Investigating Novel Methods of Insect Control

Stewart Leigh

A thesis submitted to the University of East Anglia for the degree of Doctor of Philosophy

School of Biological Sciences

University of East Anglia

Norwich, UK

October 2019

This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with the author and that use of any information derived there from must be in accordance with current UK Copyright Law. In addition, any quotation or extract must include full attribution.

Abstract

Over half of the world's population is at risk of contracting potentially fatal arboviruses such as dengue or Zika. Treatment of many of the diseases caused by these viruses is limited by the absence and lack of availability of vaccines or cures, prompting strategies to control the viral vectors and prevent disease transmission to humans. In this thesis I investigated two potential vector control strategies – "satyrization" and "genetic underdominance" to provide proof of principle. Satyrization is a form of reproductive interference whereby unidirectional hybrid mating frequency and asymmetric post-mating effects from seminal fluid protein (Sfp) transfer between species can contribute to the competitive exclusion of one species by another. Satyrization has been documented in nature whereby the dengue vector *Aedes aegypti* has been shown to suffer population reduction when in sympatry with the less virulent vector *Ae. albopictus*. In this thesis I investigated the underlying mechanics and drivers of satyrization in a series of experiments conducted within closely related members of the *Drosophila melanogaster* species subgroup. This confirmed the widespread presence of satyrization and demonstrated a potential link between hybrid mating and asymmetric effects of Sfps. I also found that resistance to satyrization did not quickly evolve between *D. melanogaster* and *D. simulans* as active satyrization was still present between the species after 12 generations in sympatry, in contrast to what has been reported between *Aedes* mosquitoes. My underdominance research focussed on a "killer-rescue" strategy. This comprises a lethal *Minute* phenotype caused by knockouts (KO) of key haploinsufficient Ribosomal protein (*Rp*) genes, and a transgenic *Rescue* gene on a separate chromosome that contains functional copies of the KO *Rp* genes to nullify the costly effects of the *Minute* phenotype. This allows the lethal KO *Rp* alleles to spread through a population for multiple generations. I developed and built this *Rescue* gene in *D. melanogaster* and quantified the fitness costs associated with *Minute*, to then theoretically model and simulate the spread of an underdominant release. My model showed that a single 1:1 release of this underdominant killer-rescue system could provide insect control for up to 20 generations. However, the *Rescue* construct in the form that I developed requires further development before it is fit for purpose as it would not express effectively in female carriers, and very few males carried the transgene. This informed the next steps required for redesigning the *Rescue* constructs. Overall my thesis research produced a promising evaluation of the potential use of satyrization and genetic underdominance systems for insect control, and opened up important new avenues for further study.

Access Condition and Agreement

Each deposit in UEA Digital Repository is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of the Data Collections is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form. You must obtain permission from the copyright holder, usually the author, for any other use. Exceptions only apply where a deposit may be explicitly provided under a stated licence, such as a Creative Commons licence or Open Government licence.

Electronic or print copies may not be offered, whether for sale or otherwise to anyone, unless explicitly stated under a Creative Commons or Open Government license. Unauthorised reproduction, editing or reformatting for resale purposes is explicitly prohibited (except where approved by the copyright holder themselves) and UEA reserves the right to take immediate 'take down' action on behalf of the copyright and/or rights holder if this Access condition of the UEA Digital Repository is breached. Any material in this database has been supplied on the understanding that it is copyright material and that no quotation from the material may be published without proper acknowledgement.

Table of Contents

Abstract **2**

Table of Contents **3**

List of Tables **6**

List of Figures **9**

Acknowledgments **17**

Chapter 1 - Genetic Insect Control: Current Use and Future Prospects **19**

1.1 Abstract 20

1.2 Introduction and Rationale for the Development of New Genetic Control Methods 21

1.3 Insect Control via Satyrization 31

1.4 Control via Gene Drive through Underdominance 36

1.5 *Wolbachia*-Induced Cytoplasmic Incompatibility for Control 41

1.6 Gene Drive or Population Suppression through Gene Editing using the CRISPR-Cas9 system 45

1.7 Discussion 49

1.8 Thesis Roadmap 51

1.9 Conclusion 52

Chapter 2 - Evidence of Reproductive Interference between *D. melanogaster* and *D. simulans* **53**

2.1 Abstract 54

2.2 Introduction 55

2.3 Methods 57

2.4 Results 61

2.5 Discussion 78

2.6 Conclusions 84

Chapter 3 - Quantifying the Effect of Heterospecific Seminal Fluid Proteins on Post-Mating Responses within the *D. melanogaster* Species Subgroup **86**

3.1 Abstract 87

3.2 Introduction 88

3.3 Methods 92

3.4 Results 95

3.5 Discussion 101

3.6 Conclusions 107

Chapter 4 - Testing for Pre-Mating and Post-Mating Resistance to Satyrization via Experimental Evolution 109

4.1 Abstract 110

4.2 Introduction 111

4.3 Methods 114

4.4 Results 119

4.5 Discussion 127

4.6 Conclusions 132

Chapter 5 - Design and Construction of an Underdominant Killer-Rescue Insect Control Strategy in *D. melanogaster* 134

5.1 Abstract 135

5.2 Introduction 136

5.3 Development of the Underdominant Killer-Rescue Strategy in *D. melanogaster* 141

5.4 Discussion 155

5.5 Conclusion 157

Chapter 6 - Simulating the Release of an Underdominant Killer-Rescue Strategy for Insect Control 158

6.1 Abstract 159

6.2 Introduction 160

6.3 Methods 163

6.4 Results 166

6.5 Discussion 172

6.6 Conclusion 179

Chapter 7 - Conclusions 180

7.1 Introduction 181

7.2 Main Findings and Implications 181

7.3 Future Research 184

7.4 Where does this Work fit into the Broader Field of Control? 187

7.5 Final Conclusions 187

References 189

List of Tables

Table 2.1: Frequency of courtships and copulations (average per female per treatment), mating duration and latency (mins), fecundity and offspring of conspecific and reciprocal heterospecific hybrid matings between *D. melanogaster* and *D. simulans*. Mean fecundity and progeny averaged over the 3 post mating vials as well as the individual vial data are shown. Two of the "% Hatched Progeny" entries are >100% due an egg miscount that resulted in more offspring being counted than eggs. 63

Table 2.2: Progeny sex ratios from mated females in conspecific and heterospecific crosses between *D. melanogaster* and *D. simulans*. Data for the progeny in each of the three post mating vials are given. *D. simulans* (♂) x *D. melanogaster* (♀) produced no progeny in Vial 1 and Vial 2 at 25°C or in Vial 1 at 22°C. 71

Table 2.3: Mating latency and duration (mins), fecundity and offspring of conspecific and reciprocal heterospecific first matings and conspecific second matings between *D. melanogaster* and *D. simulans*.. 73

Table 4.1: Number of hybrid matings in the initial baseline mating tests. 50 virgin males and females from each species were placed into each of the four conspecific and heterospecific treatments listed below. Wing clipping was used to identify individuals in each treatment. Spot-checks of mating behaviour were performed every 20 minutes for 3h over three consecutive days and the total frequency of matings presented is the total for this whole assay period. Also shown are the relative proportion of matings observed within each treatment in relation to the total number of matings. 120

Table 4.2: The frequency of heterospecific matings in allopatric and sympatric populations in the experimental evolution regimes. Assay (generation) refers to assay numbers

1-5 conducted in generations 3, 6, 9, 10 and 12, respectively. The number and % (of total matings) of heterospecific matings (*D. melanogaster* (♂) x *D. simulans* (♀) or the reciprocal) are shown. One sympatric treatment was lost early in the experiment (sympatric 1). 122

Table 5.1: Components of the injection mix used to create the transgenic rescue lines of *D. melanogaster* with an insertion of the *Rescue* plasmid. The final volume was made up to 30µl. 144

Table 5.2: Two rounds of *D. melanogaster* microinjection. The first at the Pirbright Institute and the second by the Fly Facility. Date, number of injected *D. melanogaster*, number and % of the G_0 survivors and the number and % of G_0 lines positive for the transgene are shown. 145

Table 5.3: Number of individual *Drosophila* PCR screened for the presence of the transgene. Males and females were screened separately, as denoted below. The plus symbol indicates individuals positive for the transgene, the minus symbol those that were negative for the transgene. The percentage of positives and negative individuals in each sex from each line is shown. 148

Table 5.4: The sex ratio of offspring from parents positive for carrying the *Rescue* gene as confirmed from PCR screening, compared to offspring from parents who are negative for carrying the *Rescue* gene. Shown are the number and percentage of males and females from each parent pair. Also shown are the transgenic line from which each parental pair was derived. 149

Table 5.5: Components and cycling conditions used in the qRT-PCR. Shown are the components used in the qRT-PCR, the concentration, and the quantity of each component that was added. The temperature and time of each step and the number of cycles used in the denaturation, annealing and extension step are also shown. 151

Table 6.1: The relative fitness values of the alleles and genotypes in the model. Shown are the relative fitness costs of carrying each allele, the fitness categories of each genotype, the genotypes that are to be produced in each generation and the relative fitness of each genotype based on the allele fitness. The simulation was run with a range of five fitness costs for carrying the *Rescue* allele, as shown below. 165

Table 6.2: The results of the 24h developmental delay experiment. Progeny production over 10 days since the introduction of *Sb* flies is shown. WT refers to offspring sired from WT parents, *Sb* refers to offspring sired from *Stubble* parents and the vial refers to the vial from which each offspring was counted. Three vials were placed into each mating cage for the adults to lay eggs into. The percentage at the bottom of each table shows the overall percentage of offspring that were from WT and *Sb* parents. 169

List of Figures

Figure 1.1: Sterile insect technique (SIT) in the field. A: Males are irradiated in a laboratory setting to induce germline damage, rendering them sterile (green). B: Sterilized males are released into a population of wildtype mosquitoes. An excess number of released insect is required to compensate for the reduction in competitive fitness caused by the irradiation and by the negative impacts of laboratory adaptation. C: Offspring produced from matings between sterile males and wildtype females are inviable due to the male gametic sterility, reducing the pest population size......... 24

Figure 1.2: The mode of action by HEGs. The target site is cleaved and replaced by the endonuclease and gene of interest. Homology based repair uses this as a template to fix it within the homologous chromosome (adapted from a diagram by Champer et al., 2016). 29

Figure 1.3: A simplified overview of the two-locus underdominant killer-rescue strategy. Mutants are made containing lethal genes (in blue) caused by the knockout (KO) of essential genes and *Rescue* genes (in green) that are composed of functional versions of the KO genes. When released in to the wild, mating with wildtypes creates a variety of lethal and non-lethal genotypes. (A) Offspring that inherit just wildtype alleles are viable and survive. (B) Offspring that inherit the KO alleles without the *Rescue* alleles are inviable. (C) Offspring that inherit the KO alleles with the *Rescue* alleles are viable and competitively fit, allowing KO alleles, the *Rescue* alleles, and cargo genes linked to the *Rescue* gene that express a desired trait to spread to the next generation.. 38

Figure 1.4: *Wolbachia* spread through a population by cytoplasmic incompatibility. *Wolbachia* infected *Aedes* are shown in blue. Females that mate with infected males will suffer embryonic lethality, whereas *Wolbachia* infected females will produce

viable progeny regardless if the male is *Wolbachia* infected or not. All progeny from *Wolbachia* infected females will also be *Wolbachia* infected, quickly spreading *Wolbachia* infection in a population within several generations, as well as any attached *Wolbachia* effects (adapted from a diagram by eliminatedengue.com, 2016) 42

Figure 2.1: Number of courtships observed between conspecific and heterospecific matings between *D. melanogaster* and *D. simulans* at 25°C (top) and 22°C (bottom); crosses shown as male x female. Courtship behaviour was sampled by spot checks conducted every 20 minutes for three hours after lights on over three consecutive days. Shown are box plots (median, 25-75% IQ range and outliers). Sample sizes for each cross are indicated. Different letters indicate statistically significant differences between groups 64

Figure 2.2: Number of copulations observed between conspecific and heterospecific matings between *D. melanogaster* and *D. simulans* at 25°C (top) and 22°C (bottom); crosses shown as male x female. Copulation behaviour was sampled by spot checks conducted every 20 minutes for three hours after lights on over three consecutive days. Different letters indicate statistically significant differences between groups. Boxplots defined as in Figure 2.1. 65

Figure 2.3: Mating latency (mins) between conspecific and heterospecific matings between *D. melanogast*er and *D. simulans* at 25°C (top) and 22°C (bottom); crosses shown as male x female. Mating latency was sampled by observing mating behaviour for three hours after initial introduction of *Drosophila* pairs into the first vial. Different letters indicate statistically significant differences between groups. Boxplots defined as in Figure 2.1. 66

Figure 2.4: Mating duration (mins) observed between conspecific and heterospecific matings between *D. melanogaster* and *D. simulans* at 25°C (top) and 22°C (bottom); crosses

shown as male x female. Mating duration was sampled by observing mating behaviour for three hours after initial introduction of Drosophila pairs into the first vial. Different letters indicate statistically significant differences between groups. Boxplots defined as in Figure 2.1. 68

Figure 2.5: Number of eggs observed between conspecific and heterospecific matings between *D. melanogaster* and *D. simulans* at 25°C (top) and 22°C (bottom); crosses shown as male x female. Matings were inferred either by observation of mating itself or by the presence of offspring in the hybrid mating vials. Different letters indicate statistically significant differences between groups Boxplots defined as in Figure 2.1. 69

Figure 2.6: Progeny number observed between conspecific and heterospecific matings between *D. melanogaster and D. simulans* at 25°C (top) or 22°C (bottom); crosses shown as male x female. Mated pairs are defined as such through either physical observation of mating or the presence of offspring. Different letters indicate statistically significant differences between groups. Boxplots defined as in Figure 2.1 70

Figure 2.7: Mating latency (mins) during the first (FM, white) and second (SM, grey) matings between *D. melanogaster* and *D. simulans* at 25°C (top) and 22°C (bottom); crosses shown as male x female. The x-axis labels represent the groups of the original first mating however all females in the second mating were paired with a conspecific male. Mating latency was sampled by observing mating behaviour for three hours after initial introduction of *Drosophila* pairs into the first vial. Different letters indicate statistically significant differences between groups. Boxplots as defined in Figure 2.1. 75

Figure 2.8: Mating duration (mins) during the first (FM, white) and second (SM, grey) matings between *D. melanogaster* and *D. simulans* at 25°C (top) and 22°C (bottom); crosses

shown as male x female. The x-axis labels represent the groups of the original first mating however all females in the second mating were paired with a conspecific male. Mating duration was sampled by observing mating behaviour for three hours after initial introduction of *Drosophila* pairs into the first vial. Different letters indicate statistically significant differences between groups. Boxplots as defined in Figure 2.1 76

Figure 2.9: Mating incidence and mating latency (mins) observed between conspecific and heterospecific matings of *D. melanogaster* and *D. simulans* that had been treated with tetracycline for seven generations to remove any potential *Wolbachia* infection; crosses shown as male x female. Mating latency was sampled by observing mating behaviour for three hours after initial introduction of *Drosophila* pairs into the first vial. Different letters indicate statistically significant differences between groups. Boxplots defined as in Figure 2.1 77

Figure 3.1: The *D. melanogaster* species subgroup showing all nine known members and their evolutionary relationships. Species in the blue circles were used in this study. Figure modified from Llopart et al., 2005. 91

Figure 3.2: Percentage of females that mated over the 3h remating assay period, 24h following injection with either saline (red), *D. melanogaster* Sfps (blue) or *D. simulans* Sfps (black). Injections were into *D. melanogaster* females (left) and *D. simulans* females (right). Shown in the shaded areas are the 95% confidence intervals for each treatment, asterisks indicate significant differences (p<0.05). 96

Figure 3.3: Percentage of females that mated over the 3h remating assay period 24h following injection with either saline (red), *D. melanogaster* Sfps (blue) or *D. sechellia* Sfps (black). Injections were into *D. melanogaster* females (left) and *D. sechellia* females (right).Shown in the shaded areas are the 95% confidence intervals for each treatment, asterisks indicate significant differences (p<0.05). 97

Figure 3.4: Percentage of females that mated over the 3h remating assay period 24h following injection with either saline (red), *D. melanogaster* Sfps (blue) or *D. teissieri* Sfps (black). Injections were into *D. melanogaster* females (left) and *D. teissieri* females (right). Shown in the shaded areas are the 95% confidence intervals for each treatment, asterisks indicate significant differences (p<0.05). 98

Figure 3.5: Percentage of females that mated over the 3h remating assay period 24h following injection with either saline (red), *D. melanogaster* Sfps (blue) or *D. yakuba* Sfps (black). Injections were into *D. melanogaster* females (left) and *D. yakuba* females (right). Shown in the shaded areas are the 95% confidence intervals for each treatment, asterisks indicate significant differences (p<0.05). 99

Figure 3.6: Percentage of females that mated over the 3h remating assay period 24h following injection with either saline (red), *D. melanogaster* Sfps (blue) or *D. erecta* Sfps (black). Injections were into *D. melanogaster* females (left) and *D. erecta* females (right). Shown in the shaded areas are the 95% confidence intervals for each treatment, asterisks indicate significant differences (p<0.05). 100

Figure 4.1: The frequency of heterospecific matings between *D. melanogaster* and *D. simulans* in sympatric and allopatric populations over 12 generations of experimental evolution. Red circles refer to populations that were maintained in allopatry, blue squares to populations maintained in sympatry, Green “base” triangles refer to the results of the initial baseline mating assay. Also displayed are the regression lines for each treatment showing the change in heterospecific mating frequency across generations. 121

Figure 4.2: Percentage of *D. melanogaster* and *D. simulans* females from the two allopatric treatments that mated over the 3h remating assay period 24h following injection with either saline (red), *D. melanogaster* Sfps (blue) or *D. simulans* Sfps (black).

Shown in the shaded areas are the 95% confidence intervals for each treatment. Asterisks indicate significant differences (p<0.05). 125

Figure 4.3: Percentage of *D. melanogaster* and *D. simulans* females from the two allopatric treatments that mated over the 3h remating assay period 24h following injection with either saline (red), *D. melanogaster* Sfps (blue) or *D. simulans* Sfps (black). Shown in the shaded areas are the 95% confidence intervals for each treatment. Asterisks indicate significant differences (p<0.05). 126

Figure 5.1: The mechanism of the two-locus killer-rescue underdominance strategy for insect control. WT individuals that are homozygous carriers of functional endogenous *Rp* genes mate with individuals homozygous for the *Rp-Killer* and *Rescue* transgene. G_1 offspring are double heterozygotes, all carrying only one functional copy of the *Rp* genes, *Rp-Killer* genes and *Rescue* transgene which nullifies the *Minute* phenotype. G_1 gametes containing *Rp-Killer* genes and no *Rescue* transgene will create inviable or competitively unfit offspring when paired with most other gamete combinations, as shown in the Punnett square. On average, 5/16 of the progeny in this population will die, but 11/16 will suffer no serious deleterious effects, allowing the *Rescue* construct and *Rp-Killer* genes to persist in the population. 10/16 will be viable and will inherit at least one copy of the *Rescue* gene as well as any cargo genes attached to the *Rescue* gene. 140

Figure 5.2: The ribosomal protein gene *Rescue* construct for *D. melanogaster*. It is designed to contain functional but synonymously modified versions of all three target *Rp* genes, including the promoter, CDS and 3'UTR regions (arrows represent 5' & 3' direction of the sense strand for each gene). The construct also contains an attP and a LoxP site for further integration of genetic material such as cargo genes via site-specific recombinase and a HR5-IE1 dsRed2 fluorescent marker for identifying transgenics. The construct was synthesised at Genewiz and cloned into a piggyBac

vector for injection into *D. melanogaster* embryos. Marked with black arrows are the location and orientation of the primers used to screen the dsRed2 gene in identifying carriers of the transgene. Also marked are unique restrictions between each gene of interest for site directed excisions and insertions. 142

Figure 5.3: Colony PCR of the synthesised *Rescue* construct ligated into the pBac-HIS-attP-HR5IE1-dsRed-LoxP vector. Product size was ~800bp, positive samples that were incubated in LB broth are highlighted by the blue arrows. 143

Figure 5.4: Summary results from the qRT-PCR showing the patterns of *Rp* expression detectable from WT *D. melanogaster* and transgenic *D. melanogaster* females containing the *Rescue* plasmid. Primers were designed within and to amplify the CDS of endogenous *RpS19a* and *RpS26* genes and the CDS of the recoded *RpS19a* gene 152

Figure 5.5: Quantitative Real Time PCR (qRT-PCR) amplification plots carried out on TG1 (red), TG2 (yellow), WT1 (light green), WT2 (dark green) cDNA, and a negative control (blue) containing no cDNA. The three graphs show the three different sets of primers used to amplify a different target gene. These primers were designed to amplify (A) Endogenous *RpS26* (B) Endogenous *RpS19a* (C) Transgenic *RpS19a*. WT and TG cDNA is strongly amplified with the endogenous *RpS26* and *RpS19a* (though amplification of TG1 is low when amplified with endogenous *RpS19a* primers) compared to the negative control (A + B). The recoded *RpS19a* primers did not seemingly amplify any of the WT or TG cDNA samples, presenting an amplification profile similar to the negative controls (C) indicating either no expression or very low expression of the *Rescue* gene. 153

Figure 5.6: Quantitative Real Time PCR (qRT-PCR) melt curve plots carried out on TG1 (red), TG2 (yellow), WT1 (light green), WT2 (dark green) cDNA, and a negative control (blue) containing no cDNA. The three graphs show the three different sets of primers

used to amplify a different target gene. These primers were designed to amplify (A) Endogenous *RpS26* (B) Endogenous *RpS19a* (C) Transgenic *RpS19a*. These melt curves again show strong amplification of all DNA samples from primers designed around the endogenous *RpS26* and *RpS19a* genes compared to the negative control (A + B). The recoded *RpS19a* primers did not seemingly amplify any of the WT or TG cDNA samples, presenting an amplification profile similar to the negative controls (C) indicating either no expression or very low expression of the Rescue gene. ... 154

Figure 6.1: Cumulative line graphs showing the rate and percentage of individuals that pupated (A) and eclosed (B) from WT (Black), *RpS21* knockout (Red) and *RpS26* knockout (blue) *D. melanogaster* lines. Pupation and eclosion was measured at set intervals three times a day until all eclosion had ceased. 167

Figure 6.2: Line graph showing the cumulative number of eclosed adults expressing the *Sb* (Red) and the WT (Blue) phenotype when *Sb D. melanogaster* were placed into the population cage 24h (A) and 48h (B) after WT *D. melanogaster*. Eclosed offspring were counted every day for 10 days. 168

Figure 6.3: Simulation of the spread of the *Rp-Killer* (A) and the *Rescue* (B) allele in a controlled 1:1 killer-rescue:WT release (i.e. 50 killer-rescue males and 50 wildtype females). The *Minute* genotypes confer a fitness reduction of 95% compared to WT fitness. The *Rescue* allele has been altered in each simulation to confer a relative fitness reduction of 25% (purple), 20% (blue), 15% (green), 10% (orange) per copy. Each line in the graph is a mean of 20 runs of the simulation. Simulation of the spread of the *Rp-Killer* (C) and the *Rescue* (D) allele in a controlled 1:1 killer-rescue:WT release (i.e. 50 killer-rescue males and 50 wildtype females. The *Minute* genotypes confer a fitness reduction of 95% compared to WT fitness. The *Rescue* allele confers a fitness reduction of 5% (black) per copy. All 20 runs of the simulation are displayed. 171

Acknowledgments

First and foremost I would like to say an impossibly large thank you to my primary supervisor **Professor Tracey Chapman** for her inexhaustible supply of advice, patience and support throughout my PhD. Tracey has always made time for me for the entirety of the project no matter how busy she was and I'll always be grateful for her guidance.

