaics of *Ostrinia scapulalis* moths infected with Wolbachia' *Insect Biochem. Mol. Biol.* **40***,* 847-854.

Sutrisno, N., (1998) *Potential use of F1 sterility and the parasitoid Cotesia plutellae to control diamondback moth Plutella xylostella (Lepidoptera: Plutellidae)* in Proceedings of FAO/IAEA Final Research Coordination Meeting, Evaluation of Population Suppression by Irradiated Lepidoptera and Their Progeny (Penang, Malaysia).

Sutrisno, S., and Hoedaya, M. (1993) *The sterile insect technique and transmission of inherited sterility to control the diamondback moth, Plutella xylostella (L.), and the cabbage webworm, Crocidolomia binotallis Zell*. in Proceedings of FAO/IAEA International Conference on Area-Wide Control of Insect Pests integrating the Sterile Insect and related nuclear and other techniques (Vienna, Austria).

Suzuki, M.G., Ohbayashi, F., Mita, K., and Shimada, T. (2001) 'The mechanism of sex-specific splicing at the *doublesex* gene is different between *Drosophila melanogaster* and *Bombyx mori*' *Insect Biochem. Mol. Biol.* **31***,* 1201-1211.

Suzuki, M.G., Imanishi, S., Dohmae, N., Nishimura, T., Shimada, T., and Matsumoto, S. (2008) 'Establishment of a novel in vivo sex-specific splicing assay system to identify a trans-acting factor that negatively regulates splicing of *Bombyx mori dsx* female exons' *Mol. Cell. Biol*. **28***,* 333-343.

Tabashnik, B.E., Brevault, T., and Carriere, Y. (2013) 'Insect resistance to *Bt* crops: lessons from the first billion acres' *Nat. Biotechnol.* **31***,* 510-521.

Tabashnik, B.E., Gassmann, A.J., Crowder, D.W., and Carriere, Y. (2008) 'Insect resistance to *Bt* crops: evidence versus theory' *Nat. Biotechnol.* **26***,* 199-202.

Tabashnik, B.E., and Gould, F. (2012) 'Delaying Corn Rootworm Resistance to *Bt* Corn' *J. Econ. Entomol.* **105***,* 767-776.

Tabashnik, B.E., Sisterson, M.S., Ellsworth, P.C., Dennehy, T.J., Antilla, L., Liesner, L., Whitlow, M., Staten, R.T., Fabrick, J.A., Unnithan, G.C.*, et al.* (2010) 'Suppressing resistance to Bt cotton with sterile insect releases' *Nat. Biotechnol.* **28***,* 1304-U119.

Talekar, N.S., and Shelton, A.M. (1993) 'Biology, ecology, and management of the diamondback moth' *Annu. Rev. Entomol.* **38***,* 275-301.

Tan, A., Fu, G., Jin, L., Guo, Q., Li, Z., Niu, B., Meng, Z., Morrison, N.I., Alphey, L., and Huang, Y. (2013) 'Transgene-based, female-specific lethality system for genetic sexing of the silkworm, *Bombyx mori*' *Proc. Natl. Acad. Sci. U. S. A.* **110***,* 6766-6770.

Tang, J.D., Collins, H.L., Metz, T.D., Earle, E.D., Zhao, J.Z., Roush, R.T., and Shelton, A.M. (2001) 'Greenhouse tests on resistance management of *Bt* transgenic plants using refuge strategies' *J. Econ. Entomol.* **94**, 240-247.

Tang, J.D., Gilboa, S., Roush, R.T., and Shelton, A.M. (1997) 'Inheritance, Stability, and Lack-of-Fitness Costs of Field Selected Resistance to *Bacillus thuringiensis* in Diamondback Moth (Lepidoptera: Plutellidae) J. Econ. Entomol. **90**, 732-741.

Tao, X., Xue, X., Huang, Y., Chen, X., and Mao, Y. (2012) 'Gossypol-enhanced P450 gene pool contributes to cotton bollworm tolerance to a pyrethroid insecticide' *Mol. Ecol.* **21***,* 4371-4385.

Thomas, D.D., Donnelly, C.A., Wood, R.J., and Alphey, L.S. (2000) 'Insect population control using a dominant, repressible, lethal genetic system' *Science* **287***,* 2474-2476.