I would also like to say a massive thank you to my other supervisors **Prof. Luke Alphey** and **Dr. Martin Taylor**, for their time, advice, and direction of my project. To **Dr. Lewis Spurgin** who allowed me to use and modify his simulation model for my work in Chapter 6 and spent many hours talking me through how it worked and how to fix any problems I encountered while using it. And to **Dr. Sanjay Basu**, who looked after me at The Pirbright Institute, and despite his busy schedule was always there to teach me new techniques and give general guidance.

I would like to give thanks to NERC EnvEast for their funding and giving me the opportunity to come to UEA and carry out this work. They have organised wonderful cohort retreats during my time here which has taught me a lot about life in science and allowed me to get to know many other wonderful people who been incredibly fun to spend time with, in particular **Kate**, **Sarah**, **Kevin**, **Duncan**, **Luli**, **Ulysse**, **David**, **Stuart**, **Debbie**, **Alice** and **Oliver**. Additionally I would like to thank my CASE partner The Pirbright Institute for allowing me to visit and carry out key elements of my thesis research, and their financial contribution. I'd also like to thank the Norwich Research Park Seed Corn Fund who provided me with additional funding for my thesis.

I have been bowled over by the welcome and kindness I have received from the members of the Chapman lab who have been incredibly lovely and have given lots of advice, help and

assistance with experiments: **Alice**, **Mike**, **Emily**, **Wayne**, **Nick**, **Aaron**, **Jessy**, **Damian** and **Janet**.

The people at The Pirbright Institute where I was placed for seven months to work on Chapter 5 of this thesis were so welcoming and willing to help me at a moment's notice with my never-ending questions. **Rebecca** and **David,** my housemates, helped me feel at home straight away when moving to Pirbright. Members of the Alphey lab **Phil**, **Ruth, Katharina**, **Jessica M**, **Jessica P**, **Priscilla**, **Matt**, **Tim** and **Victoria** have all been fantastically kind and were always there for providing advice and help with my work. Other Pirbright members **Derek**, **Barry**, **Isobel**, **Tom**, **Phoebe** and **Laura** were great to spend time with and made my time at Pirbright a very enjoyable one.

The School of Biological Sciences at UEA has been a fantastic place to work and I'd like to thank everyone in the department for making my PhD as wonderful an experience as possible. Writing can be long and tedious, but I have been very fortunate to have been doing it in the Fat Hen lab with **Dave M**, **Ryan**, **Sarah**, **Sara**, **Miriam**, **David C** and **Liam.** I would like to also give a special thanks to **Jen**, **Kris**, **Jessie**, **Siobhan**, **Charli**, **Ram**, **James**, **Tom**, **Ellie**, **Ellen**, **Laurie**, **Becky**, and **Jenn** for making Norwich a great place to live and work.

And finally a thank you to my parents **Olive** and **David Leigh** for raising me, for always been there for me and for supporting me in anything and everything. They have always motivated me to go as far as I can and I would never have made it to this stage if it were not for their kindness and for instilling a love of learning that has stayed with me throughout my life.

Chapter 1

Genetic Insect Control: Current Use and Future Prospects

1.1 **Abstract**

Insect vectors are responsible for millions of deaths every year resulting from the transmission of disease. For example, more than half of the world's population is at risk of contracting at least one arbovirus due to the range expansion of prominent disease vectors such as *Aedes* and *Anopheles* mosquitoes. Most of these diseases have no widely available vaccine or cure. Therefore, control of the insect vectors of disease is vitally important. Many techniques are currently in use and working effectively. However, there is a need to ensure currently used strategies remain viable while developing new methods that are more effective while also being sustainable and long lasting. Here I present a review of control methods for reducing populations of insect vectors. I first discuss efficacy and issues associated with insecticide use. I then describe species-specific control methods currently at the vanguard of insect control, some of which are already used in the field, some that are undergoing trials and some that are still under development. The techniques discussed are: Sterile Insect Technique (SIT), Release of Insects carrying a Dominant Lethal (RIDL), satyrization, underdominance, *Wolbachia* and Gene Drives using CRISPR-Cas9. In each case I describe the mechanism of action, the benefits and potential drawbacks of each, and the general direction of future improvements for each strategy. I conclude with a brief summary of the work that I have completed in this thesis and the insect control methods that have formed the focus for my investigations, namely satyrization and underdominance.

1.2 **Introduction and Rationale for the Development of New Genetic Control Methods**

Insect pests are a global problem affecting human livelihoods and health, through damage to economically important crops and via the spread of disease. Along with plant pathogens and weeds, insect pests contribute to the destruction of 40% of crops grown globally for food production, food that would otherwise be sufficient to feed around 3 billion people (Pimentel, 2009). Billions of people around the globe are also at risk of contracting insect-borne diseases with over a million fatalities each year (World Health Organization, 2014). It is a significant challenge to tackle such insect pests due to their short generation time and high reproductive potential which can maintain insect population sizes at a level that may be difficult to manage in control programmes, as well as their significant evolutionary potential which can allow insect populations to evolve rapid resistance to implemented control strategies.

The focus of this review is on methods to control insects that act as vectors for serious threats to human health. For example, dengue disease infects over 390 million people per annum across the world with many of these infections being severe and resulting in ~25,000 annual deaths (Castillo et al., 2016; Bhatt et al., 2013). Though malaria remains a hugely important problem across the globe, vector-borne diseases such as dengue are of increasing concern due to the rapid increase of cases and the lack of effective treatment. Dengue fever is a viral pandemic in tropical areas across the world, roughly half of the world's population are at risk of infection (Dighe et al., 2019; Zhang et al., 2015). Dengue fever manifests serious symptoms such as shock syndrome and haemorrhagic fever leading to high levels of mortality in many countries (Arauz et al., 2015; Gibson et al., 2013). It is carried and transmitted by *Aedes* mosquitoes, mainly *Aedes aegypti,* and has a range spanning the tropics but with a recent expansion into Europe and the Americas. It is likely that the range of *Aedes* will gradually

expand further due to climactic shifts and, as there is neither a widely available vaccine nor cure for dengue fever, there is a pressing need for additional *Ae. aegypti* control mechanisms (Dighe et al., 2019; Kraemer et al., 2015).

To date, *Aedes* control has been tackled primarily using population suppression methods. *Aedes* control strategies include: removing areas of standing water (in which mosquitoes breed and their larvae grow), "fogging" whereby an insecticide is sprayed around public areas, or by setting up lethal oviposition traps to lure and kill adult mosquitoes with insecticides. These methods have variable efficacy. Removing standing water around human homes and settlements and setting up oviposition traps may be effective in protecting residents (Suman, 2019). However, such measures generally provide only short-term solutions as the mosquito populations within such areas are not effectively eliminated. There is some evidence that insecticide fogging can be effective in the short term, as part of an integrated pest control strategy to target and kill adults within a local area. However, by itself its reach is not sufficiently comprehensive to eliminate an entire local pest population and the target species is often observed to 'bounce back' within several days (Esu et al., 2010). Fogging is also environmentally damaging and non-species specific – it often unintentionally kills off-target insects such as beneficial pollinators (Abeyasuriya et al., 2017). The evolution of resistance to insecticides is an additional and prevalent problem. *Ae. aegypti* resistance to insecticides such as pyrethroids and organophosphates has been documented in countries across the world such as Nigeria (Ayorinde et al., 2015), Papua New Guinea (Demok et al., 2019), and Singapore, where *Ae. aegypti* average mortality caused by pyrethroids is just 13% in some strains (Koou et al., 2014). For these reasons, additional and complimentary methods of insect control are required that are cheap, environmentally benign, that provide strong species-specificity, and have fewer negative off-target effects.

Genetic strategies for insect vector control can be divided into two broad categories, those relying on population suppression (e.g. the Sterile Insect Technique (SIT), Release of Insects carrying a Dominant Lethal (RIDL), some applications of underdominance and gene drives) versus trait or population replacement (e.g. Cytoplasmic Incompatibility (CI), satyrization, other applications of gene drives and underdominance). Population suppression aims to eliminate or severely reduce the size of a local pest population, whereas replacement aims to control insects through the replacement of one species or population with a less harmful one. For example, satyrization can be used to replace a disease vector species with a closely related but less virulent vector species, or gene drives can be used to create and release a mutant strain of a target vector containing genes that prevent virulence, and spread these to wild populations.

Species-Specific Population Suppression

SIT

The best known of these genetic methods is SIT (Knipling, 1955), in which 'sterile' males are mass reared and released to mate with wild females and reduce the population size (Figure 1.1). Sterility is usually achieved by irradiating pupae with X-rays or ionising radiation, with the aim of disrupting the rapidly-dividing male germ line. Offspring that inherit the damaged germ cells from the father are inviable. SIT is species-specific in its direct effects as its targeting relies on the mate recognition system of the released male. This eliminates concerns regarding direct off-target effects, though there may be unintentional damage to the ecosystem from altering the population dynamics of an established species. SIT has shown to be successful for eliminating insect pests such as Tsetse fly in Zanzibar (Vreysen et al., 2000), Mediterranean fruit fly in Hawaii (McInnis et al., 1994), and New World screwworm in North America (Wyss, 2000). SIT has also been successful for eliminating *Aedes* mosquitoes in Reunion Island (Oliva et al., 2012) and regions of Italy (Bellini et al., 2013).

Classically, the main drawback associated with SIT is the somatic tissue damage that arises as a consequence of irradiation. This generally reduces the performance of SIT males, putting

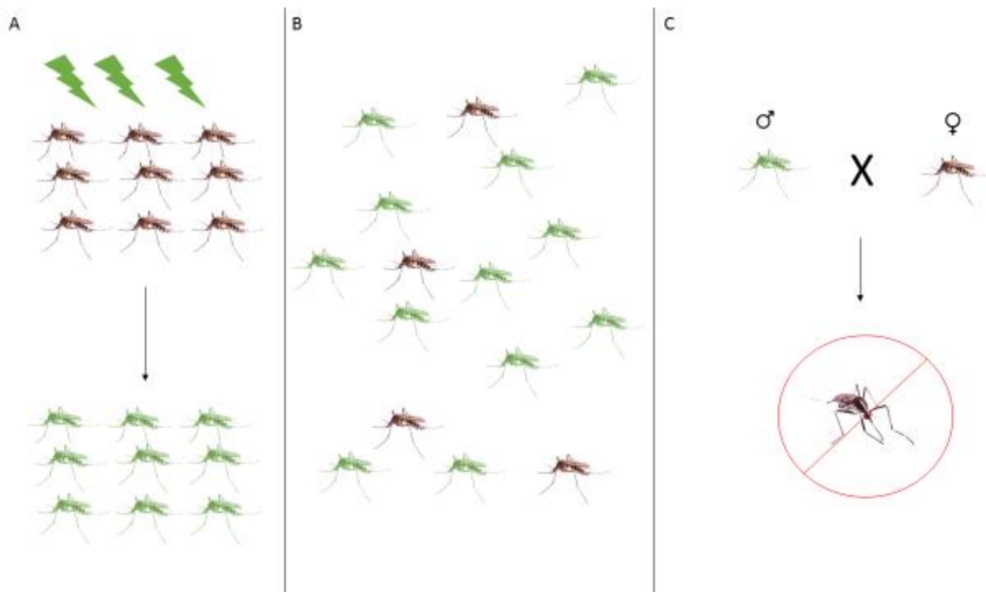


Figure 1.1: Sterile insect technique (SIT) in the field. **A**: Males are irradiated in a laboratory setting to induce germline damage, rendering them sterile (green). **B**: Sterilized males are released into a population of wildtype mosquitoes. An excess number of released insect is required to compensate for the reduction in competitive fitness caused by the irradiation and by the negative impacts of laboratory adaptation. **C**: Offspring produced from matings between sterile males and wildtype females are inviable due to the male gametic sterility, reducing the pest population size.

them at a competitive disadvantage compared to wildtype (WT) males (Rodriguez et al., 2013; Oliva, 2013). Irradiation dosage can be reduced to enhance SIT male fitness, but this comes with the danger of incomplete sterility, allowing some viable progeny to develop from matings between SIT males and WT females (Mastrangelo et al., 2018).

Release strategies of SIT must be considered for maximum efficiency and minimum danger to the public. Bi-sex releases, whereby both sterile males and sterile females are released into target populations are simpler to rear in the laboratory or factory but are less effective than releases of exclusively sterile males, due to some sterile males mating with sterile

females rather than target females. This is evidenced from field releases in Guatemala which showed that male only releases of medfly are 3-5 times more effective for control than bi-sex programmes (Rendón et al., 2004). Releasing female mosquitoes into wild populations is also undesirable as even sterilized females may be able to bite and transmit disease, potentially increasing disease incidence for a short time (Alphey et al., 2013).

Sexing for male-only releases has historically been laborious. The most common method has been to sex based on pupae size (male pupae are smaller than female pupae) but individual variation results in some size overlap between sexes (Papathanos et al., 2009) and therefore this method of sexing is inefficient. Attempts to find new methods to improve the resolution of sex sorting have been partially successful. For example, studies investigating feeding SIT mosquitoes with toxic blood (blood containing insecticides so that it is lethal to ingest) have shown that toxic blood can kill the majority of females in a cage, providing some sex sorting potential, though some males also die through coming into contact with the toxic blood (Lowe et al., 1981). More recently there have been developments of high-throughput methods, such as by using sex-specific fluorescent markers which do not impact mating fitness. This allows for the quick and easy separation of males from females at an early larval stage. However, the instruments used for high-throughput screening are currently very expensive (Marois et al., 2012) and can cost tens if not hundreds of thousands of dollars.

Unfortunately, mosquitoes seem particularly radiation-sensitive, rendering them relatively intractable to control by SIT (Helinski et al., 2009). Advances in radiation dosage and targeting in recent years have achieved some success in preventing somatic tissue damage in laboratory and field experiments (Munhenga et al., 2016; Ageep et al., 2014; Madakacherry et al., 2014). However despite these advances, 1:1 fitness of SIT and WT males has not yet been achieved (partly due to additional fitness costs arising from laboratory adaptation) and a large excess of SIT individuals must always be sterilized and released to 'flush out' and

compete with WT males. The degree of 'overflooding' required has obvious cost, infrastructure and efficiency implications. SIT is not a self-sustaining strategy and requires multiple, successive releases of SIT insects over a programme period. Collectively, these disadvantages can make SIT a costly control strategy for pests such as mosquitoes.

RIDL

Next-generation SIT-based methods, such as the development of the Release of Insects with a Dominant Lethal (RIDL) have been successful in alleviating some of the potential drawbacks associated with SIT. RIDL works by introducing a dominant lethal gene through a population via released insects. The viability of individuals carrying this lethal gene is maintained in the presence of a dietary component that is freely available in reared laboratory conditions but not in the wild. Thus offspring that inherit the gene in the wild are killed (Thomas et al., 2000). An example is the tet-off tTAV (tetracycline-repressible Transcriptional ActiVator) system in which individuals carry a lethal transgene construct containing a tetO promoter that drives the expression of a tTAV gene. tTAV expresses a toxic protein that is lethal in high doses. The toxic tTAV protein binds to the tetO promoter to drive further tTAV protein expression in a positive feedback loop. When constitutive tTAV expression reaches high enough levels, the toxicity of the tTAV protein will kill carriers of the lethal transgene. Tetracycline breaks this loop as it binds to the tTAV protein, preventing the tTAV protein from binding to tetO and therefore repressing further tTAV expression. Thus, RIDL individuals that are reared on tetracycline are viable. Using this, RIDL males can be reared in the laboratory on diets containing tetracycline then released into the wild to mate. Offspring from these crosses inherit the tetracycline-repressible lethal gene and die as there is no tetracycline in the wild, which removes the repressive effect and allows the expression and toxic build-up of the tTAV protein (Phuc et al., 2007). RIDL males do not require irradiation and thus avoid somatic damage arising from this source prior to release. This means that the mating performance of

RIDL males may be less compromised than standard SIT males and more comparable to that of WT males (Massonnet-Bruneel et al., 2013). RIDL has been successfully used for reducing *Ae. aegypti* populations in field trials. For example, sustained releases of RIDL *Ae. aegypti* males for a year in an urban area of Brazil Islands reduced wild *Ae. aegypti* populations by up to 95% (Carvalho et al., 2015).

RIDL has shown to be effective but has limitations. As with other 'sterile-male' methods, sustained effective control requires repeated releases over a long period of time. As for other control strategies, RIDL could also become subject to evolved resistance, e.g. via mutations in functional RIDL genes, decreasing RIDL effectiveness (Alphey et al., 2011). Additionally, RIDL is a genetically modified (GM) based method of control that requires regulatory permission prior to implementation. The appropriate permissions may be opposed or take time to be granted before they can be widely used (Black et al., 2011).

Species-Specific Population Replacement

Gene Drives - HEG

An alternative approach to population suppression is 'population replacement' whereby a target population is replaced with another population. This can take the form of a genetically modified or pathogen infected population of the same species, or another closely related species entirely that fulfils a similar niche while being less harmful to human health.

The definition of a gene drive according to the National Academy of Science, Engineering and Medicine is: "***A system of biased inheritance in which the ability of a genetic element to pass from a parent to its offspring through sexual reproduction is enhanced***" (National Academies of Sciences, Engineering and Medicine, 2016). The biased inheritance of gene drives can be exploited to push through genes throughout an entire population that produce a desired effect such as lowering fitness for population suppression (Medina et al., 2018), or

making a desired population express a particular phenotype such as disease resistance (Deredec et al., 2008) for population replacement.

One example of this is genetic engineering using a Homing Endonuclease Gene (HEG) drive system (Traver et al., 2009). HEG works by using an endonuclease joined to a desired gene which is designed to excise and replace a target gene within the organisms' genome. Following replacement, homology directed repair uses the new gene as a template to create a copy in the homologous chromosome, permanently establishing it within the genome of the organism (Figure 1.2). HEGs are thus heritable, allowing them to spread within an insect population relatively quickly if there are short generation times and high mating frequency (e.g. as occurs in *Aedes* mosquitoes and the *Drosophila* model system). The aim of a gene drive system would therefore be to create a gene with the power to either kill a proportion of the individuals that carry it, or prevent carriers from being able to transmit disease, while allowing the target gene to sustain and spread throughout a population for multiple generations (Esvelt et al., 2014). A concern with population replacement methods is the possibility of inserted transgenes genes escaping outside their release context (Marshall, 2009). Hence, regulatory and ethical concerns have prompted safeguarding in gene drive systems to make them reversible or self-limiting (Reed et al., 2018; Goldim, 2015; Quétier, 2015).

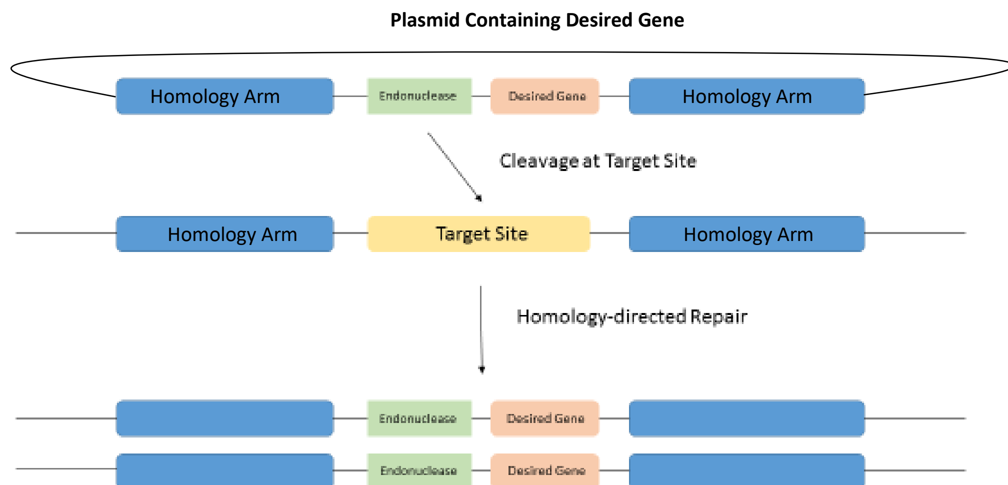


Figure 1.2: The mode of action by HEGs. The target site is cleaved and replaced by the endonuclease and gene of interest. Homology based repair uses this as a template to fix it within the homologous chromosome (adapted from a diagram by Champer et al., 2016).

Gene Drives - CRISPR-Cas9

The development of CRISPR-Cas9 for gene editing in the last few years have made gene drives easier than ever to develop and applications have moved towards engineering gene drive systems using the technology (Gantz et al., 2015). CRISPR-Cas9 uses the Cas9 endonuclease to edit genes designed against a guide RNA, directing the Cas9 enzyme where to cut. CRISPR is more efficient and cheaper than other HEG methods, and its specificity allows site-directed cleavage at almost every gene within the genome (Hammond & Galizi, 2017; Belhaj et al., 2015). This specificity allows for fine tuning of genes and for multiple genes to be targeted and modified to provide extra safeguards to be built into the gene drive system that can make it self-limiting or reversible (e.g. adding a small fitness cost to carrying transgenes so that the transgenes will eventually be removed from the gene pool in natural populations).

The brief summary above highlights that there are existing techniques with proven control potential. However, given the ever-shifting nature of agricultural pests, vector-borne diseases and regulatory frameworks, there is a pressing need for additional new techniques that can provide complementary or alternative methods of control by using a diversity of mechanisms that can be applied in a manner predicted to slow the evolution of resistance to control.

Drosophila as a Model Organism

Though the targets of control strategies are pests of medical and agricultural importance, test cases built for proof of principle in other, more experimentally tractable species such as *Drosophila melanogaster*, can be extremely useful. *Drosophila* is an excellent model organism to use in developing existing techniques, and investigating novel ones, due to their short generation time, ease of rearing in a laboratory setting and the extensive genetic toolkits available (Beckingham et al., 2005). *Drosophila* has been of key utility in the early stages of developing and understanding control methods such as RIDL (Alphey, 2002), that was subsequently successfully applied in *Aedes aegypti* in field tests carried out in the Cayman Islands (Harris et al., 2012; Harris et al., 2011). *D. melanogaster* was also involved in early development of CRISPR-Cas9 genome editing in insects which showed that targeted alleles could efficiently be modified using CRISPR-Cas9 and that these modifications were heritable (Gratz et al., 2013). Because of its effective use as a model organism, I used *Drosophila* species, in the work described in the following chapters, to investigate satyrization and genetic underdominance insect control techniques.

There are several methods that could be explored to provide mechanisms for insect control. I discuss a selection of methods at the forefront of vector control technology below.

1.3 **Insect Control via Satyrization**

Closely-related species undergoing divergence are often incompletely reproductively isolated and hence can occasionally mate and hybridize. The incomplete divergence can lead to a form of reproductive interference, a phenomenon whereby the fitness of one species (or population) is adversely affected due to pre-mating or post-mating processes being interrupted by members of another. This can take many different forms across taxa (Gröning & Hochkirch, 2008). In the context of insect control, reproductive interference can reduce the fitness of a species by preventing them from mating properly and is often referred to as satyrization (Ribeiro and Spielman, 1986). Satyrization describes fitness costs arising from hybrid mating, contributing to competitive exclusion and replacement of one species by another closely related species. Satyrization is hypothesised to be due to processes that occur both before and after mating. Pre-mating drivers of satyrization involve incomplete mating barriers between two closely related species, allowing one species to court and mate with another. Post-mating effects and fitness costs can arise due to the actions of Seminal fluid proteins (Sfps) transferred during mating. These proteins are transferred in the ejaculate of the male and induce many changes in the female e.g. stimulating oogenesis and reducing remating receptivity (Wolfner, 1997). These post-mating effects may also occur when the females of one species receive costly Sfps from the male of another. Offspring produced from these hybrid matings are often either inviable or sterile and their production is therefore a total waste of time and energy for the female, drastically lowering her fitness. Seminal fluid proteins that stimulate oogenesis will cause the female to produce more of these evolutionarily null offspring. Rendering the female refractory to further mating will prevent her from mating with a conspecific male and thus prevent the recovery of lost fitness by producing healthy offspring. Because of the fitness costs arising from hybrid mating, satyrization can contribute to interspecific competition between two closely related species where one species can gain a competitive advantage through the imposition of asymmetric

mating costs in the species with which they can hybridize. This can facilitate population replacement of one species by the other (Tripet et al., 2011).

The extent of satyrization in nature is unknown, but some examples are recognised. For example, in crosses between *D. mauritiana* and *D. simulans*, Carracedo et al. (2000) demonstrated that *D. mauritiana* males will readily mate with *D. simulans* females, resulting in infertile male offspring. However, *D. mauritiana* females exert strong mate choice and only mate with conspecifics. Hence the reciprocal costs of hybrid matings are significantly different. A second example comes from *Ae. aegypti* and *Ae. albopictus*. When *Ae. albopictus* males mate with *Ae. aegypti* females, *Ae. aegypti* females become refractory to further mating (Tripet et al., 2011). This mating is unidirectional, *Ae. albopictus* females will not mate with *Ae. aegypti* males and hence suffer no fitness costs through hybrid mating. This asymmetry can lead to competitive exclusion and indeed in locations in which both of these species live in sympatry, populations of *Ae. aegypti* decline, apparently due to significant asymmetric female mating costs, leading to replacement by *Ae. albopictus* (Tripet et al., 2011). Both species are vectors of disease. However, *Ae. aegypti* is a more competent vector of dengue (Alto et al., 2014), Zika (Hugo et al., 2019), and yellow fever (Johnson et al., 2002) than *Ae. albopictus*. Therefore, satyrization between these two species can potentially reduce disease transmission.

Mechanism of Satyrization

Using satyrization as a method for insect vector control involves introducing a population of a benign species into the habitat of the target species or by increasing the population size of a benign species in populations where they already live in sympatry with the target species. The creation of infertile hybrid males, or other fitness costs associated with hybridization for one species but not the other, would reduce the target population over time and replace it with the benign species. Theoretically, population suppression should occur even if hybrid

mating was bi-directional and satyrization costs were exactly equal between two species. However, modelling has shown that the stronger that satyrization asymmetry is (i.e. mating between satyr males and target females is unidirectional and post-mating effects arising from hybrid mating are asymmetrical), the faster that competitive exclusion of the target species by the satyr species can occur and therefore the stronger the control effect is (Kishi & Nakazawa, 2013). Therefore, strategies involving satyrization should aim to introduce satyr species into populations of a target species where strong asymmetry in terms of hybrid mating fitness costs is found.

Using *Ae. aegypti* and *Ae. albopictus* satyrization as an example: *Ae. aegypti* is an important vector of dengue fever. Introducing *Ae. albopictus* into populations of *Ae. aegypti* is predicted to reduce *Ae. aegypti* numbers due to the refractory effect that occurs with hybrid mating, rendering the *Ae. aegypti* females unable to mate and produce viable or fertile progeny.

Replacement of *Ae. aegypti* by *Ae. albopictus* would not eradicate dengue fever, but would result in far fewer human sufferers (Bargielowski et al., 2013). *Ae. albopictus* is itself a vector of arboviruses such as dengue though it is not as competent a vector as *Ae. aegypti* (Alto et al., 2014) which means that the application of additional, complementary strategies to eventually rid the area of *Ae. albopictus* would also be required.

An alternative strategy to bi-sex releases would be to introduce satyr males only. This would be less damaging in the long run as it would not involve the release of females which bite, transmit disease, and lay eggs to sustain the satyr population. However, this would be more labour intensive as the satyr species would not be able to establish itself in the target population and releases would have to be made every generation in order to maintain the hybrid mating pressure.

Benefits and Drawbacks of Satyrization for Insect Control

A benefit of satyrization is its ease and relative freedom from regulations surrounding GM, although there is a danger of invasive spread when releasing non-native species into an area. As for any other strategy, it is also important to consider the likelihood of resistance evolution. Experiments in *Aedes* mosquitoes have shown that satyrization costs are high, facilitating the selection of resistance alleles in *Ae. aegypti* females that are in sympatry with *Ae. albopictus* males (Bargielowski & Lounibos, 2014). However, resistance genes are also costly and are often not maintained outside of sympatry where satyrization is likely to occur (Bargielowski et al., 2019). Therefore, allopatric *Ae. aegypti* populations should be susceptible to satyrization as there will be no selection to maintain satyrization resistance genes at a high frequency in the population. However, once *Ae. albopictus* populations are introduced into *Ae. aegypti*, the rapid resistance to satyrization in sympatry may lower its effectiveness and make it harder for *Ae. albopictus* to competitively exclude *Ae. aegypti*. Hence, an effective release plan for this strategy should be developed to periodically cycle the use of satyrization in a way that can minimise the development of resistance, or use satyrization alongside other insect control methods in order to kill females in a population where resistance to satyrization is selected for (REX Consortium, 2013).

Future Prospects for Satyrization

It would be useful to develop a metric for identifying satyrization targets. Effective satyrization leading to competitive exclusion requires that fitness costs are asymmetric between two different species (Kishi & Nakazawa, 2013) as a result of hybrid mating, either through unidirectional hybrid mating, asymmetric fitness effects of cross species Sfps, or both. Identification that unidirectional hybrid mating occurs could therefore be a good initial indicator for promising satyrization targets. Fitness costs arising from historical hybrid mating between two species may promote resistance to hybrid mating (Bargielowski et al., 2013) or to heterospecific post-mating effects in one species, therefore asymmetries in the

frequency of hybrid mating (i.e. hybrid mating being unidirectional) may be an indicator that some degree of satyrization is active between two species. In Chapters 3 and 4 of this thesis I explore this in greater detail, looking for indicators of satyrization and how quickly resistance to hybrid mating might be evolving.

A potential drawback with satyrization is the health and ecological concern of replacing a target vector species with another. Satyrization works by introducing populations of one species to drive competitive exclusion of a target species. Therefore, in the context of *Aedes* mosquitoes, satyrization would utilize releases of *Ae. albopictus* to competitively exclude and replace populations of *Ae. aegypti*. However, *Ae. albopictus* are themselves vectors of diseases such as dengue and Zika, though they are less competent disease vectors compared to *Ae. aegypti* (Liu et al., 2017; Alto et al., 2014). Satyrization strategies that involve releases of large numbers of a disease vector into an area would be controversial. *Ae. albopictus* may even experience a boost in population numbers from controlled releases and disease transmission may therefore be higher in the immediate days after *Ae. albopictus* releases. Future work to examine how to improve satyrization for field releases may consider male only releases or the development of an engineered strategy (e.g. releases of mutant transgenic *Ae. albopictus* lines that have increased resistance to transmittable disease) that will replace the disease vector with a completely benign species, while minimising public health risks.

As with all insect control methods, recall strategies also need to be designed in case of unforeseen consequences. With regards to satyrization, there is the immediate increased risk of disease arising from increasing mosquito frequency for a short while, though this could be partially alleviated with male only releases (Beech et al., 2009). There is also the risk of unintended ecological effects of replacing one species from another. In the *Aedes* example, *Ae. aegypti* and *Ae. albopictus* inhabit a similar niche. However, it is naïve to suggest that we

comprehensively understand their role in the ecosystem and can predict what the ecological effects of replacing one species with another would be. Therefore, it would be important to develop a plan to revert the population dynamics back to pre-control levels to undo any unintended damage that might occur. This could be done, for example, by adding more *Ae. aegypti* back into satyrization target populations, or by transforming *Ae. albopictus* for release with a gene that would be susceptible to RNAi knockdown as a reversible failsafe.

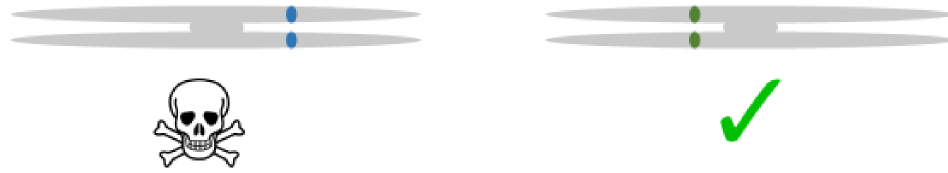
1.4 Control via Gene Drive through Underdominance

Classically, underdominance describes a one-locus phenomenon where heterozygotes at a single locus are less fit than the two homozygotes. Here I will talk about a two-locus form of underdominance, broadly describing a scenario where two different parental strains are homozygous for different alleles at two unlinked loci. Hybrid offspring from matings between these two parental strains produce a variety of hybrid genotypes with combinations of heterozygosity and homozygosity at each of the two loci, each allelic combination being less fit than the two parental strains (Edginton & Alphey, 2018; Reed et al., 2013). The principle of implementing underdominance using gene drives for control populations has been discussed for many years (Huang et al., 2007; Magori & Gould, 2006; Davis et al., 2001; Curtis, 1968). However, it has recently undergone renewed evaluation due to the increased ability to construct strains of model and non-model insects carrying genetic modifications. This is providing new mechanisms by which underdominance control strategies can be implemented.

Mechanism of Underdominance-Based Control

Artificially induced underdominance can be used for population suppression or population replacement. One example is the underdominant killer-rescue strategy (Figure 1.3). This is a two-locus underdominance strategy whereby one or more fitness related alleles are knocked out (KO) that are important for reproductive success and an unlinked *Rescue* gene or genes

(functional copies of the KO alleles created with a slight fitness cost) is inserted onto a separate chromosome to nullify the KO effects. A cargo gene, such as a gene that makes insect vectors refractory to disease so that they cannot transmit disease to humans, can also be linked to the *Rescue* gene. Transgenic homozygotes are released into a WT population, passing on various combinations of KO and *Rescue* alleles to offspring. Offspring that have one or two copies of the KO allele without the sufficient number of *Rescue* alleles to rescue viability will express the costly phenotype and die. The *Rescue* gene, masking the costly effect of the KO alleles, allows both *Rescue* and KO genes to be driven into a population for multiple generations to achieve control before the high cost of the KO alleles causes the KO alleles to decline in frequency and fall out of the gene pool. The *Rescue* allele will initially spread within the target population, spreading the cargo gene with it, as there is a selective advantage for individuals to inherit the *Rescue* allele while the KO alleles are high in frequency in the gene pool. However, the fitness costs associated with the *Rescue* gene forces it to decline in frequency and eventually to be removed from the gene pool. At this stage it no longer becomes advantageous for individuals to carry it in the absence of the KO alleles – this is the property that allows for the underdominant killer-rescue strategy to be self-limiting (Edgington & Alphey, 2018).



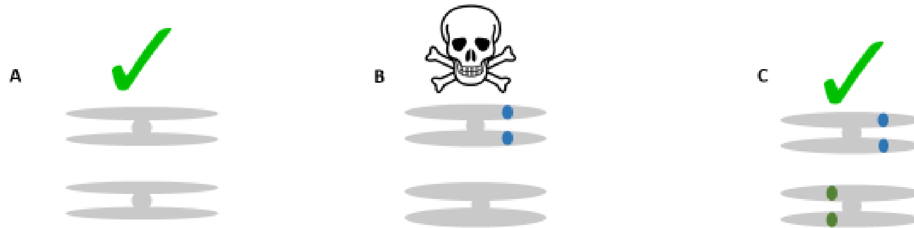


Figure 1.3: A simplified overview of the two-locus underdominant killer-rescue strategy. Mutants are made containing lethal genes (in blue) caused by the knockout (KO) of essential genes and *Rescue* genes (in green) that are composed of functional versions of the KO genes. When released in to the wild, mating with wildtypes creates a variety of lethal and non-lethal genotypes. (A) Offspring that inherit just wildtype alleles are viable and survive. (B) Offspring that inherit the KO alleles without the *Rescue* alleles are inviable. (C) Offspring that inherit the KO alleles with the *Rescue* alleles are viable and competitively fit, allowing KO alleles, the *Rescue* alleles, and cargo genes linked to the *Rescue* gene that express a desired trait to spread to the next generation.

Another example of a two-locus underdominance strategy was devised by Davis et al. in 2001. It consists of two unlinked transgenes, each transgene containing one lethal gene and a suppressor for the complementary lethal gene that is present on the other transgene. In this scenario, individuals that inherit just one of these transgenes will die as the lethal gene will express in the absence of the suppressor on the other transgene which is not inherited. Individuals that inherit both transgenes survive as the linked suppressor for each lethal gene on the complimentary transgene are both inherited, preventing the toxic effects of the lethal genes (Davis et al., 2001). The method is reversible (i.e. by releasing WT individuals into the

population) and also allows for geographic isolation of the control programme as the transgenes can become diluted upon mating with WT individuals, preventing the build-up of sufficient homozygote transgenics that would lead to the fixation of the allele in another location.

Benefits and Drawbacks of Underdominant Control

Fixation of artificial transgenes remains a controversial subject and responsible gene drive strategies aim to be reversible or self-limiting to avoid unforeseen negative consequences of pushing genes to fixation (e.g. transmission of transgenes to off-target species or populations) (Harvey-Samuel et al., 2017). An underdominance system could be reversed by introducing the WT allele back into the population and flushing out the transgenic allele. This reversal means that the technology is relatively safe as there is no risk of transgene escape, or other potential dangers such as causing harm to humans when bitten by an insect carrier of the transgene. Reversibility also means there is a low risk of transgene escape.

One advantage of the underdominant killer-rescue system is that it is self-limiting. The *Rescue* gene would increase in frequency initially while the KO alleles are in the gene pool at high frequency. As the KO alleles decline in frequency, the *Rescue* gene is no longer selected for. Fitness costs associated with carrying the *Rescue* gene itself then results in the *Rescue* gene declining in frequency. This means the underdominant killer-rescue system will not be maintained in the population, producing a fail-safe in case of any adverse effects, though it could be sustained for as long as was required through periodic releases of killer-rescue males.

A potential disadvantage in the killer-rescue strategy is the possibility of resistance through genome duplication, to create another copy of the gene that would function normally, or through upregulation of the *Rescue* allele in heterozygotes, which would negate the fitness costs of the KO alleles.

Future Prospects for Underdominance Control

Much of this work is in the early stages, with constructs currently being designed and transformed into target organisms and simulation modelling conducted to predict the spread of underdominant alleles. However, no field work has yet been carried out to measure the spread of these transgenic alleles or quantify the levels of population reduction as compared to WT populations. This will be crucial to evaluate which underdominance methods are the most effective in killing pest populations for a low cost and ecologically sound strategy.

Some underdominant killer-rescue strategies currently under development target the Ribosomal Protein (*Rp*) genes, as they produce the *Minute* phenotype in their heterozygous state, and are characterised by significant fitness costs due to low fertility and developmental delay (Marygold et al., 2007). Reeves et al. (2014) suggested that the potential of underdominance using *Rp* genes for control could be enhanced through the development of enhanced transgenes e.g. snoRNAs (small nucleolar RNAs). snoRNAs chemically modify RNA molecules, but their contribution to fitness is currently unclear. *RpL14*, an *Rp* gene found in *Aedes* mosquitoes, codes for its own particular snoRNAs which are knocked down along with the gene, causing unknown fitness effects. Quantifying these effects could allow for targeting specific snoRNA knockdowns to attain a more controlled reduction in fitness. This could allow the design of constructs that exhibit lethality at particular life stages, for example, to increase larval competition against WT larvae before lethality at eclosion. It would also allow for the design of more efficient *Rescue* constructs to raise homozygote transgenic fitness further relative to that of the WT.

1.5 ***Wolbachia*-Induced Cytoplasmic Incompatibility for Control**

It is estimated that 66% of all known insect species are infected with some strain of *Wolbachia* (Serbus et al., 2008). Parasitic and intracellular bacteria of the genus *Wolbachia* are of significant fundamental and applied interest because of their widespread distribution and ability to spread through populations due to the property of cytoplasmic incompatibility (CI) (Hoffman et al., 2015). CI causes a modification in the sperm such that an infected male can only successfully fertilize the eggs of an infected female. Infected females can receive and utilise sperm from both infected and uninfected males and can outcompete uninfected females. *Wolbachia* is maternally transmitted, so all offspring from an infected female will also be infected, increasing the frequency of *Wolbachia* infection throughout the population (Figure 1.4) (Sinkins, 2004). Along with cytoplasmic incompatibility, infection of insect hosts with different *Wolbachia* strains confers a variety of fitness effects on the host such as male killing, feminization and lifespan reduction which are useful for population suppression of insect vectors (Hoffman et al., 2015; Hoffman, 2014). *Wolbachia* infection can also increase pathogen resistance in some species such as *Aedes*. The exact mechanism for disease resistance effect is not yet known but CI could be exploited to spread the disease resistance effect throughout a population and prevent that population from picking up viruses such as dengue and Zika virus and transmitting them to humans.

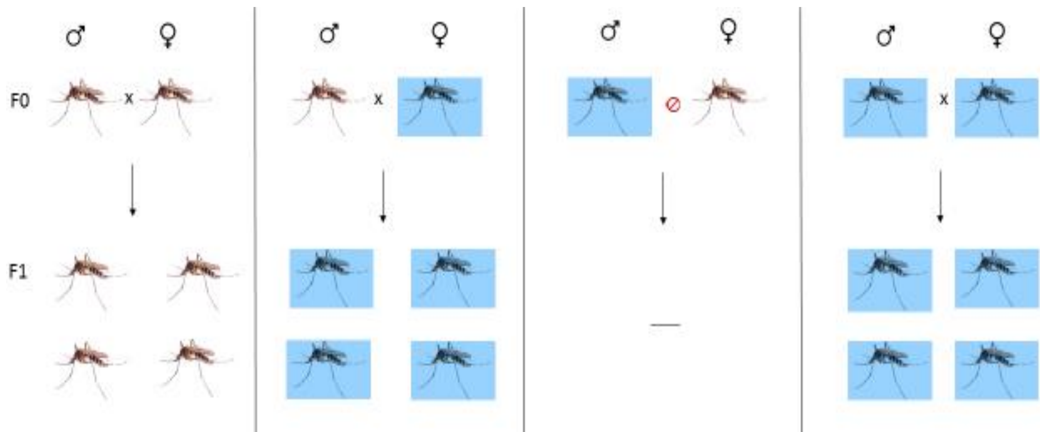


Figure 1.4: *Wolbachia* spread through a population by cytoplasmic incompatibility. *Wolbachia* infected *Aedes* are shown in blue. Females that mate with infected males will suffer embryonic lethality, whereas *Wolbachia* infected females will produce viable progeny regardless if the male is *Wolbachia* infected or not. All progeny from *Wolbachia* infected females will also be *Wolbachia* infected, quickly spreading *Wolbachia* infection in a population within several generations, as well as any attached *Wolbachia* effects (adapted from a diagram by eliminatedengue.com, 2016)

Mechanism of Control via *Wolbachia* Infection

CI can be exploited in a manner similar to SIT in a strategy known at Incompatible Insect Technique (IIT). As previously described, male carriers of *Wolbachia* that mate with non-infected females do not produce viable progeny (Figure 1.4). Therefore, *Wolbachia* infected males can be released into target populations that either carry a different strain of *Wolbachia*, or no *Wolbachia* at all. Progeny produced from matings between released males and target females that contain incompatible strains of *Wolbachia* will be inviable, supressing the target population (Kittayapong, 2018).

Alternatively, the spread of *Wolbachia* through CI can be used to spread desirable traits. A strain of *Wolbachia* called '*w*MelPop' infection upregulates the immune system in *Ae. aegypti* which shortens lifespan and increases resistance to dengue infection (Kambris et al., 2009). *Aedes* adults have a window of 8-12 days from initial infection by dengue virus before

they are able to transmit it to humans. During this period *w*MelPop can be sustained within the population through *Aedes* mating and the shortened lifespan of *Wolbachia*-infected mosquitoes may limit the available window for transmitting dengue (Hugo et al., 2014). This offers the potential for a two-pronged control technique in spreading a self-sustaining suppression of dengue, as CI would be sufficiently effective to rapidly infect populations and sustain itself while increasing dengue resistance and shortening the adult lifespan of *Aedes*.