Thomas, J.L., Da Rocha, M., Besse, A., Mauchamp, B., and Chavancy, G. (2002) '3xP3-EGFP marker facilitates screening for transgenic silkworm *Bombyx mori* L. from the embryonic stage onwards' *Insect Biochem. Mol. Biol*. **32***,* 247-253.

Thompson, D.G., Solomon, K.R., Wojtaszek, B.F., Edginton, A.N., and Stephenson, G.R. (2006) 'The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities' *Ecol. Appl.* **16***,* 2022-2027.

Thresher, R.E. (2007) *Genetic options for the control of invasive vertebrate pests* in Prospects and constraints in Managing Vertebrate Invasive Species. http://digitalcommons.unl.edu/nwrcinvasive/ Accessed 2014.

Tilman, D. (1998) 'The greening of the green revolution' *Nature* **396***,* 211-212.

Tobin, P.C., Berec, L., and Liebhold, A.M. (2011) 'Exploiting Allee effects for managing biological invasions' *Ecol. Lett*. **14***,* 615-624.

Tothova, A., and Marec, F. (2001) 'Chromosomal principle of radiation-induced F1 sterility in *Ephestia kuehniella* (Lepidoptera: Pyralidae)' *Genome* **44***,* 172-184.

Uchino, K., Sezutsu, H., Imamura, M., Kobayashi, I., Tatematsu, K., Iizuka, T., Yonemura, N., Mita, K., and Tamura, T. (2008) 'Construction of a piggyBac-based enhancer trap system for the analysis of gene function in silkworm *Bombyx mori*' *Insect Biochem. Mol. Biol.* **38***,* 1165-1173.

Underwood, N. (2010) 'Density dependence in insect performance within individual plants: induced resistance to *Spodoptera exigua* in tomato' *Oikos* **119***,* 1993-1999.

Wallace, H. (2014) *GeneWatch UK comment on Animal and Plant Health Inspection Service Docket No. APHIS–2014 0056: Environmental Assessment for the Field Release of Genetically Engineered Diamondback Moths.* http://www.genewatch.org/uploads/f03c6d66a9b354535738483c1c3d49e4/GeneWatch_APHIS_2014_0056_GE_DBM.pdf, Accessed 2014.

Walters, M.L., Staten, R.T., and Roberson, R.C. (2000) *Pink bollworm integrated management using sterile insects under field trial conditions, Imperial Valley California* in Proceedings: Area-Wide control of Fruit flies and Other Insect Pests. (Penang, Malaysia).

Walters, M., Morrison, N.I., Claus, J., Tang, G., Phillips, C.E., Young, R., Zink, R.T., and Alphey, L. (2012) 'Field Longevity of a Fluorescent Protein Marker in an Engineered Strain of the Pink Bollworm, Pectinophora *gossypiella* (Saunders)' *PLoS ONE* **7**, e38547.

Wang, X.G., Duff, J., Keller, M.A., Zalucki, M.P., Liu, S.S., and Bailey, P. (2004) 'Role of *Diadegma semiclausum* (Hymenoptera : Ichneumonidae) in controlling *Plutella xylostella* (Lepidoptera : Plutellidae): Cage exclusion experiments and direct observation' *Biocontrol Sci. Technol*. **14**, 571-586.

Wang, X.P., Fang, Y.L., and Zhang, Z.N. (2005) 'Effect of male and female multiple mating on the fecundity, fertility, and longevity of diamondback moth, *Plutella xylostella* (L.)' *J. Appl. Entomol.* **129**, 39-42.

Wang, X., Chen, X., and Yang, Y. (2012) 'Spatiotemporal control of gene expression by a light-switchable transgene system' *Nat. Methods.* **9**, 266-U64.

Wee, S.L., Suckling, D.M., Burnip, G.M., Hackett, J., Barrington, A., and Pedley, R. (2005) 'Effects of substerilizing doses of gamma radiation on adult longevity and level of inherited sterility in *Teia anartoides* (Lepidoptera : Lymantriidae)' *J. Econ. Entomol.* **98**, 732-738.

Wei, X., Potter, C.J., Luo, L., and Shen, K. (2012) 'Controlling gene expression with the Q repressible binary expression system in *Caenorhabditis elegans*' *Nat. Methods* **9**, 391-395.

Whalon, M. (2008) *Global pesticide resistance in arthropods* (Wallingford: CABI).

White, A.L., and Boutin, C. (2007)' Herbicidal effects on nontarget vegetation: Investigating the limitations of current pesticide registration guidelines' *Environ. Toxicol. Chem.* **26**, 2634-2643.