It is important to note that different *Wolbachia* strains have different effects in different host species. In *Ae. albopictus*, for example, *Wolbachia* can confer some level of resistance to dengue fever, but from a low level of infection within the host. However, the same *Wolbachia* strain in *Ae. aegypti* confers much higher levels of resistance, as the strain infects the host tissues at higher density (Martinez et al., 2015). This may make it difficult to apply *Wolbachia* to multiple vector species in the absence of fine-tuning each *Wolbachia* to each species in question. The '*w*MelPop-CLA' strain is currently considered to have good control potential in *Ae. aegypti* (Ritchie et al., 2015). However, to reduce the impact of evolved resistance, other effective strains should also be sought.

There is no clear explanation for many of the effects of *Wolbachia* on their insect hosts, although there are several possibilities. For example, *Wolbachia* infection upregulates the immune system, which could increase protection against viral infection causing the increase in *Aedes* resistance to dengue infection. There may also be competition between viruses and *Wolbachia* for resources within the host, e.g. for cholesterol (Iturbe-Ormaetxe et al., 2011), shortening host lifespan and fitness. Discovering the molecular basis by which *Wolbachia* interferes with host fitness and virus transmission is of prime importance for control efforts, but is challenging because of the inability to grow *Wolbachia* in culture.

Benefits and Drawbacks of the use of *Wolbachia* for Control

The major benefit of *Wolbachia* infection for control is its ability to spread easily through a population via cytoplasmic incompatibility. This potentially allows the spread of desired phenotypes through specific strains (Bull & Turelli, 2013).

There is some variability regarding the ability of *Wolbachia* to spread through *Aedes* populations. Trials using the '*w*Mel' strain in *Ae. aegypti* have indeed been successful, showing that the fitness costs associated with *Wolbachia* infection are small, which can allow a successful spread of *Wolbachia* throughout localised populations (Walker et al., 2011; Hoffman et al., 2011). Another example of successful *Wolbachia* spread through *Aedes* populations comes from a study by Dutra et al. (2016). In this study, five urban sites in Brazil were chosen to explore the potential for *Wolbachia* gene drive by infecting *w*Mel into *Ae. aegypti*. *Wolbachia* was able to establish itself successfully in all five sites with minimal detrimental side-effects in *Ae. aegypti* from the infection.

However, a counter example by Nguyen et al. (2015) found that fitness costs associated with infection of another *Wolbachia* strain "*w*MelPop-PGYP" were too high to establish *w*MelPop-PGYP in *Aedes* populations in Australia and Vietnam. It is possible that the effectiveness of *Wolbachia* for control could be correlated with the density of the infection within the tissue of the host. Higher density could produce stronger effects though this may also cause higher fitness costs which could make it difficult to spread *Wolbachia* through a population. For example, the disease resistance effects of *Wolbachia* infection may incur large deleterious fitness effects for the host (Martinez et al., 2015; Joshi et al., 2014). It is also reported that infection with some *Wolbachia* strains actually increases host fitness (Brelsfoard & Dobson, 2011). It is likely that not all *Wolbachia* strains will be useful in combating disease vectors and each must be individually assessed for the desired phenotypes they produce and the ability to establish that strain throughout a population. It is likely therefore that fitness costs

are dependent on both the strain of *Wolbachia* used and the species of the host. Future research should examine these dynamics to fully optimise the system.

The introduction of *Wolbachia* into a mosquito population may require raising the baseline population level of mosquitoes in order for *Wolbachia* to be established. In regions where malaria and dengue fever are rife there may be legitimate worries about increasing vector numbers, even temporarily, as this could increase the rate of infection before benefits are evident (Ritchie et al., 2013). Implementing *Wolbachia* as a method of population control therefore requires several regulatory and ethical challenges to be overcome (Hoffman et al., 2015).

Future Prospects for *Wolbachia* Control

As different strains seem to confer different effects in different hosts, it is necessary to optimise individual *Wolbachia* strains for control. In *Ae. aegypti*, the most likely useful *Wolbachia* candidates are those that reduce disease transmission and those that shorten host insect lifespan. Testing the logistics and fitness costs of each strain is needed (Lambrechts et al., 2015). It may even be possible for strains to be combined to induce a superinfection of *Wolbachia* by more than one strain. The advantages of this could be to spread specific *Wolbachia* strains through already infected populations. Infection of hosts by multiple *Wolbachia* strains could also result in beneficial stacking of traits whereby the effects of each strain will manifest itself in the infected host (Watanabe et al., 2011).

1.6 Gene Drive or Population Suppression through Gene Editing using the CRISPR-Cas9 system

Gene editing refers to the modification of DNA in order to change or knockout the function of a particular gene. CRISPR-Cas9 gene editing technology allows for efficient, heritable and

easy gene editing (Ma et al., 2010), and offers many potential avenues for control via both gene drive and population suppression.

Mechanisms of Gene Editing Techniques for Control

The CRISPR-Cas9 system is found in many strains of bacteria (44%) and archaea (95%) (Grissa et al., 2007; Kunin et al., 2007) where it functions to confer effective immune responses against attack by bacteriophages. CRISPR elements (or Clustered Regularly Interspaced Short Palindromic Repeats) are separated by short runs of 'spacer' DNA. This spacer DNA is genetic material taken from bacteriophages during infection and stored within the CRISPR cassette to provide a type of immunological 'memory'. When a bacterium suffers another infection, spacer DNA is transcribed into CRISPR RNA (crRNA) which can be used as a guiding system by a Cas9 enzyme to target the foreign invader, cleave it at a complementary pairing site and destroy it (Horvath & Barrangou, 2010).

This system has been exploited, using an RNA guidance system, to induce precise gene editing that is both easier and more efficient than previous gene editing technologies (Cong et al., 2013). Single guide RNA (sgRNA) can be constructed using crRNA for recognition and trans-CRISPR RNA (tracrRNA) for binding and activating the Cas9 enzyme (Hsu et al., 2014; Chylinski et al., 2013; Jinek et al., 2012). sgRNAs are designed to be complementary to the desired target DNA sequence for modification, Cas9 then binds to this sequence and cleaves it.

CRISPR-Cas9 gene editing can be conducted in two ways: The Non-Homologous End Joining (NHEJ) based technique, which involves cutting a double stranded site, creating indels and knocking out genes due to incorrect repair. The second pathway is the Homology Directed Repair (HDR) technique, which involves a designed DNA template being introduced with the Cas9 enzyme to act as a new template at the cleavage site, exploiting the DNA repair systems

to fix the other strand using the new introduced template and hence overwriting the original gene (Sternberg et al., 2014; Zhang et al., 2014).

In the context of insect control, CRISPR gene editing can be used to knockout, insert or modify a target gene which can then spread through the population via gene drive (Hammond et al., 2016). It has been demonstrated that CRISPR works in mosquito species (Basu et al., 2015; Dong et al., 2015; Kistler et al., 2015), which opens up an exciting new avenue for novel control routes. Many efforts are currently focused on using HDR to drive genes that cause population suppression (Kyrou et al., 2018; Dhole et al., 2018) but HDR could also be used for population replacement techniques. It should be possible to drive through a population a gene that confers resistance to parasites or viruses to reduce transmission risk to humans. NHEJ could also be used to knock out resistance genes in current control methods in order to prolong their effectiveness, or to knock out genes associated with vector competence, to reduce the ability of vectors to carry and transmit disease (Gabrieli et al., 2014).

Benefits and Drawbacks of Gene Editing for Insect Control

Gene editing technology has become widely celebrated due to its accuracy, robustness, ease and low cost (Cong et al., 2013; Mali et al., 2013). As this technology literally rewrites DNA, the changes are heritable, allowing edits made in insect vectors to be sustained indefinitely within a population. This is advantageous, as the release of insects for control is needed only infrequently, reducing costs and labour in comparison to methods such as classical SIT. However, the ability of gene drives to spread quickly through populations brings ethical concerns, such as the potential damaging ecological effects from rapid population decline, unintentional human health effects from being bitten by genetically modified mosquitoes, and the possibility of accidental leakage of gene from a target population into a non-target population due to migration or dispersal (Rode et al., 2019; Mumford, 2012). Current

recommendations specify gene drives must be reversible or self-limiting to minimise these risks (Dhole et al., 2018).

Functionally, a significant concern for applications of CRISPR-Cas9 is the potential for off-target mutagenesis (de Bruin et al., 2015). The sgRNA targets a specific gene through complementary base pairing. However, similar DNA sequences may also be targeted by the Cas9 complex, potentially knocking out or modifying other essential genes (Fu et al., 2013). If such a system were to be used in population control of insect vectors and off-target mutagenesis occurred, the transgenic individuals would be unsuitable for population control. There is evidence that this can be alleviated through controlled dosage of CRISPR-Cas9 and genetic screening to highlight any potential off-targets (Ansai & Kinoshita, 2014; Fu et al., 2013). Furthermore, pleiotropy, the phenomenon whereby genes can affect multiple phenotypic traits, could result in unexpected phenotypic effects when targeting specific genes for knockdown or insertion (Li & Shen, 2019). This may severely impact the effectiveness of a gene editing strategy if pleiotropic effects also mean that the edited population are too unfit to spread the edited gene throughout a population. For example, a gene knockdown that makes an individual more resistant to picking up and transmitting a disease would be useless if it also makes the individual so unfit that the gene cannot effectively spread when released into a wild population.

There may also be constraints about which DNA sites are suitable for modification, potentially limiting target choice. The Cas9 enzyme requires a 2-5nt Protospacer Adjacent Motif (PAM) sequence immediately downstream of the target area in order to function. PAM is an essential part of Cas9 editing as it separates which DNA is to be cleaved from that which is not. Genes of interest that do not contain a PAM site therefore cannot be targeted for gene editing, which may limit the applications of CRISPR to particular genes. Future work may seek to overcome this limitation in order to edit any gene of interest (Wei et al., 2015).

Future Prospects for Control by Gene Editing

The potential for CRISPR is significant for the treatment of human disease and for modifying the genetic code of animal or plant species to combat various pests and diseases. Future developments include the opportunity to create genetic 'trigger' mechanisms, to allow lethal genes to drive through a population without immediately killing hosts bearing the genetic modifications. This could work by pushing two genes through a population using the CRISPR-Cas9 system – one with an inactive version of the lethal gene and another with the activator for that gene linked up to a genetic trigger, perhaps a pheromone detector. After allowing the genes to spread within a population for several generations until it reaches fixation, a specific pheromone could be introduced into the habitat, triggering the activator and turning on the lethal gene, killing large swathes of the population simultaneously. However, there are significant ethical concerns regarding the use of gene editing, especially when using them for gene drives, for example, the risk of propagating genes with unintended side effects through a population (Goldim, 2015).

Regarding the development of gene editing tools in the broader sense, there is also the concern that powerful gene editing technology can reawaken a form of eugenics through non-essential or cosmetic genetic modifications (e.g. choosing a child's eye colour before it is born). As such, the potential for gains through this powerful technology are subject to careful limitation by ethical debate and legislation (Ishii, 2015; Pollack, 2015).

1.7 **Discussion**

The potential control methods described above can be grouped into insect suppression or replacement approaches. SIT and RIDL induce population suppression as they aim to reduce pest numbers directly within populations. Satyrization is primarily a population replacement or substitution method as it aims to introduce a transgenic strain or a new species into a population. *Wolbachia*, underdominance, and gene drives using CRISPR can be either

suppression or substitution depending on how they are used. For example *Wolbachia* can be used for IIT or for spreading resistance against viruses.

There is growing debate about which of insect suppression or replacement offers the most acceptable and effective control potential. Janet Fang (2010) posed the question to biologists and ecologists studying mosquitoes, of whether there would be any significant negative effects if all mosquito species were eradicated, concluding with “probably not.” Mosquitoes have been shown to pollinate (Lahondère et al., 2019) and are prey for many species of fish and birds (Dida et al., 2015; Louca et al., 2009). However, many researchers proposed that any niche left vacant would quickly be filled and, even if not, it was deemed a small price to pay for the eradication of prominent disease vectors. It is also true that the native habitat of *Ae. aegypti* lies in parts of Africa such Kenya and Uganda (Mattingly, 1967). The relatively recent habitat introductions to the Americas and parts of Asia (Bracco et al., 2007; Slosek, 1986) suggest that mosquito elimination in those areas would produce minimal ecological effects as it is unlikely that mature ecological dependencies would yet have evolved. It may even be bioremedial whereby removing a recently established invasive species could be beneficial for the ecosystem.

Whether this is an overly naïve stance, due to the complex nature of ecosystems and the lack of knowledge to accurately judge what would happen if a species was suddenly removed, is not yet clear. There are also significant bioethical concerns regarding what right we might have to eliminate species because they are inconvenient. Practically speaking, we also lack the capability to destroy multiple large populations of rapidly breeding species. Substitution methods may offer a way of editing traits (Ansai & Kinoshita, 2014) and inserting replacement, hereditary genes to render insect disease vectors harmless to humans without removing them (Yi et al., 2014). Since evolutionary resistance is inevitable, it is also necessary to implement new methods in a way that minimizes the risks of resistance. A

recent review suggested that various methods of population control, applied cyclically and in multiple locations could prevent populations from being subject to single selection pressures over time and hence slow resistance evolution (Leftwich et al., 2015). It would be beneficial to test such methods in cage and field trials.

1.8 **Thesis Roadmap**

In my thesis research, I have investigated the existence and form of satyrization in closely related *Drosophila* species as a test case, and explored underdominant killer-rescue based gene drives. I initiated my satyrization study by observing the frequency of hybrid matings between two closely related *Drosophila* species and the fitness costs that arise from these hybrid matings, which can be indicative of satyrization. I then investigated the role of seminal fluid proteins in satyrization within the *D. melanogaster* species subgroup to identify evolutionary drivers and consequences of satyrization. I concluded by setting up populations of two satyrizing species of *Drosophila* in allopatry and sympatry for 12 generations to test whether pre- or post-mating resistance to satyrization evolved as predicted.

For my underdominance investigations, I took a genetic approach to assess whether the underdominant killer-rescue strategy can be used as a method of population control. Together with collaborators from The Pirbright Institute, I developed and constructed an underdominant killer-rescue system in *D. melanogaster* as a test case. This underdominant killer-rescue system includes three targeted ribosomal protein genes (*RpS19a, RpS21* and *RpS26*) and a genetically engineered *Rescue* construct for all 3 *Rp* genes. Specific *Rp* knockouts produce a *Minute* phenotype, characterized by delayed development and a reduction in female fertility, ultimately leaving individuals that express *Minute* competitively inviable. Introducing the transformed lines into WT populations would lead to a proportion of offspring inheriting non-functional copies of *Rp* and no copies of the *Rescue* construct, causing the *Minute* phenotype and resulting in their inviability. Some offspring will receive

KO *Rp* genes and a copy of the *Rescue* construct, allowing them to be competitively viable and spread the KO *Rp* genes to the next generation. I concluded by constructing a parameterised theoretical model of this system, to simulate a controlled release of the underdominant killer-rescue strategy developed. In this, I used parameters that I obtained from experimental data, to monitor the frequency of KO *Rp* alleles, *Rescue* alleles, and WT alleles over multiple generations.

1.9 **Conclusion**

There are multiple effective routes for combating disease vectors. I have discussed them in turn and described the pros and cons of each. The development of insect vector control is continually ongoing and no single strategy is likely to be able to eradicate entire *Aedes* populations in dengue stricken areas or offer a permanent solution, due to the high reproductive value and evolutionary potential of the host to adapt to control methods. There are also important, ongoing debates concerning whether substitution or suppression are the most logistical and ethically sound strategies. The potential for employing complementary methods for control is significant and the correct application of each will undoubtedly aid in reducing the occurrence of mosquito-borne diseases in vulnerable areas.

Chapter 2

Evidence of Reproductive Interference between *D. melanogaster* and *D. simulans*

2.1 **Abstract**

Satyrization is a form of reproductive interference that results in asymmetric fitness costs from reciprocal hybrid matings between two closely related species or between divergent populations within species. It is of increasing interest as a potential method of insect control, to complement existing approaches. Asymmetric fitness costs of hybrid mating can lead to competitive exclusion. Hence, a species could be introduced into the habitat of a target pest with which it can hybridize and control achieved if the asymmetric costs of hybrid matings cause a greater reduction in fitness of the pest species over the introduced species. However, the prevalence of, and mechanisms underlying, asymmetric fitness costs associated with hybrid matings are under-researched. Evidence from mosquitoes highlights a potential role for incompatibility between the actions of the seminal fluid proteins (Sfps) of males and their receptors in females. Here I tested for the presence of asymmetric fitness costs in hybrid matings between two widely used fruit fly models, *Drosophila melanogaster* and *Drosophila simulans,* which hybridize in nature. Despite a wealth of research into the mating of these species and their hybrids, such costs have not previously been tested for. I also tested directly for the involvement of Sfps in mediating any asymmetric costs observed. Males and females of *D. simulans* and *D. melanogaster* were placed in conspecific and heterospecific pairs and mating, courtship and fertility recorded. I found that *D. simulans* females mated significantly more frequently with *D. melanogaster* males than was observed for the reciprocal hybrid cross. *D. simulans* females suffered reduced fitness by producing unfertilised eggs and infertile and inviable progeny as a result of heterospecific matings to *D. melanogaster* males. Receptivity tests conducted 24h after conspecific and heterospecific matings showed that hybrid mating caused *D. simulans* females to significantly reduce their remating receptivity, reducing their opportunity to receive fertile sperm from a conspecific male. This showed that *D. simulans* are susceptible to the refractory post-mating response of *D. melanogaster* Sfps. Together these results provide evidence for satyrization in matings between *D. melanogaster* and *D. simulans.* Overall, *D. simulans* females mated more frequently with *D. melanogaster* males than was true for the reciprocal cross, suffered potential costs in the production of inviable hybrid offspring from hybrid matings, and were less likely to mate again due to the refractory effects of *D. melanogaster* Sfps on *D. simulans* females. The results contribute to a growing realisation of the importance and unappreciated prevalence of satyrization and thus its potential for insect control.

2.2 **Introduction**

Satyrization is a type of reproductive interference that is characterised by asymmetric mating costs resulting from hybrid mating between two closely related species. The result can be a competitive advantage for one species over the other (Ribeiro & Spielman, 1986). The frequency of satyrization in the wild is not well known, nor have the mechanisms underlying it been thoroughly established. However, recent evidence from mosquitoes suggests that Seminal fluid proteins (Sfps) are involved in determining reproductive asymmetries (Tripet et al., 2011). Sfps are a cocktail of proteins that form the non-sperm part of the male ejaculate. They cause a variety of effects in females such as a higher oviposition rate and a reduced receptivity to further mating (Rubinstein & Wolfner, 2013; Chapman & Davies, 2004; Chapman et al., 2003). Approximately 10% of the genes encoding seminal fluid proteins are known to evolve rapidly (Swanson et al., 2001). It has been suggested that such proteins may therefore have unexpected effects when transferred into the female of a different species (Dapper & Wade, 2016).

Previous work on quantifying asymmetric fitness costs between *Drosophila* hybrid pairs suggests that a contributor to satyrization may be the production of infertile or inviable offspring. The fitness costs of producing such hybrids may exert strong selection in sympatric species to evolve pre-mating isolation barriers (Coyne & Orr, 1989). With regards to crosses between *D. simulans* and *D. melanogaster*, Jamart et al. (1995) found that *D. melanogaster* females were incompatible with *D. simulans* males, the cross producing inviable males and a 100% female, infertile progeny. The reciprocal cross is reported to be hard to achieve (Ellis & Carney, 2009). However, in successful hybrid matings that do occur, 100% male progeny are produced (Barbash, 2010). Differences in the frequency of reciprocal hybrid matings have been described, with Sturtevant (1920), Sperlich (1962) and Moulin et al. (2004) all finding that the *D. simulans* (♂) x *D. melanogaster* (♀) cross occurs more readily than the reciprocal.

However, there can also be variability, with Barker (1962) reporting instead that *D. melanogaster* (♂) x *D. simulans* (♀) crosses were more frequent. Variation in the relative frequency of *D. melanogaster* x *D. simulans* reciprocal hybrid matings could be due to age (Moulin et al., 2004), the specific strains used (Barker, 1962), or to the presence of the intracellular symbiont *Wolbachia*. *Wolbachia* produces a spectrum of effects on reproduction across different arthropod species, including feminization and cytoplasmic incompatibility (Stouthamer et al., 1999) some of which can select for mating directionalities. The exact effects of *Wolbachia* infection depend on the host species and *Wolbachia* strain. For example, a strain known as *w*Mel can increase viral resistance in *D. melanogaster* (Teixeira et al., 2008), whereas another strain, *w*Dm*popcorn*, shortens *D. melanogaster* lifespan (Fry et al., 2004). *Wolbachia* infection is thought to be present in at least 30% of laboratory stocks (Clark et al., 2005).

The magnitude of fitness costs arising from *Drosophila* hybrid matings between reciprocal species pairs has not yet been quantified, and this is the omission I address here. As all hybrid progeny are sterile or infertile between *D. melanogaster* and *D. simulans,* the fitness effects primarily arise as opportunity and energetic costs, via 'time out' of the mating pool or through wasted progeny production. I tested for unidirectional mating and asymmetric fitness costs to provide evidence of satyrization between these two species. I investigated mating receptivity and fitness costs arising from reciprocal matings between *D. melanogaster* and *D. simulans* in comparison to conspecific pairings. Fitness costs were measured by quantifying fecundity as an index of reproductive investment and energy expenditure by females arising from the production of hybrid offspring. Hybrid offspring are either inviable or infertile and thus represent an evolutionary dead end. Production of these hybrid offspring represents a potential energy cost for the female. The final step was to test the involvement of Sfps in altering remating receptivity in hybrid matings. Seminal fluid proteins are known to evolve quickly (Sirot et al., 2015) and thus may not function as

expected in females of a different species. A role for Sfps in determining costs of hybridization would be apparent if prior mating with a heterospecific male reduced mating receptivity to the same degree as a prior mating with a conspecific male.

2.3 **Methods**

Overview and Rationale for Experimental Methods

I first conducted two replicate experiments in which I measured the consequences of single pair conspecific and heterospecific matings between *D. melanogaster* and *D. simulans.* I conducted these replicated experiments at 25°C and 22°C, to check that any asymmetric fitness costs observed were not caused by performing experiments at the optimal temperature of one or other species, respectively. 25°C is within the optimal range for *D. melanogaster* and is the temperature under which the stocks have been maintained for many years (Crill et al., 1996; Cohet & David, 1978). However, the optimal temperature of *D. simulans* is more variable and strain-dependent, with many strains having increased fitness at temperatures lower than 25°C (Austin & Moehring, 2013; Morin et al., 1996; Montchamp-Moreau, 1983). The stocks of *D. simulans* I used here had been kept for several years at 22°C. To examine the role of seminal fluid-mediated effects in satyrization, I next compared conspecific mating receptivity in females having initially experienced heterospecific or conspecific matings, both at 25°C and 22°C. Finally, to test for any involvement of *Wolbachia* infection on mating asymmetries, I treated laboratory stocks of *D. melanogaster* and *D. simulans* with tetracycline (e.g. O'Shea & Singh, 2015; Ikeya et al., 2009) for seven generations to cure them of any potential *Wolbachia* infection and then repeated the mating tests to investigate whether the characteristics of hybrid mating had changed.

Sample Collection

All experiments were conducted in a constant temperature (CT) room at 25°C, 60%RH and 12h:12h light:dark photoperiod**.** The experiments tested conspecific and heterospecific reciprocal matings between *D. melanogaster* (Dahomey wild type strain) and *D. simulans* (obtained from the San Diego Stock Center). *D. melanogaster* and *D. simulans* eggs were collected by placing one red grape juice agar plate (550ml H_2O, 25g agar, 300ml red grape juice, 21ml 10% w/v Nipagin solution per batch of medium) into each of the population stock cages for each species. Plates were left for three hours, taken out of the cages, yeast removed and then incubated. After 24 hours, first instar larvae of each species were picked from the plates and placed 100 larvae per vial (75 x 25 mm), containing Sugar Yeast Agar (SYA) medium (30 ml 10% w/v Nipagin solution, 3ml propionic acid, 15g agar, 50g sugar and 100g brewer's yeast per Litre of medium). This procedure standardised the larval development across and within species and minimised any environmentally-induced variation in body size. Virgin adult females and males were collected using ice anaesthesia and separated by sex. The sex segregated flies were then stored, 10 to a vial, for 3-6 days until use in the experiments.

Frequency and Fitness Effects of Hybrid and Conspecific Matings between *D. melanogaster* and *D. simulans* at 25°C

At 3-6 days old, flies were allocated at random to one of the four following experimental treatments: *D. melanogaster* (♂) x *D. simulans* (♀) (N=39)*; D. simulans* (♂) x *D. melanogaster* (♀) (N=40)*; D. melanogaster* (♂) x *D. melanogaster* (♀) (N=40) and *D. simulans* (♂) x *D. simulans* (♀) (N=40). One male and one female from each species were placed into a vial and were observed for three hours, during which spot checks were also performed every 20 minutes to score courtship and copulation frequency. The spot checks of behaviour were then repeated for the same three hours over the following two days. After matings had occurred, the pairs were retained in the vial and transferred to a fresh vial every two days

for six days in total. The eggs from each vacated vial were counted and the vials then incubated until the progeny hatched. Progeny were then frozen for later counting.

Frequency and Fitness Effects of Hybrid and Conspecific Matings between *D. melanogaster* and *D. simulans* at 22°C

I then tested to see if hybrid mating frequencies were as common at 22°C, the preferred temperature of *D. simulans,* as they were at 25°C, the preferred temperature of *D. melanogaster.* The experimental set up was the same as above except that the experiment itself was carried out at 22°C. The sample size for this experiment was: *D. melanogaster* (♂) x *D. simulans* (♀) (N=39)*; D. simulans* (♂) x *D. melanogaster* (♀) (N=39)*; D. melanogaster* (♂) x *D. melanogaster* (♀) (N=39) and *D. simulans* (♂) x *D. simulans* (♀) (N=39).

Effects of Hybrid and Conspecific Matings on Fecundity and Female Remating Receptivity in *D. melanogaster* and *D. simulans* at 25°C

Here I tested receptivity of females that had been previously mated to a heterospecific male in comparison to a conspecific male. Females were each placed into a vial with a conspecific or heterospecific male that had been placed in the vial the day before. Pairs were given three hours to mate and the time they were placed into the vial, the time that copulation started and the time that copulation ended were recorded. After mating had occurred, the male was immediately removed and the female retained in the vial for 24 hours. Any females that did not mate in this first mating were immediately discarded. A remating receptivity test with conspecific males was then set up in the afternoon of the following day, 24 hours after the end of the first matings. Females that had mated previously were each placed in a new vial containing a conspecific male and were observed over three hours. I recorded the time they were placed into the vial and the time that copulation started and ended. No matings between *D. simulans* (♂) x *D. melanogaster* (♀) were observed during the first mating and so

no females from this treatment were available for the second mating. Excess heterospecific pairs were set up to try to ensure that there were sufficient mated females for the remating tests. The sample size for each treatment was *D. simulans* (♂) x *D. simulans* (♀) 1st mating = 30, 2nd mating = 25; *D. melanogaster* (♂) x *D. melanogaster* (♀) 1st = 52, 2nd = 48; D. *melanogaster* (♂) x *D. simulans* (♀) 1st = 136, 2nd = 31; *D. simulans* (♂) x *D. melanogaster* (♀) 1st = 82, 2nd = 0.

Effects of Hybrid and Conspecific Matings on Fecundity and Female Remating Receptivity in *D. melanogaster* and *D. simulans* at 22°C

The above assay was then repeated at 22°C. The experimental set-up was the same as above. The sample sizes for the second, conspecific, mating were: The sample size for each treatment was *D. simulans* (♂) x *D. simulans* (♀) 1st mating = 50, 2nd mating = 49; *D. melanogaster* (♂) x *D. melanogaster* (♀) 1st = 49, 2nd = 48; D. *melanogaster* (♂) x *D. simulans* (♀) 1st = 100, 2nd = 39; *D. simulans* (♂) x *D. melanogaster* (♀) 1st = 161, 2nd = 1.

Effects of *Wolbachia* Removal via Tetracycline Treatment on the Incidence of Hybrid Mating in *D. melanogaster* and *D. simulans*

Subpopulations of wild type Dahomey *D. melanogaster* and *D. simulans* were fed on SYA food containing 100µg/ml tetracycline for seven generations to cure them of any potential *Wolbachia* infection (Alexandrov et al., 2007). After the 7th generation, after which any potential *Wolbachia* infection would have been cured (Rottschaefer & Lazzaro, 2012), adults were collected, separated by sex and stored at 10 flies per vial for 4-6 days post-eclosion to reach sexual maturity. The day before the mating assay, males were placed singly into SYA vials containing a small amount of yeast paste to encourage mating. On the day of the mating test, a female was placed into a vial of the corresponding treatment (see below) and

observed for three hours. The time that the female was placed into the vial, the time to start mating, and the time to finish mating were all recorded. All pairs were discarded after three hours whether they had mated or not. Treatments were: *D. melanogaster* (♂) x *D. simulans* (♀) (N=40); *D. simulans* (♂) x *D. melanogaster* (♀) (N=25); *D. melanogaster* (♂) x *D. melanogaster* (♀) (N=40); *D. simulans* (♂) x *D. simulans* (♀) (N=25).

Statistical Analysis

Courtship frequency, mating duration, egg number and progeny number data were analysed by performing a one-way ANOVA and Tukey's HSD post-hoc test. Copulation frequency and mating latency were analysed by performing a Kruskal-Wallis (KW) and Dunn's post-hoc test (p-values adjusted with the Benjamini-Hochberg method) to test for significance differences between treatments for each dependant variable. Sex ratios were calculated in each experiment from counting the number of adult males and females from all vials in each experiment. All analysis was carried out in R v3.2.2. All data were visualised using box plots constructed using R (R Core Team, 2012).

2.4 Results

Courtship Frequency of Hybrid and Conspecific Matings between *D. melanogaster* and *D. simulans*

At 25°C and at 22°C there was significant variation between the conspecific and heterospecific pairs in terms of courtship behaviour (Figure 2.1). At 25°C, males of both species courted conspecific females significantly more than heterospecific females ($F_{(2,179)}$ = 51.42; p<0.001). At 22°C, *D. simulans* males again courted conspecific females significantly more than heterospecific females ($F_{(1,154)}$ = 18.4; p<0.001) but this time there was no

difference in the frequency of courtship between either species of female courted by *D. melanogaster* males.

Copulation Frequency of Hybrid and Conspecific Matings between *D. melanogaster* and *D. simulans*

Conspecific mating was significantly more frequent than heterospecific mating at both 25°C ($H_{(1)} = 62.33$; $p<0.001$) and 22°C ($H_{(1)} = 37.94$; $p<0.001$) (Figure 2.2). Post-hoc analysis showed that *D. simulans* females mated heterospecifically significantly more frequently than *D. melanogaster* females at 22°C ($Z_{(1)} = -2.83$; $p=0.007$) but not 25°C ($Z_{(1)} = -1.53$; $p=0.15$).

Mating Latency in Hybrid and Conspecific Matings between *D. melanogaster* and *D. simulans*

Conspecific pairs initiated mating significantly quicker than *D. melanogaster* (♂) x *D. simulans* (♀) pairs at both 25°C ($H_{(1)} = 18.39$; $p<0.001$) and 22°C ($H_{(1)} = 17.82$; $p<0.001$) (Figure 2.3). *D. melanogaster* (♂) x *D. simulans* (♀) hybrid matings were also rarer than conspecific matings, with successful mating occurring only 1/5th – 1/3rd as often as the number of conspecific mating pairs. No data could be collected for the reciprocal hybrid cross as no mating occurred during the three hour observation window.

Table 2.1: Frequency of courtships and copulations (average per female per treatment), mating duration and latency (mins), fecundity and offspring of conspecific and reciprocal heterospecific hybrid matings between *D. melanogaster* and *D. simulans*. Mean fecundity and progeny averaged over the 3 post mating vials as well as the individual vial data are shown. Two of the "% Hatched Progeny" entries are >100% due an egg miscount that resulted in more offspring being counted than eggs.

	22°C				25°C			
	D. melanogaster (♂) x *D. simulans* (♀)	*D. simulans* (♂) x *D. simulans* (♀)	*D. simulans* (♂) x *D. melanogaster* (♀)	*D. melanogaster* (♂) x *D. melanogaster* (♀)	*D. melanogaster* (♂) x *D. simulans* (♀)	*D. simulans* (♂) x *D. simulans* (♀)	*D. simulans* (♂) x *D. melanogaster* (♀)	*D. melanogaster* (♂) x *D. melanogaster* (♀)
Mean Observed Courtships	7.80	5.26	0.33	9.92	3.92	4.68	0.43	8.40
Mean Observed Copulations	0.72	2.00	0.08	0.97	0.26	1.40	0.00	0.93
Mean Mating Latency (minutes)	72	22	n/a	15	65	11	n/a	13
Mean Mating Duration (minutes)	16	23	n/a	23	14	22	n/a	22
Mean Total Egg Number	43.50	47.73	32.31	55.16	49.17	66.92	52.00	97.42
Mean Total Hatched Progeny	11.00	26.42	7.75	54.03	9.89	46.32	9.00	88.42
% Hatched Progeny (Vial 1)	0.00	50.41	19.39	97.30	15.24	71.04	0.00	90.10
% Hatched Progeny (Vial 2)	52.98	61.70	33.06	102.19	20.94	73.34	0.00	92.68
% Hatched Progeny (Vial 3)	12.50	55.39	20.98	93.13	24.01	65.01	128.57	89.48

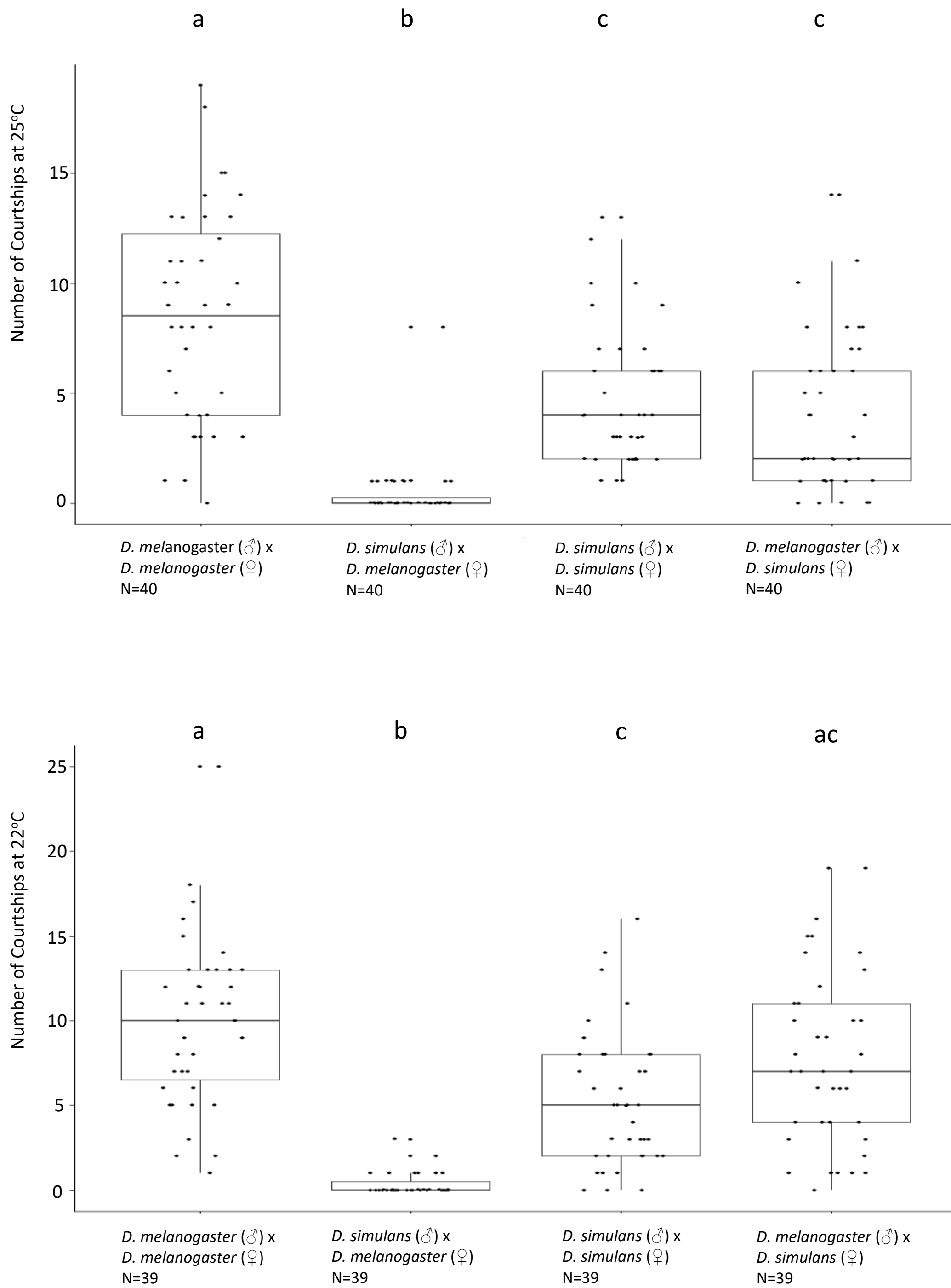


Figure 2.1: Number of courtships observed between conspecific and heterospecific matings between *D. melanogaster* and *D. simulans* at 25ºC (top) and 22ºC (bottom); crosses shown as male x female. Courtship behaviour was sampled by spot checks conducted every 20 minutes for three hours after lights on over three consecutive days. Shown are box plots (median, 25-75% IQ range, whiskers (1.5 x IQR), and outliers). Sample sizes for each cross are indicated. Different letters indicate statistically significant differences between groups

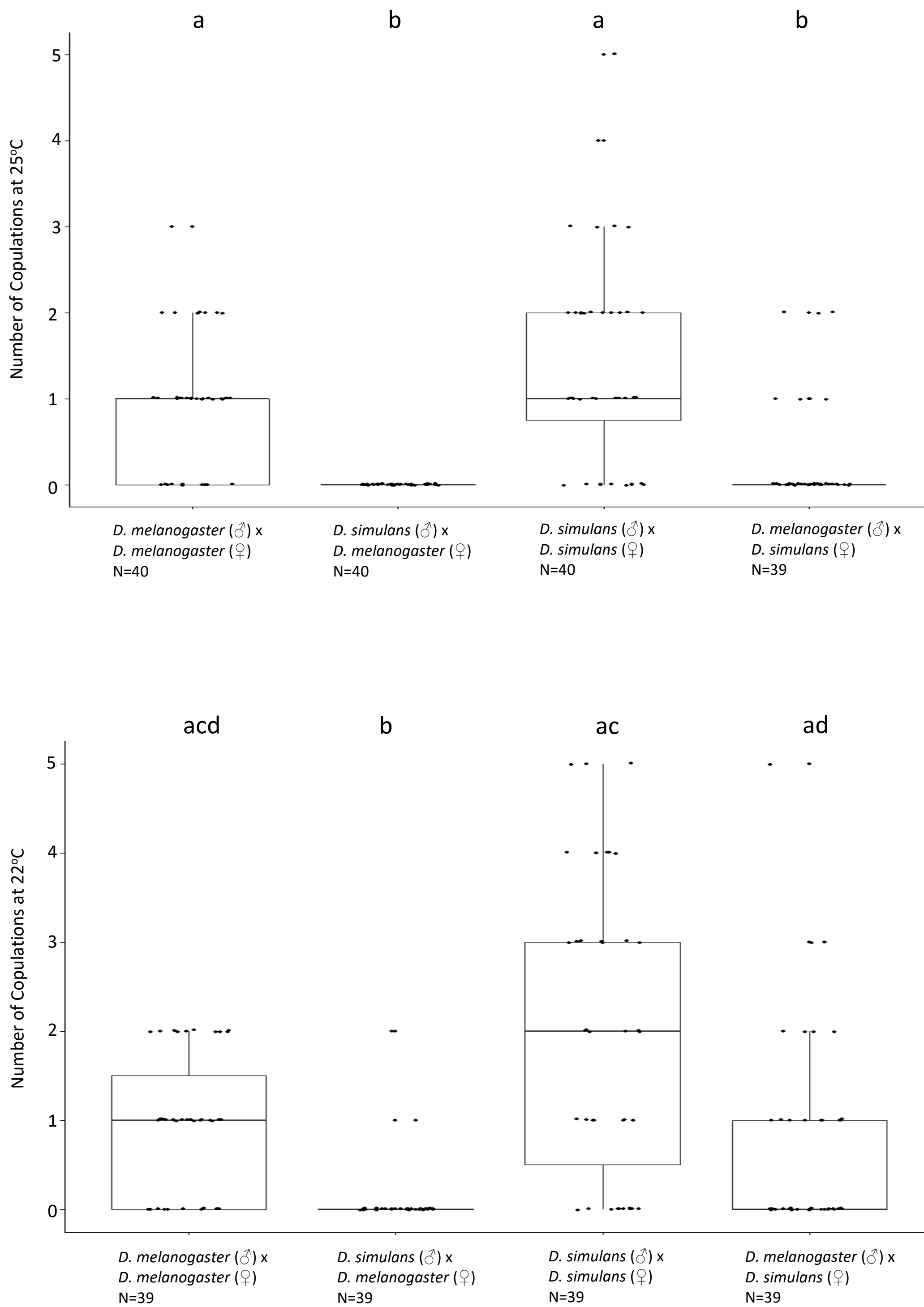


Figure 2.2: Number of copulations observed between conspecific and heterospecific matings between *D. melanogaster* and *D. simulans* at 25ºC (top) and 22ºC (bottom); crosses shown as male x female. Copulation behaviour was sampled by spot checks conducted every 20 minutes for three hours after lights on over three consecutive days. Different letters indicate statistically significant differences between groups. Boxplots defined as in Figure 2.1.

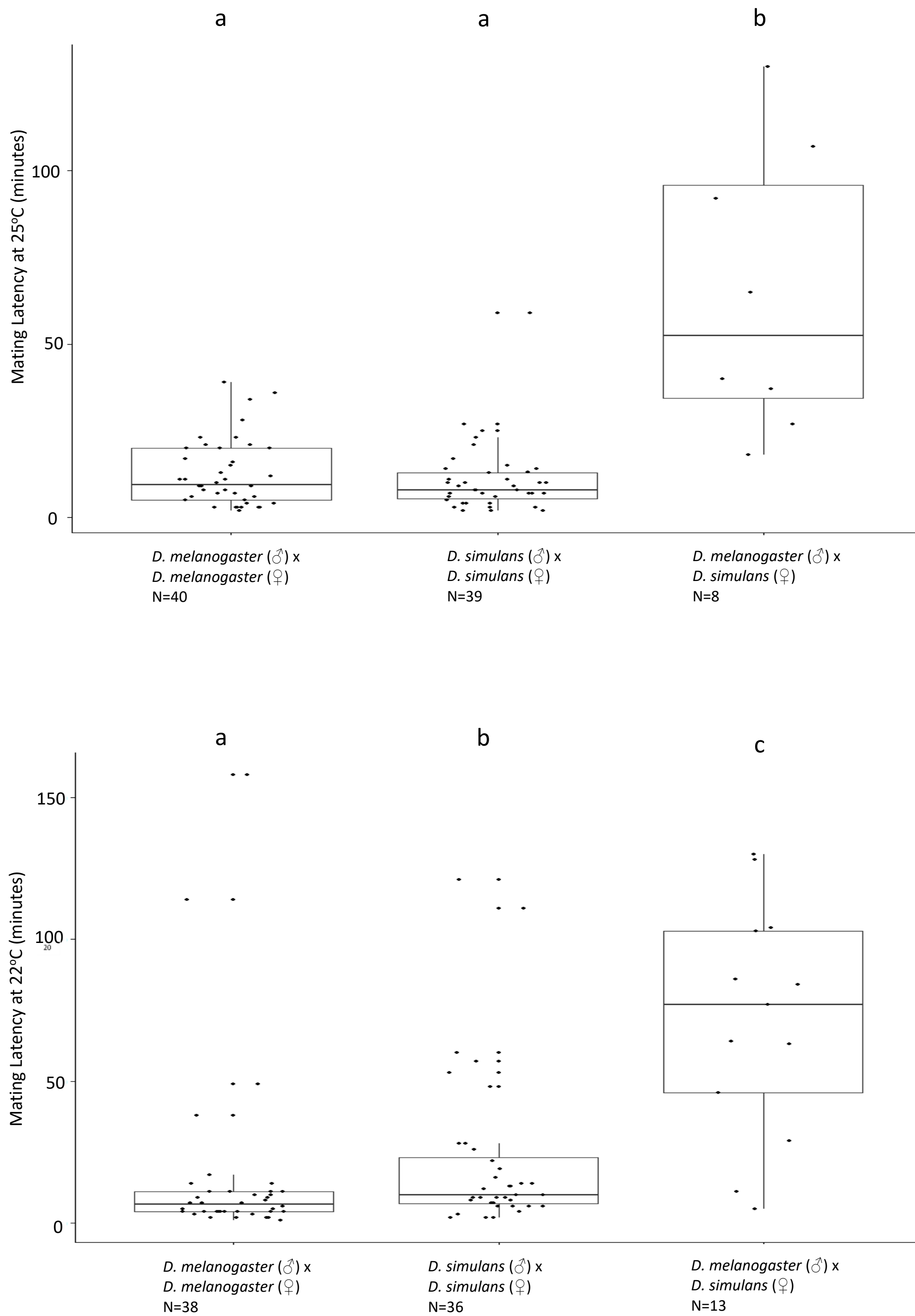


Figure 2.3: Mating latency (mins) between conspecific and heterospecific matings between *D. melanogast*er and *D. simulans* at 25ºC (top) and 22ºC (bottom); crosses shown as male x female. Mating latency was sampled by observing mating behaviour for three hours after initial introduction of *Drosophila* pairs into the first vial. Different letters indicate statistically significant differences between groups. Boxplots defined as in Figure 2.1.