White, S.M., Rohani, P., and Sait, S.M. (2010) 'Modelling pulsed releases for sterile insect techniques: fitness costs of sterile and transgenic males and the effects on mosquito dynamics' *J. Appl. Ecol*. **47**, 1329-1339.

Whitten, M., and Mahon, R. (2005) *Misconceptions and Constraints* In Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management, Dyck, V. A., Hendrichs, J. and Robinson, A. S. eds., (Springer) pp. 601-626.

Wimmer, E.A. (2003) 'Applications of insect transgenesis' *Nature Rev. Genet.* **4**, 225-232.

Windbichler, N., Papathanos, P.A., and Crisanti, A. (2008) 'Targeting the X Chromosome during Spermatogenesis Induces Y Chromosome Transmission Ratio Distortion and Early Dominant Embryo Lethality in *Anopheles gambiae*' *PLoS Genetics* **4**, e1000291.

Woodruff, R.C. (1992) 'Transposable DNA Elements and Life-History Traits .1. Transposition of P-Dna Elements in Somatic-Cells Reduces the Life-Span of *Drosophila melanogaster*' *Genetica* **86***,* 143-154.

Woodruff, R.C., Thompson, J.N., Barker, J.S.F., and Huai, H. (1999) 'Transposable DNA elements and life history traits: II. Transposition of P DNA elements in somatic cells reduces fitness, mating activity, and locomotion of *Drosophila melanogaster*' *Genetica* **107***,* 261-269.

Xu, J., and Wang, Q. (2014) 'Ejaculate economics: an experimental test in a moth' *Biol. Letters* **10***,* 20131031.

Yang, R., Xia, D., Gu, W., and Zhang, Y. (1998) *Field trails in South China to control the diamondback moth, Plutella xylostella (Lepidoptera: Plutellidae) using radiation-induced sterility* in Proceedings of FAO/IAEA Final Research Coordination Meeting, Evaluation of Population Suppression by Irradiated Lepidoptera and Their Progeny (Penang, Malaysia) pp 101-109

Yonemura, N., Tamura, T., Uchino, K., Kobayashi, I., Tatematsu, K., Iizuka, T., Tsubota, T., Sezutsu, H., Muthulakshmi, M., Nagaraju, J., and Kusakabe, T. (2013) 'phiC31-integrase-mediated, site-specific integration of transgenes in the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae)' *Appl. Entomol. Zool.* **48***,* 265-273.

You, M., Yue, Z., He, W., Yang, X., Yang, G., Xie, M., Zhan, D., Baxter, S.W., Vasseur, L., Gurr, G.M.*, et al.* (2013) 'A heterozygous moth genome provides insights into herbivory and detoxification' *Nat. Genet.* **45***,* 220-225.

Zalucki, M.P., Shabbir, A., Silva, R., Adamson, D., Liu Shu-Sheng, and Furlong, M.J. (2012) 'Estimating the Economic Cost of One of the World's Major Insect Pests, *Plutella xylostella* (Lepidoptera: Plutellidae): Just How Long Is a Piece of String?' *J. Econ. Entomol.* **105***,* 1115-1129.

Zervas, G.A., and Economopoulos, A.P. (1982) 'Mating Frequency in Caged Populations of Wild and Artificially Reared (Normal Or Gamma-Sterilized) Olive Fruit-Flies *Dacus oleae* (gmelin) (Diptera, Tephritidae)' *Environ. Entomol.* **11***,* 17-20.

Zhao, J.Z., Cao, J., Collins, H.L., Bates, S.L., Roush, R.T., Earle, E.D., and Shelton, A.M. (2005) 'Concurrent use of transgenic plants expressing a single and two *Bacillus thuringiensis* genes speeds insect adaptation to pyramided plants' *Proc. Natl. Acad. Sci. U. S. A.* **102***,* 8426-8430.

Zhao, J.Z., Cao, J., Li, Y.X., Collins, H.L., Roush, R.T., Earle, E.D., and Shelton, A.M. (2003) 'Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution' *Nat. Biotechnol.* **21***,* 1493-1497.

Zygouridis, N.E., Argov, Y., Nemny-Lavy, E., Augustinos, A.A., Nestel, D., and Mathiopoulos, K.D. (2014) 'Genetic changes during laboratory domestication of an olive fly SIT strain' *J. Appl. Entomol.* **138***,* 423-432.