Mating Duration in Hybrid and Conspecific Matings between *D. melanogaster* and *D. simulans*

D. melanogaster (♂) x *D. simulans* (♀) matings were significantly shorter than conspecific matings at 25°C ($F_{(1,85)}$ = 9.65; p>0.001) and 22°C ($F_{(1,85)}$ = 18.51; p>0.001) (Figure 2.4). No data could be collected for the *D. simulans* (♂) *x D. melanogaster* (♀) treatment as they did not mate during the 3h observation window.

Fecundity in Hybrid and Conspecific Matings between *D. melanogaster* and *D. simulans*

There were significant differences between treatments at 25°C ($F_{(3,91)}$ = 31.07; p<0.001) and 22°C ($F_{(3,79)}$ = 10.36; p<0.001) (Figure 2.5). Post-hoc analysis shows conspecifically mated *D. simulans* (♀) produced significantly more eggs than heterospecifically mated *D. simulans* (♀) at both temperatures (25°C: p<0.05; 22°C: p<0.05). It was not possible to make any inferences in the number of eggs laid between conspecific *D. melanogaster* pairs and the *D. simulans* (♂) x *D. melanogaster* (♀) treatment due to low statistical power from to the low sample size in this cross.

Progeny Number in Hybrid and Conspecific Matings between *D. melanogaster* and *D. simulans*

Fewer progeny on average were produced at 22°C than 25°C. However, the pattern between treatments was the same at both temperatures. Conspecific matings produced significantly more progeny than heterospecific matings at 25°C ($F_{(1,93)}$ = 68.9; p<0.001) and 22°C ($F_{(1,81)}$ = 47.3; p<0.001) (Figure 2.6).

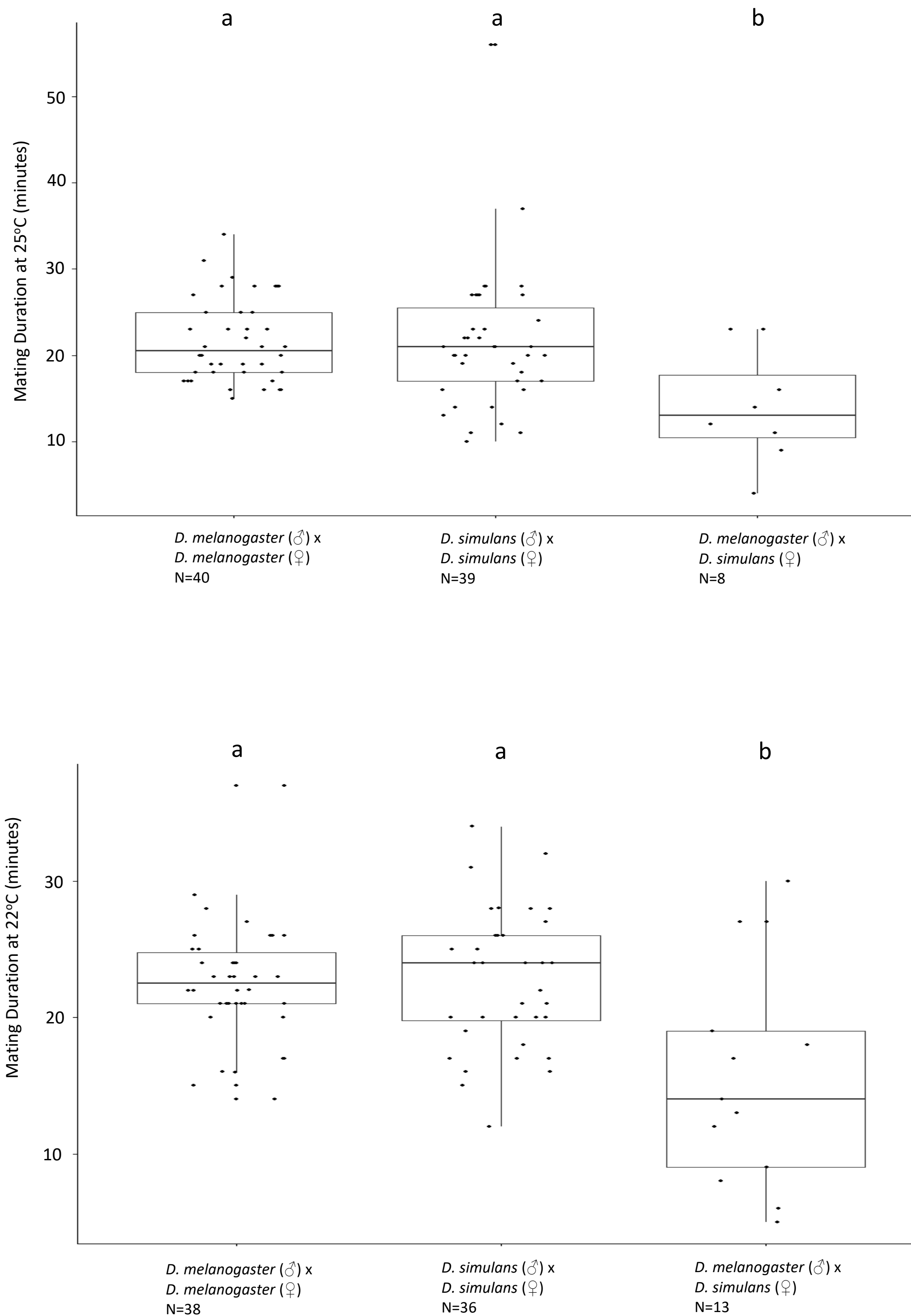


Figure 2.4: Mating duration (mins) observed between conspecific and heterospecific matings between *D. melanogaster* and *D. simulans* at 25°C (top) and 22°C (bottom); crosses shown as male x female. Mating duration was sampled by observing mating behaviour for three hours after initial introduction of Drosophila pairs into the first vial. Different letters indicate statistically significant differences between groups. Boxplots defined as in Figure 2.1.

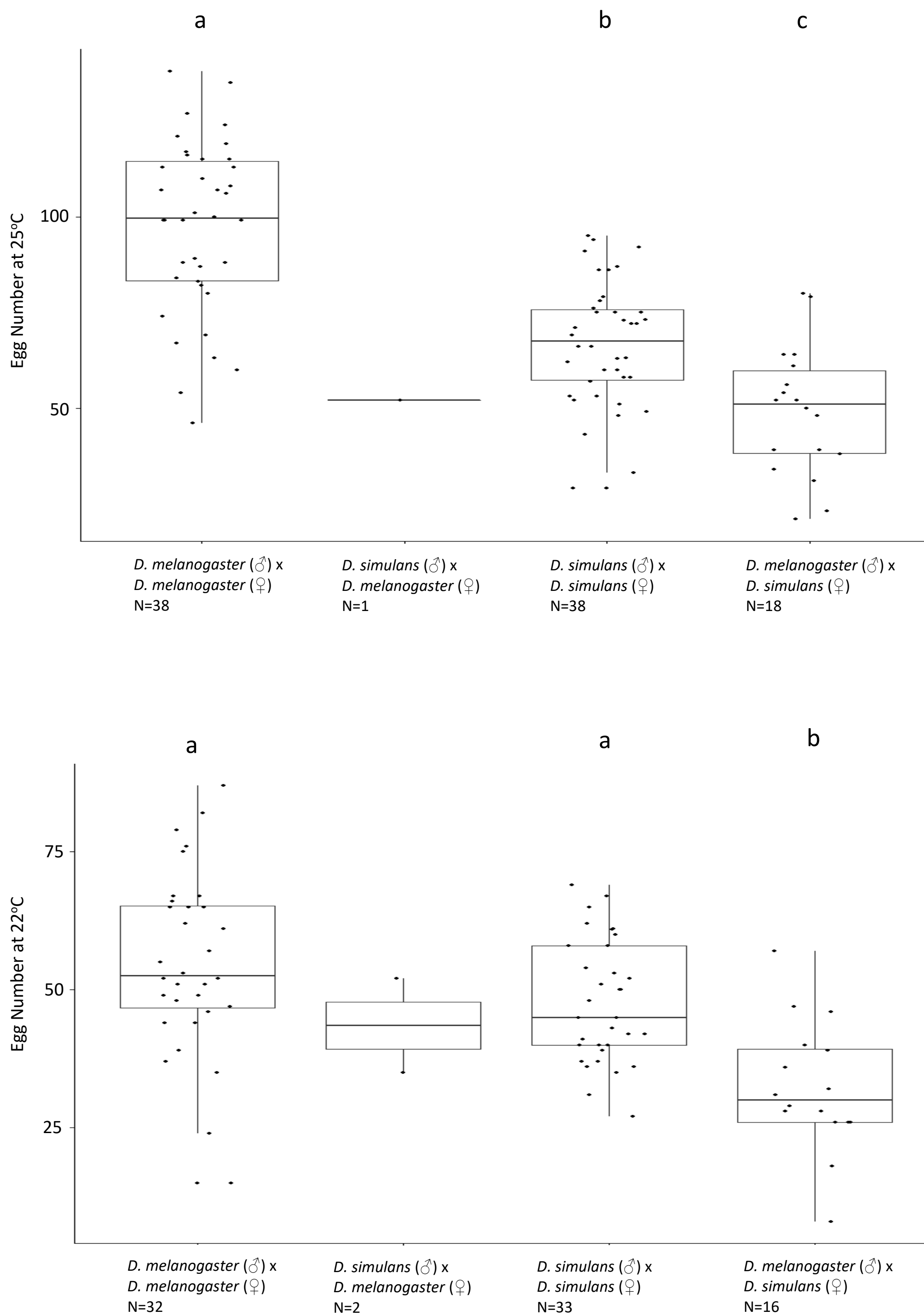


Figure 2.5: Number of eggs observed between conspecific and heterospecific matings between *D. melanogaster* and *D. simulans* at 25ºC (top) and 22ºC (bottom); crosses shown as male x female. Matings were inferred either by observation of mating itself or by the presence of offspring in the hybrid mating vials. Different letters indicate statistically significant differences between groups Boxplots defined as in Figure 2.1.

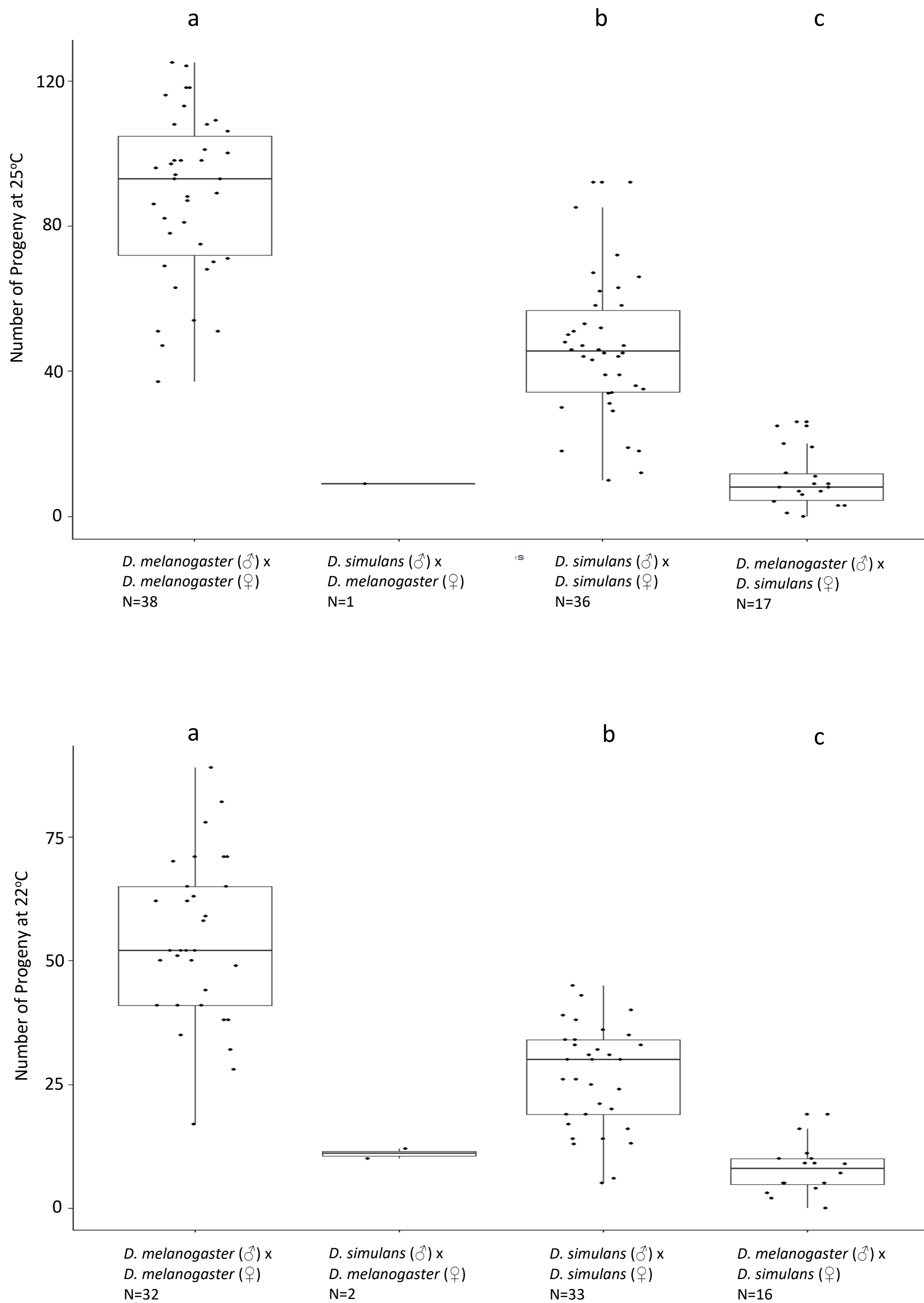


Figure 2.6: Progeny number observed between conspecific and heterospecific matings between *D. melanogaster and D. simulans* at 25ºC (top) or 22ºC (bottom); crosses shown as male x female. Mated pairs are defined as such through either physical observation of mating or the presence of offspring. Different letters indicate statistically significant differences between groups. Boxplots defined as in Figure 2.1

Progeny Sex Ratio in Hybrid and Conspecific Matings between *D. melanogaster* and *D. simulans*

Table 2.2: Progeny sex ratios from mated females in conspecific and heterospecific crosses between *D. melanogaster* and *D. simulans*. Number and percentage of male and female progeny in each of the three post mating vials are given. *D. simulans* (♂) x *D. melanogaster* (♀) produced no progeny in Vial 1 and Vial 2 at 25°C or in Vial 1 at 22°C.

		Vial 1 (♂:♀)	**Vial 2 (♂:♀)**	**Vial 3 (♂:♀)**	**Total (♂:♀)**
25°C	*D. melanogaster (♂) x D. melanogaster (♀)*	921:970 (49%:51%)	423:416 (50%:50%)	308:322 (49%:51%)	1652:61 (49%:51%)
	D. simulans (♂) x D. *melanogaster (♀)*	0	0	9:0 (100%:0%)	9:0 (100%:0%)
	D. simulans (♂) x *D. simulans (♀)*	436:526 (45%:55%)	211:223 (49%:51%)	163:201 (45%:55%)	810:950 (46%:54%)
	D. melanogaster (♂) x *D. simulans (♀)*	76:0 (100%:0%)	52:0 (100%:0%)	76:0 (100%:0%)	204:0 (100%:0%)
22°C	*D. melanogaster* (♂) x *D. melanogaster* (♀)	446:434 (51%:49%)	242:218 (53%:47%)	184:205 (47%:53%)	872:857 (50%:50%)
	D. simulans (♂) x *D. melanogaster* (♀)	0	3:13 (19%:81%)	0:6 (0%:100%)	3:18 (14%:85%)
	D. simulans (♂) x *D. simulans* (♀)	156:195 (44%:56%)	111:157 (41%:59%)	90:151 (37%:63%)	357:503 (42%:58%)
	D. melanogaster (♂) x *D. simulans* (♀)	46:1 (98%:2%)	36:0 (100%:0%)	41:0 (100%:0%)	123:1 (99%:1%)

The sex ratios for the conspecific crosses showed that an equal number of males and females were produced (Table 2.2). There was only one data point from matings between *D. simulans* (♂) x *D. melanogaster* (♀) at 25°C, which was a male. More progeny were produced from *D. simulans* (♂) x *D. melanogaster* (♀) at 22°C and these were mostly female. The *D. melanogaster* (♂) x *D. simulans* (♀) cross produced almost exclusively male progeny at 25°C and 22°C.

Effects of Heterospecific and Conspecific Mating on Female Remating Receptivity in *D. melanogaster* and *D. simulans* at 25°C and 22°C

Hybrid matings were unidirectional with matings exclusively occurring between *D. melanogaster* (♂) x *D. simulans* (♀) direction but the reciprocal cross of *D. simulans* (♂) x *D. melanogaster* (♀) occurring very rarely. Therefore *D. simulans* are the only species receiving the cost of hybrid mating. This effect was found at both 25°C and 22°C, which demonstrated that temperature acclimatisation did not significantly affect these results.

A summary of the data is shown in Table 2.3 and each measured variable will now be discussed in turn.

Table 2.3: Mating latency and duration (mins), fecundity and offspring of conspecific and reciprocal heterospecific first matings and conspecific second matings between *D. melanogaster* and *D. simulans*.

	22°C				25°C			
	D. melanogaster (♂) x *D. simulans* (♀)	*D. simulans* (♂) x *D. simulans* (♀)	*D. simulans* (♂) x *D. melanogaster* (♀)	*D. melanogaster* (♂) x *D. melanogaster* (♀)	*D. melanogaster* (♂) x *D. simulans* (♀)	*D. simulans* (♂) x *D. simulans* (♀)	*D. simulans* (♂) x *D. melanogaster* (♀)	*D. melanogaster* (♂) x *D. melanogaster* (♀)
Mean Mating Latency – First Mating (minutes)	77.00	15.92	18.00	13.15	58.19	14.07	n/a	13.38
Mean Mating Duration – First Mating (minutes)	14.62	28.02	25.00	23.00	15.02	21.70	n/a	18.46
Mean Egg Number – First Mating	16.21	18.35	17.00	24.79	13.33	15.85	n/a	24.60
% Mated – First Mating	39.00	98.00	0.62	97.96	34.56	93.33	0	92.31
Mean Mating Latency – Second Mating (minutes)	51.00	83.00	n/a	90.71	65.00	99.67	n/a	96.71
Mean Mating Duration – Second Mating (minutes)	32.64	29.67	n/a	18.74	26.09	23.33	n/a	20.53
Mean Egg Number – Second Mating	23.93	22.9	n/a	27.43	13.18	10.33	n/a	15.18
% Mated – Second Mating	35.90	20.41	0.00	14.78	39.29	12.00	n/a	36.96

Mating Latency

During the first mating, *D. melanogaster* (♂) x *D. simulans* (♀) took significantly longer to mate at 25°C ($H_{(2)}$ = 42.22; $p<0.001$) and 22°C ($H_{(3)}$ = 63.83; $p<0.001$) than the two conspecific treatments (Figure 2.7). For the second mating no significant differences in mating latency were seen between any of the treatments at 25°C ($H_{(2)}$ = 2.38; $p=0.31$) or 22°C ($H_{(2)}$ = 2.32; $p=0.31$) demonstrating that the post-mating refractory effect from previous *D. melanogaster* mating affected *D. simulans* females as significantly as *D. melanogaster* females. This indicated significant fitness costs for *D. simulans* females as they were less likely to remate with a conspecific male following a hybrid mating and therefore less likely to produce viable offspring. No matings were seen between D. simulans (♂) x D. melanogaster (♀)

Mating Duration

D. melanogaster (♂) x *D. simulans* (♀) pairs mated for a significantly shorter time than the conspecific treatments at 25°C ($F_{(2,119)}$ = 19.78; $p<0.001$) and 22°C ($F_{(3,133)}$ = 31.5; $p<0.001$). The second mating by contrast showed no significant difference between any of the treatments at 25°C ($F_{(2,26)}$ = 1.7; $p<0.2$) though *D. melanogaster* females mated for a significantly shorter time than *D. simulans* females at 22°C ($F_{(2,22)}$ = 7.24; $p<0.003$) (Figure 2.8). Thus, *D. simulans* females mated for the same length of time with a conspecific male during the second mating, independent of the first mating. No conclusions could be established for the *D. simulans* (♂) x *D. melanogaster* (♀) cross due to a low sample size

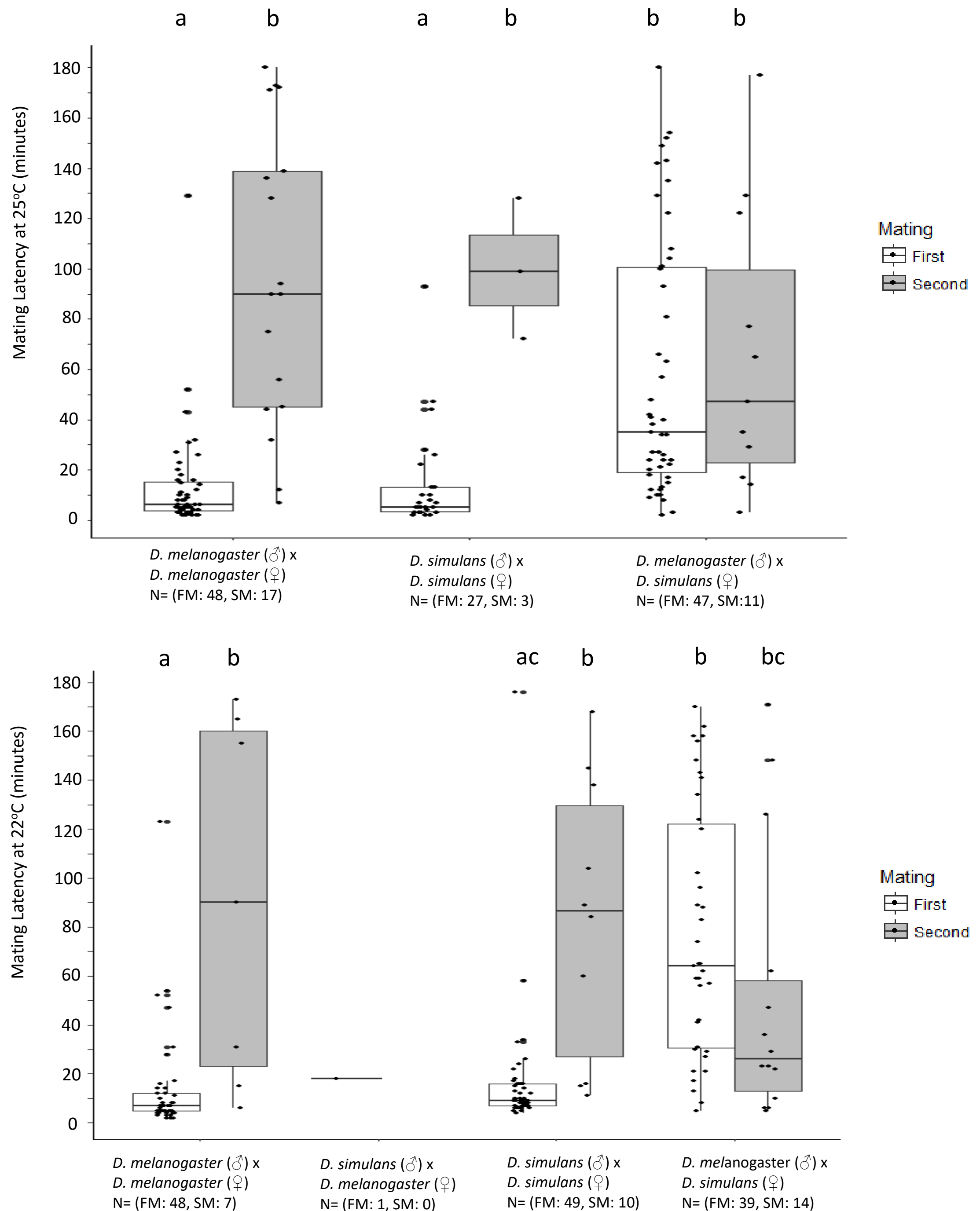


Figure 2.7: Mating latency (mins) during the first (FM, white) and second (SM, grey) matings between *D. melanogaster* and *D. simulans* at 25ºC (top) and 22ºC (bottom); crosses shown as male x female. The x-axis labels represent the groups of the original first mating however all females in the second mating were paired with a conspecific male. Mating latency was sampled by observing mating behaviour for three hours after initial introduction of *Drosophila* pairs into the first vial. Number of mated females in the first and second matings are shown. Different letters indicate statistically significant differences between groups. Boxplots as defined in Figure 2.1.

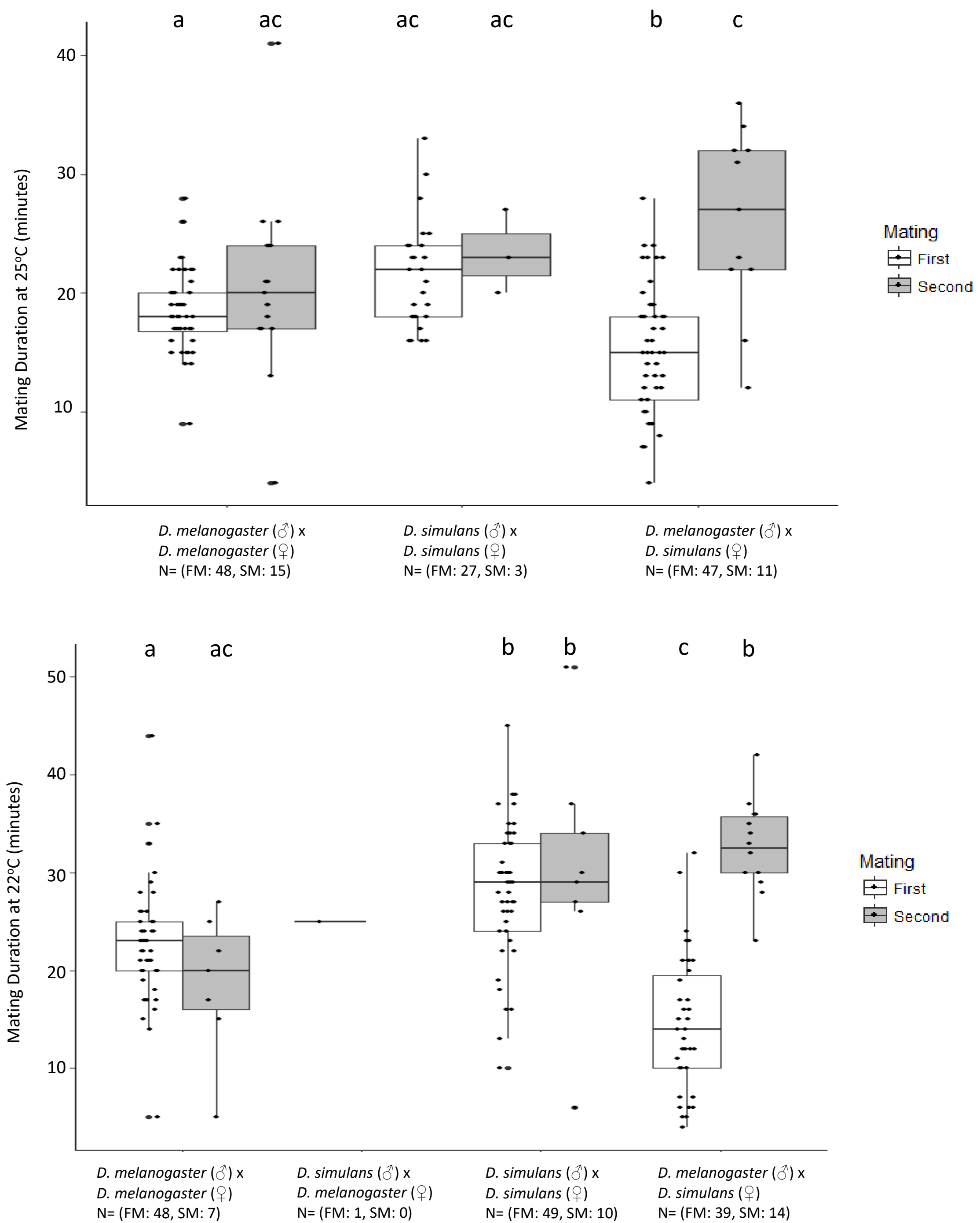


Figure 2.8: Mating duration (mins) during the first (FM, white) and second (SM, grey) matings between *D. melanogaster* and *D. simulans* at 25°C (top) and 22°C (bottom); crosses shown as male x female. The x-axis labels represent the groups of the original first mating however all females in the second mating were paired with a conspecific male. Mating duration was sampled by observing mating behaviour for three hours after initial introduction of *Drosophila* pairs into the first vial. Number of mated females in the first and second matings are shown. Different letters indicate statistically significant differences between groups. Boxplots as defined in Figure 2.1

Testing for Incidence and Directionality of Hybrid Mating in Tetracycline Treated *D. melanogaster* and *D. simulans*

Consistent with the previous experiments, unidirectional mating was seen between *D. melanogaster* males and *D. simulans* females (here in 37.5% of the total opportunities for such matings) (Figure 2.9). Mating in the reciprocal direction was not observed. Additionally the significantly reduced mating receptivity in hybrid matings that was observed in the previous experiments was also found here, with *D. melanogaster* (♂) x *D. simulans* (♀) matings taking significantly longer to initiate than either of the conspecific treatments ($H_{(2)}$ = 34.88; p<0.001).

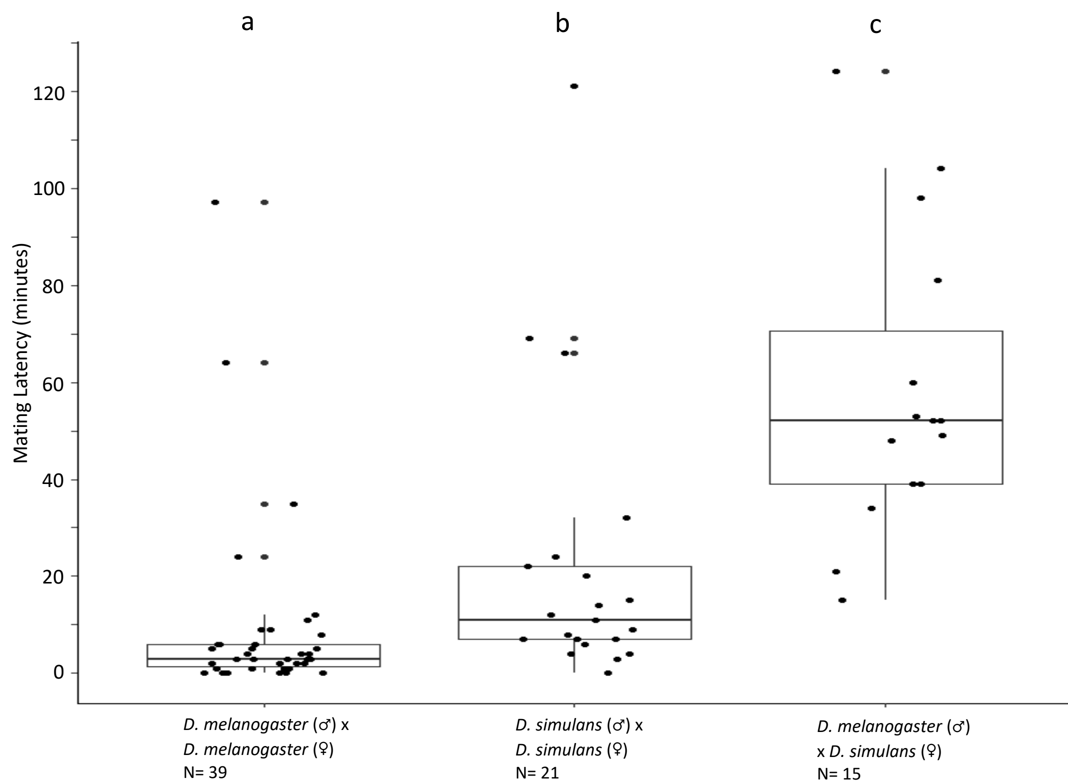


Figure 2.9: Mating incidence and mating latency (mins) observed between conspecific and heterospecific matings of *D. melanogaster* and *D. simulans* at 25°C that had been treated with tetracycline for seven generations to remove any potential *Wolbachia* infection; crosses shown as male x female. Mating latency was sampled by observing mating behaviour for three hours after initial introduction of *Drosophila* pairs into the first vial. Different letters indicate statistically significant differences between groups. Boxplots defined as in Figure 2.1

2.5 Discussion

Collectively, my results revealed evidence for satyrization between *D. melanogaster* and *D. simulans.* Hybrid matings occurred unidirectionally, with *D. melanogaster* males frequently mating with *D. simulans* females but the reciprocal cross being very rare. *D. simulans* females that mated with *D. melanogaster* males suffered significant fitness costs in terms of reduced mating receptivity and production of inviable or sterile (Barbash, 2010) hybrid offspring.

Pre-Mating Evidence for Satyrization

For practical, control applications of satyrization in the wild, fitness effects of hybrid matings are required to be asymmetric in reciprocal crosses in order for one species to outcompete the other (Kishi & Nakazawa, 2013). The frequency of hybrid matings can play a significant role in this as the initial step in determining such costs. *D. simulans* males exhibited almost no courtship behaviour towards heterospecific *D. melanogaste*r females and only one such pair was observed to mate. *D. melanogaster* males showed a reduction in courtship behaviour towards *D. simulans* females compared to females of their own species but courtship was frequently present. This may represent incomplete species recognition in *D. melanogaster* males, facilitating the occurrence of hybrid matings.

This result is consistent with previous studies describing incompatibilities in courtship behaviour between *D. melanogaster* and *D. simulans*, in which *D. melanogaster* has been observed to court *D. simulans* females (Ellis & Carney, 2009). This is thought to be attributable to differences in the cuticular hydrocarbon composition and courtship song inter-pulse intervals, two factors widely believed to allow individuals to distinguish between sex and species (Arthur et al., 2013; Sharma et al., 2012; Takahashi et al., 2012). The significantly fewer courtships in heterospecific in comparison to conspecific pairings indicated some, albeit incomplete, influence of mate recognition systems. The heterospecific

courtships may represent asymmetric opportunity costs of time and energy. For example, *D. melanogaster* males were more likely to spend time courting the wrong female than *D. simulans* males were. However, the cost of courting and mating with a female of the wrong species is likely to represent only minimal costs for males, in terms of a few minutes of opportunity costs and possibly some costs arising from ejaculate production and courtship delivery. Additionally, males do not suffer the significant refractory costs observed in females, as they have the capacity to enter the mating pool almost immediately following a heterospecific mating.

Conspecific matings were significantly more frequent and were shorter to initiate than heterospecific matings. This supported the idea of incomplete mate recognition as a driver for hybrid matings between these species indicating some mate recognition control by the females. Almost all conspecific pairs mated and some pairs mated several times. Conspecific pairs of *D. simulans* mated significantly more than conspecific pairs of *D. melanogaster*. *D. simulans* (♂) x *D. melanogaster* (♀) pairs barely mated at all, with only two observed copulations in this direction. *D. melanogaster* (♂) x *D. simulans* (♀) pairs mated more frequently than the reciprocal cross. However, mating only occurred at about a third as often as the conspecific pairs. This is strong evidence for pre-mating satyrization, as the presence of unidirectional hybrid mating means that only one species suffers the fitness costs of hybridization. The unidirectionality I observed is in contrast to many (Moulin et al., 2004; Sperlich, 1962; Sturtevant, 1920) but not all (Barker, 1962) studies on *D. melanogaster* x *D. simulans* hybrids. The unidirectionality between *D. melanogaster* x *D. simulans* appears to be strain dependent, and this should be investigated in future work.

Overall, there was little difference observed in the results of the tests conducted at 22°C in comparison to 25°C. Both species were significantly less fecund at 22°C compared to 25°C but there were no significant differences between the treatments. This suggested that the

temperature of these mating assays did not have any effect on hybrid mating incidence or the fitness costs arising from it in crosses between *D. melanogaster* and *D. simulans*.

These experiments were no choice experiments and therefore the frequency of hybrid mating between *D. melanogaster* and *D. simulans* in this experiment is likely to be much higher here than is found in natural environments where females have mate choice. Additionally, I separated males and females by sex as soon as adult eclosion occurred to ensure that virgins were used in these experiments. Males induce pheromone responses in females to coerce females to mate, and the pheromone composition is different between *D. melanogaster* and *D. simulans* (Pardy et al., 2018). If females were exposed to conspecific males for longer, induced conspecific pheromone responses may have made females receptive to conspecific mating and less likely to mate heterospecifically. I addressed these issues by investigating “free choice” mating in Chapter 4 where I set up mating cages containing multiple males and females from both species to observe how often hybrid mating occurs in a more naturalistic setting. Nevertheless, the experiments in this chapter demonstrate the stark unidirectionality of hybrid mating between *D. melanogaster* and *D. simulans*, indicative of satyrization.

Post-Mating Evidence for Satyrization

Hybrid offspring from crosses of *D. melanogaster* x *D. simulans* in both directions are inviable or infertile; they have no positive fitness value. Hence, females that produce more hybrid offspring may suffer a larger fitness cost due to the energy used to produce them. Both species suffered significant fitness loses following hybrid mating in comparison to conspecific matings, in terms of energy spent on the production of inviable and infertile offspring production. The low sample size arising from the rarity of matings between *D. simulans* (♂) x *D. melanogaster* (♀) meant that there were too few data to determine how many progeny

D. melanogaster females produced from hybrid matings in comparison to conspecific matings. However, the few data obtained suggested that heterospecifically mated *D. melanogaster* females produced fewer offspring than conspecific females. Heterospecifically mated *D. simulans* produced significantly fewer eggs and offspring compared to conspecific matings due to genetic incompatibility between *D. melanogaster* and *D. simulans* (Matute et al., 2014).

Female receptivity reduction is generally beneficial for the male as a form of paternity assurance as it reduces the chance for another male to inseminate the female and flush out the initial males' sperm (Kalb et al., 1993). This can result in sexual conflict if the female becomes less likely to be inseminated with sperm from a fitter male. With hybrid matings, the cost of reduced receptivity is costly for the female as the offspring produced are either infertile or inviable. If Sfps from heterospecific males can reduce receptivity to further mating as was observed here, the female may be hindered in her ability to remate with a male of the same species and flush out the incompatible heterospecific sperm to produce fertile progeny. Satyrization would therefore occur and harm the species in which females show greater receptivity to initial hybrid matings. There was no significant difference in remating behaviour between *D. simulans* females that had first mated heterospecifically compared to those that had first mated conspecifically. This shows that the hybrid matings costs suffered by *D. simulans* females are sustained from the actions of Sfps of the wrong species, as prior mating to *D. melanogaster* males caused them to be less receptive to further mating. The effect of *D. melanogaster* Sfps on *D. simulans* females is strong evidence for satyrization, which would result in fitness costs for a population of *D. simulans* in sympatry with *D. melanogaster.*

Gilchrist & Partridge (2000) suggested that a long mating duration, beyond the period during which sperm are transferred, is a form of mate guarding, which reduces the probability of

female remating. Other literature has supported this idea, finding that males are the primary controllers of mating duration but with an effect attributable to the female's ability to decouple during the last stages of copulation (Bretman et al., 2014; Lizé et al., 2012; Bretman et al., 2011; Mazzi et al., 2009). This suggests that shorter copulation time may be beneficial to the female as it increases they time they have to remate with another male and reduces the amount of time they are vulnerable to predation (Stockley, 1997).

Concerning satyrization, no significant difference in mating duration was seen between first and second matings of conspecific pairs. However, females that had first mated with a male of a different species mated for significantly longer when presented with a male of the same species. This may be due to males preferring to mate guard a conspecific female as opposed to wasting time and energy guarding a heterospecific female who will produce infertile or inviable offspring. For the female this is also advantageous. Remating with a conspecific male after a costly hybrid mating has been shown to displace the sperm of a heterospecific male. Thus, less time spent in heterospecific matings would be advantageous for females, in order to increase the chances of finding a future conspecific mate (Jamart et al., 1995). Additionally, a second mating with a conspecific male can rescue the fitness of a female who has had a prior heterospecific mating. This increase in mate duration with a conspecific male may be beneficial for a female who has had prior heterospecific mating as the mate guarding effect can prevent another heterospecific male from mating with her again and reducing her fitness for a second time.

Hybrid Sex Ratio in Conspecific and Heterospecific Matings between *D. melanogaster* and *D. simulans*

D. melanogaster (♂) x *D. simulans* (♀) produced almost exclusively male progeny at both 25°C and 22°C, which is in agreement with the established literature (Barbash, 2010; Sturtevant,

1920). Very few matings occurred in the reciprocal direction, only one hybrid offspring could be found from *D. simulans* (♂) x *D. melanogaster* (♀) crosses at 25°C which was a male. However at 22°C, 22 offspring were produced from *D. simulans* (♂) x *D. melanogaster* (♀) crosses which were predominantly female and this was again concurrent with established literature (Barbash, 2010).

Effect of Temperature on Patterns of Satyrization

Running the experiments at both 25°C and 22°C revealed some minor differences, though the overall pattern of satyrization remained the same. Lower temperature is known to reduce the metabolic rate of *Drosophila* populations adapted to a higher temperature (Berrigan & Partridge, 1997) and as such the lower temperature would have reduced the metabolic rate of *D. melanogaster* and the energy they spent courting females or producing eggs (Dillon et al., 2007, Chapman et al., 2001).

Effect of *Wolbachia* on Patterns of Satyrization

The pattern of matings in tetracycline treated populations showed a similar profile to that observed in the absence of tetracycline. There was a high incidence of conspecific mating in both species and mating was initiated quickly. No mating occurred at all between *D. simulans* (♂) and *D. melanogaster* (♀) but heterospecific mating between *D. melanogaster* (♂) and *D. simulans* (♀) occurred at a low rate, with 15 out of 40 pairs mating over the three hour period. These heterospecific matings were significantly slower to initiate than conspecific matings.

The *D. melanogaster* and *D. simulans* used in this experiment were cured of any potential *Wolbachia* infection. This would have removed any reproductive effects associated with *Wolbachia* infection that may have affected my stocks of *D. melanogaster* and *D. simulans*. Despite this, there was no difference in the direction of hybrid mating. Therefore it is unlikely

that *Wolbachia* itself had any effect of the patterns of hybrid matings I observed here. Any variation in unidirectionality in hybrid mating between these two species across studies (Moulin et al., 2004; Barker, 1962; Sturtevant, 1920) is likely to be dependent on the strains of *D. melanogaster* or *D. simulans* used.

Future Work

Very few matings between *D. simulans* (♂) and *D. melanogaster* (♀) were observed. Future work could optimise mating assays in order to collect data from such crosses, test how *D. simulans* sperm are received and stored by *D. melanogaster* females, and identify any asymmetries in Sfp receptivity. In the following chapter, I directly injected heterospecific seminal fluid proteins into females to overcome this barrier, similar to a study carried out by Tripet et al. in 2011.

2.6 **Conclusions**

Overall, my data provided evidence for satyrization between the sister species *D. melanogaster* and *D. simulans. D. simulans* females were more receptive to heterospecific males and produced more hybrid offspring proportional to conspecific offspring than did *D. melanogaster* females. *D. simulans* wasted energy in the production of inviable and infertile offspring as well as spending longer out of the mating pool. Evidence of satyrization was also present in the receptivity-inhibiting effects of Sfps, which function in females of both species. *D. simulans* females that mated with a male of a different species were less receptive, preventing them from receiving conspecific fertile sperm. *D. simulans* females were significantly more likely to hybrid mate than *D. melanogaster* females, further accentuating the fitness costs from hybrid matings. Based on these results and some experimental evidence (Montchamp-Moreau, 1983), it would be likely that populations of *D. simulans*

might suffer a population decline through higher fitness costs of hybrid mating, if in sympatry with *D. melanogaster,* as a result of satyrization which could lead to competitive exclusion.

Chapter 3

Quantifying the Effect of Heterospecific Seminal Fluid Proteins on Post-Mating Responses within the *D. melanogaster* Species Subgroup

3.1 **Abstract**

Reproductive interference exists when there is a disruption to normal reproductive processes. This can take many forms, and when it occurs in hybrid matings with asymmetric fitness costs from hybrid mating leading to competitive exclusion of one species by another, it is known as satyrization. This competitive exclusion effect is of increasing interest in the context of pest control. Satyrization has been shown to occur in hybrid matings between *D. melanogaster* and *D. simulans* (Chapter 2), but its general prevalence is not yet clear. Here, I tested for the presence and magnitude of asymmetrical post-mating effects, which are indicative of satyrization, between *D. melanogaster* and five other species within the *D. melanogaster* species subgroup. The selected species included three with which *D. melanogaster* can hybridize (*D. simulans*, *D. sechellia*, and *D. teissieri*) and two with which it cannot (*D. yakuba* and *D. erecta*). This allowed a preliminary investigation of whether there was any association between the presence of asymmetry in post-mating effects and hybridization in this group. Reproductive asymmetries may often be mediated by the effects of seminal fluid proteins (Sfps) on post-mating female responses. Therefore, I tested the effects of conspecific and heterospecific Sfp injections in pairwise comparisons between females of *D. melanogaster* and of *D. simulans, D. sechellia, D. teissieri, D. yakuba* and *D. erecta*. Mating receptivity was assessed by observing the frequency and mating latency of females injected with conspecific Sfps, heterospecific Sfps, or saline. There were asymmetries in the post-mating responses between *D. melanogaster* x *D. simulans, D. sechellia* and *D. teissieri*. *D. melanogaster* Sfps significantly reduced the mating receptivity of *D. simulans, D. sechellia and D. teissieri* females, but Sfps from those species either had no, or a significantly weaker, effect on female *D. melanogaster* receptivity. There were no asymmetries between *D. melanogaster* x *D. yakuba* and *D. erecta* females and in these tests, Sfps reduced female receptivity of both species. Thus asymmetric responses were observed only between species that can hybridize. Why post-mating response symmetry is retained between pairs of species that no longer mate with each other is not yet clear. The results suggest differences in the rates of evolutionary change of Sfps and their receptors across species pairs, relative to general divergence. The overall significance of these effects for fitness will depend upon how often such matings occur, hence the degree of pre-mating isolation of reciprocal matings. The finding that satyrization is apparently common among diverging species suggests that it could hold significant promise for insect control via competitive exclusion.

3.2 **Introduction**

Reproductive interference describes a phenomenon whereby courtship and copulation of one species is interrupted or disturbed by another (Gröning & Hochkirch, 2008). It is described across many taxa (Shuker & Burdfield-Steel, 2017; de Bruyn et al., 2008; Seehausen et al., 1997; Landolt & Heath, 1987) and takes many forms, including signal blocking, heterospecific rivalry and hybridization (Gröning & Hochkirch, 2008). In the context of insect control reproductive interference is often referred to as satyrization (as coined by Ribeiro and Spielman (1986)). The interest in satyrization as a control method arises because if the consequences (particularly the costs) of hybrid mating are asymmetric, then there is the potential for one species to competitively exclude the other. For instance, the probability of reciprocal hybrid matings may differ because one species has evolved a more robust mate recognition system than the other. In reciprocal hybrid matings, post-mating effects may also differ, due to various types of incompatibility in the effects of seminal fluid proteins (Sfps) that may evolve more rapidly in one species than the other. Furthermore, production of potential hybrid offspring may be sterile or simply inviable, depending upon the degree of divergence. Costs can potentially occur at each stage and affect the impact of interspecific competition. Most significant for control is when fitness costs differentially affect the more harmful of two hybridizing species that occur in sympatry and inhabit a similar ecological niche. This can result in the competitive exclusion and replacement of a harmful target species by a more benign one.

Control applications of satyrization could involve releases of 'satyr' males into populations of the target pest, increasing the interspecific competition and contributing to the decline of the target. As such, it represents a relatively benign, low cost strategy that does not involve the release of GM insects. Given this, it is surprising that it is so poorly studied. However, there are concerns surrounding the use of satyrization for control, for example, the effect on

ecosystems of altering population dynamics and introducing potentially novel species would need to be fully evaluated.

Competitive exclusion by satyrization has been demonstrated in natural populations of *Aedes* mosquitoes. Unidirectional mating is observed between *Ae. aegypti* females and *Ae. albopictus* males, whereby *Ae. aegypti* females receive Sfps from *Ae. albopictus* males. These Sfps, beneficial in conspecific *Ae. albopictus* mating for increasing oogenesis and reducing female receptivity as a form of paternity assurance, have adverse effects in *Ae. aegypti. Ae. aegypti* consequently suffer infertility and are less willing to mate with a conspecific male. In addition, offspring produced from this hybrid cross are either inviable or infertile, representing a sharp reduction in fitness for the female that produces them (Tripet et al., 2011). In sympatric populations *Ae. albopictus* outcompete *Ae. aegypti* at least partially because of satyrization effects. Nascent species divergence is characterised by markers of satyrization such as incomplete mate recognition and Sfps that are functional between species, e.g. *D. melanogaster* x *D. simulans* and *Ae. aegypti* x *Ae. albopictus*. This gives a good indicator for identifying satyrization targets and drivers. However, there are still many questions surrounding the evolutionary development, mechanics, and consequences of satyrization.

Sfps are transferred to the females along with sperm during mating in many insect species and there are at least >150 in *D. melanogaster* (Sirot et al., 2009a). They have very diverse forms and multiple post-mating functions, such as stimulating ovulation, egg laying and inhibiting mating receptivity (Hollis et al., 2019). Female post-mating responses are likely to be an efficient signal to co-ordinate the many reproductive processes that occur following mating. As such, their effects are likely to benefit both sexes. However, some Sfp effects that increase a male's fitness (e.g. oogenesis stimulation and the prevention of female remating) may conflict with the fitness interests of the female, as increased egg production beyond a

certain level may incur energetic costs, lowering survival over the longer term (Chapman et al., 1995). High refractoriness may also prevent females from subsequent matings with high quality males (Wolfner, 1997). Females may be selected to mitigate these fitness costs, whereas males may be selected to more strongly express Sfps to be highly effective in inducing potentially costly post-mating responses. It is thought that this type of sexual conflict is at least part of the reason for the rapid evolution seen in many genes encoding Sfps (Haerty et al., 2007; Mueller et al., 2005; Swanson & Vacquier, 2002). This rapid evolutionary change is also observed in studies of Sfps in closely related species of *Drosophila,* in which some *D. melanogaster* Sfps are orthologous to others in the *D. melanogaster* species subgroup, but others are species-specific (Findlay et al., 2008). As a result of sexual conflict, Sfps may quickly become incompatible in females of other species, and may even shape the evolution of reproductive isolation (van Doorn et al., 2009). Therefore, we may expect to observe significant asymmetry in the post-mating effects of Sfps that are characteristic of satyrization.

There is evidence that individual seminal fluid proteins can induce post-mating responses in different species of the *D. melanogaster* species subgroup (Denis et al., 2017; Tsuda & Aigaki 2016) and that post-mating responses can be induced via injection of seminal fluid proteins directly into the abdomen. For example, Tsuda & Aigaki (2016) showed that females from multiple species within the *D. melanogaster* species subgroup expressed a post-mating response when injected with a synthesized *D. melanogaster* Sfp.

I identified asymmetric effects of Sfps in hybrid matings between *D. melanogaster* and its closely-related sister species *D. simulans*, characteristic of satyrization (Chapter 2). I found evidence for satyrization caused by imperfect pre-zygotic mating barriers and differential costs of post-mating effects due to receptivity inhibition and stimulation of evolutionarily ‘dead end’ offspring. These effects resulted in significant fitness costs of hybrid matings for

females of *D. simulans* that were higher than for *D. melanogaster*. I extended this approach here to evaluate the effect of heterospecific Sfps in females across members of the *D. melanogaster* species subgroup. I assessed female receptivity to mating after reciprocal injections of conspecific and heterospecific Sfps between *D. melanogaster* and five other members of the *D. melanogaster* species subgroup (Figure 3.1). I hypothesised that asymmetries in post-mating responses would be associated with species hybridization because costly hybrid matings would likely create selection pressures to reduce the effect of heterospecific species Sfps in females of a satyrized species, while no such selection pressures would occur in non-satyrized species.

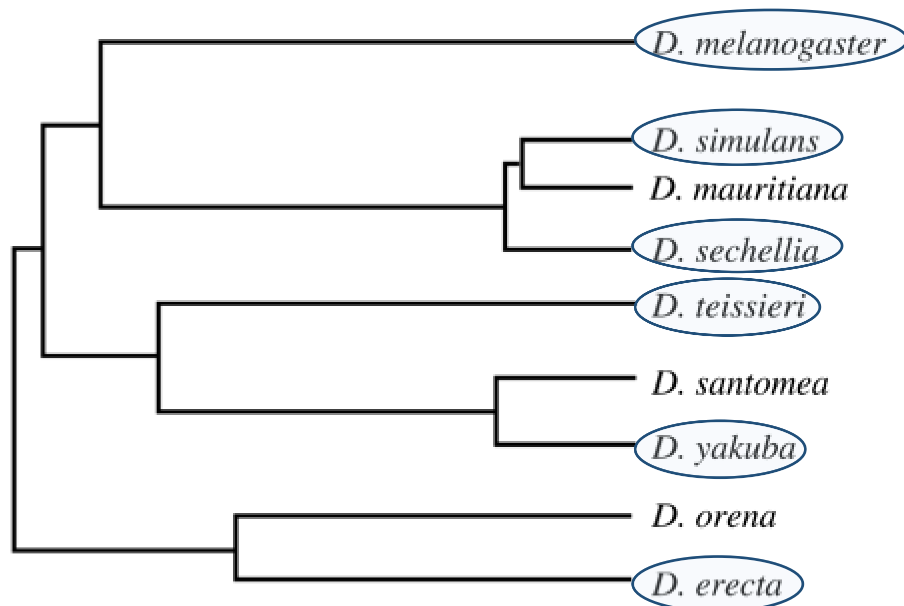


Figure 3.1: The *D. melanogaster* species subgroup showing all nine known members and their evolutionary relationships. Species in the blue circles were used in this study. Figure modified from Llopart et al., 2005.

The aim was to quantify the existence and strength of satyrization between closely related species, observe any asymmetry in post-mating responses and test for an association between post-mating asymmetries and the ability to hybridize. This will provide information regarding the evolutionary history of asymmetric reproductive interference within the *D.*

melanogaster species subgroup and provide insights into its drivers and evolutionary consequences. A detailed understanding of mechanisms of satyrization is expected to be of significant utility for developing new opportunities for control in pest species.

3.3 **Methods**

The rationale was to conduct receptivity tests in females following injection of conspecific Sfps, heterospecific Sfps or saline control. *D. melanogaster* (Dahomey) was used in each experiment as a reference against which to test all others – *D. sechellia* (KYORIN-Fly Stock No. k-s10), *D. simulans* (San Diego Stock Center), *D. erecta* (K-F Stock No. k-s02), *D. teissieri* (San Diego Stock Center) and *D. yakuba* (K-F Stock No. k-s03). As shown in Figure 3.1, these species are spread across the major clades of the *Drosophila melanogaster* species subgroup and included three species with which *D. melanogaster* can hybridize (*D. sechellia*, *D. simulans* and *D. teissieri*) plus two with which it cannot (*D. yakuba*, *D. erecta*) (Turissini et al. 2018).

Seminal Fluid Protein Collection

For tests with *D. melanogaster* x *D. simulans* / *D. erecta* / *D. yakuba* - Petri dishes containing purple grape juice (550ml H_2O, 25g agar, 300ml red grape juice, 21ml 10% w/v Nipagin solution per batch of medium) supplemented with live yeast were placed into a mating cage containing the desired species for 24h. These experiments were conducted at 25^oC under 12h:12h light:darkness photoperiod and 60% relative humidity (RH). Glass vials containing 7ml SYA (Sugar Yeast Agar: 30 ml 10% w/v Nipagin solution, 3ml propionic acid, 15g agar, 50g sugar and 100g brewer's yeast per litre of medium) were used throughout this study. Larvae were picked and placed 100 to a vial and incubated until eclosion. Males were collected upon eclosion and stored 10 per vial containing SYA medium for at least 48h to replenish any expended Sfps from prior mating.

For tests with *D. melanogaster* x *D. teissieri* / *D. sechellia* it was found that *D. teissieri* and *D. sechellia* showed low fecundity on purple agar plates and suffered high mortality at 25°C. Therefore, flies for these two experiments were cultivated in vials for 8h and 16h laying periods at 22°C. 50-100 vials were set up, each containing 8 females and 2 males of the respective species (and 4 females and 1 male for *D. melanogaster* to control for egg density across species). These experiments were conducted at 22°C in a 12h:12h light:darkness 60%RH CT incubator. Adults were first placed onto SYA vials for an 8h egg laying period, then immediately transferred to new SYA vials for 16h to lay eggs. Adult flies were removed after the egg laying period and the eggs from both oviposition periods were placed at 22°C CT to develop to adulthood after which the males were collected and isolated for at least 48h to replenish Sfps.

90-120 pairs of accessory glands were dissected from males of each species and placed into a microcentrifuge tube containing 1xPBS (Phosphate Buffer Saline) at a concentration of 3 accessory gland pairs/µl of 1xPBS. These were stored at -20°C. The day before the injection experiment, the accessory gland pairs were sonicated in 1xPBS with 5x one second pulses and centrifuged at x12,000g for 15 minutes at 4°C. The supernatant which was composed of the accessory gland homogenate, containing the Sfps, was placed into a new microcentrifuge tube and placed back into the -20°C freezer until use.

Virgin Collection

Virgin females for injection and males for the receptivity tests were collected as described above, separated into sexes using ice anaesthesia and placed into vials, 10 per vial. Adults were given 3-6 days to sexually mature before Sfp injections and receptivity tests.

Sfp Injections

On the day of the Sfp injections, virgin females were injected with 0.1µl of either 1xPBS saline, 0.1µl of conspecific Sfps or 0.1µl of heterospecific Sfps. The volume of Sfps injected represents ~1/3rd of an accessory gland pair equivalent to each female and is similar to the quantity of Sfps received in a normal mating (Sirot et al., 2009b). Immediately after injection each female was placed into a separate vial containing yeast paste to promote mating, and placed at 25°C (For experiments using *D. simulans*, *D. yakuba* and *D. erecta*) or 22°C (For experiments using *D. sechellia* and *D. teissieri*) for 24h. 80 females were injected in each treatment.

Receptivity Tests

24h post-injection, a conspecific male was placed into each vial containing a surviving female. Pairs were observed for 3h (4h for the *D. melanogaster* x *D. sechellia / D. teissieri* experiments conducted at 22°C). Time placed into the vial, time to start mating and time to stop mating was recorded and the adults were discarded after the observation period had ended or after they had mated.

Data Analysis

Female mating receptivity data were analysed using a Cox-proportional hazards regression model and the data presented as survival graphs. All analysis was performed using in R v3.2.2 (R Core Team, 2012).

3.4 **Results**

Overall, significant asymmetries in the effects of Sfps of female receptivity were seen in reciprocal injections of Sfps between *D. melanogaster* and *D. simulans, D. sechellia* and *D. teissieri. D. melanogaster* Sfps significantly reduced mating receptivity in females from all three of these species. However, the Sfps from *D. simulans, D. sechellia* and *D. teissieri* either had no effect or a significantly weaker effect than *D. melanogaster* Sfps on reducing receptivity in female *D. melanogaster*. In contrast, no asymmetries were seen in reciprocal Sfp injections between *D. melanogaster* and *D. erecta* or *D. yakuba.* In these species, the Sfps significantly reduced the receptivity of the reciprocal females, with no significant difference in the strength of the effects between conspecific and heterospecific Sfps. These results are now discussed in more detail.

D. melanogaster* x *D. simulans

D. simulans females showed significantly reduced receptivity following injection with Sfps from both *D. melanogaster* (Hazard Ratio (HR): 0.13; 95% Confidence Intervals (CI) [0.06, 0.3]; p<0.001) and *D. simulans* (HR: 0.32; 95% CI [0.18, 0.55]; p<0.001) compared to the saline control (Figure 3.2). However, *D. melanogaster* Sfps reduced the mating receptivity in *D. simulans* females significantly more than did conspecific *D. simulans* Sfps (HR: 0.41; 95% CI [0.17, 0.97]; p=0.043). Conspecific *D. melanogaster* Sfps also significantly reduced *D. melanogaster* female receptivity (HR: 0.53; 95% CI [0.35, 0.8]; p=0.002) compared to the saline control, but *D. simulans* Sfps did not (HR: 0.78; 95% CI [0.51, 1.2]; p=0.26). These data provided strong evidence of asymmetry, in which *D. melanogaster* Sfps had a significant inhibitory effect on receptivity in females of both species.

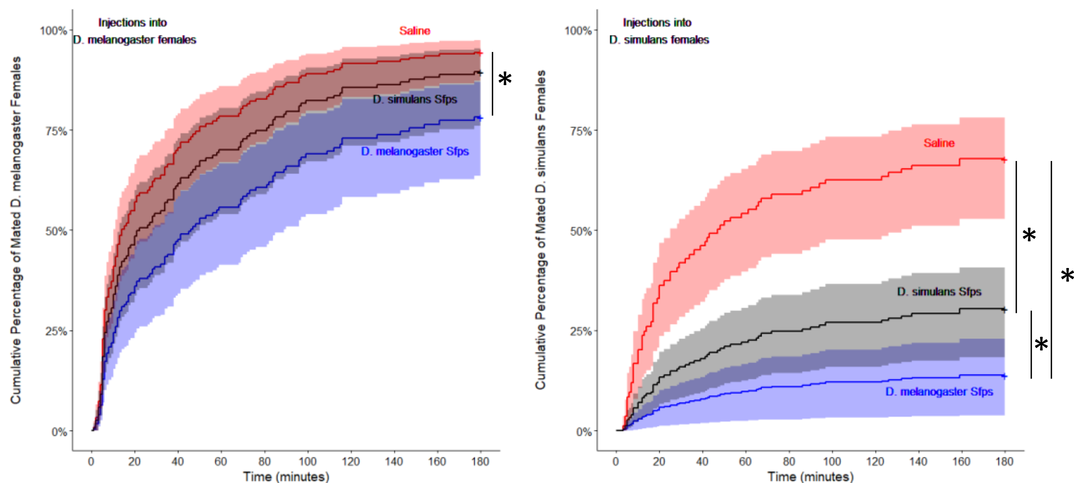


Figure 3.2: Percentage of females that mated over the 3h remating assay period, 24h following injection with either saline (red), *D. melanogaster* Sfps (blue) or *D. simulans* Sfps (black). Injections were into *D. melanogaster* females (left) and *D. simulans* females (right). Shown in the shaded areas are the 95% confidence intervals for each treatment, asterisks indicate significant differences (p<0.05).

D. melanogaster* x *D. sechellia

D. melanogaster female mating receptivity was significantly reduced by *D. sechellia* Sfps (HR: 0.35; 95% CI [0.25, 0.49]; p<0.001) and *D. melanogaster* Sfps (HR: 0.07; 95% CI [0.04, 0.11]; p<0.001) compared to the saline control. However, *D. sechellia* Sfps induced a significantly weaker refractory response than *D. melanogaster* Sfps (HR: 0.2; 95% CI [0.13, 0.32]; p<0.001) indicating an incomplete effect and an asymmetry (Figure 3.3). *D. sechellia* females conversely showed a significant reduction in mating receptivity when injected with Sfps from both *D. melanogaster* (HR: 0.02; 95% CI [0.003, 0.16]; p<0.001) and *D. sechellia* (HR: 0.1; 95% CI [0.03, 0.43]; p<0.001) compared to the saline control, with no significant difference between them.

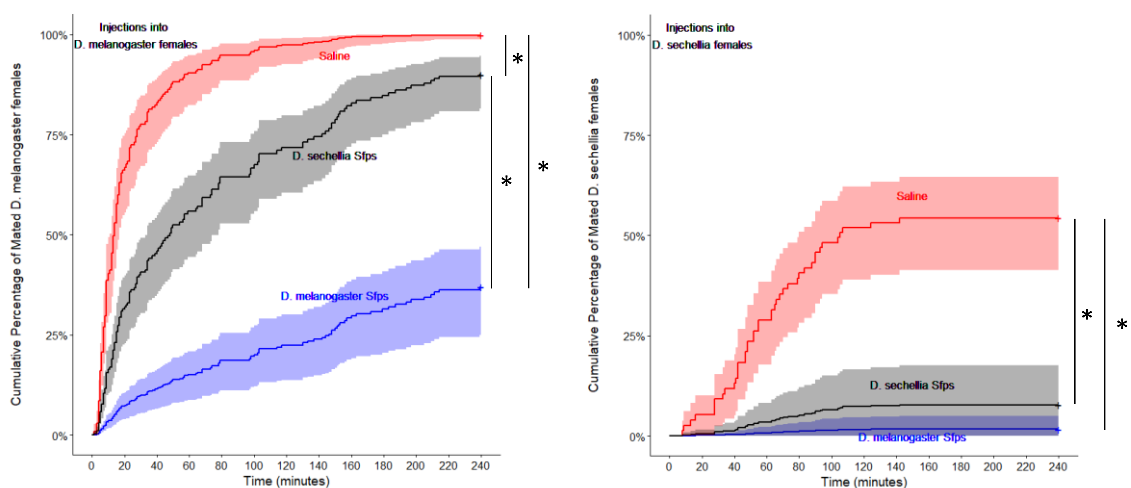


Figure 3.3: Percentage of females that mated over the 3h remating assay period 24h following injection with either saline (red), *D. melanogaster* Sfps (blue) or *D. sechellia* Sfps (black). Injections were into *D. melanogaster* females (left) and *D. sechellia* females (right).Shown in the shaded areas are the 95% confidence intervals for each treatment, asterisks indicate significant differences (p<0.05).

D. melanogaster* x *D. teissieri

D. melanogaster females significantly reduced mating receptivity when injected with conspecific Sfps (HR: 0.29; 95% CI [0.19, 0.44]; p<0.001) but not when injected with *D. teissieri* Sfps (HR: 0.82; 95% CI [0.56, 1.2]; p=0.31) compared to the saline control. However, *D. teissieri* females exhibited significantly reduced mating receptivity when injected with both *D. melanogaster* Sfps (HR: 0.14; 95% CI [0.05, 0.35]; p<0.001) and *D. teissieri* Sfps (HR: 0.21; 95% CI [0.1, 0.46]; p<0.001) compared to the saline control (Figure 3.4). As with the tests with *D. sechellia* and *D. simulans*, there was a significant asymmetry in female post-mating receptivity responses.

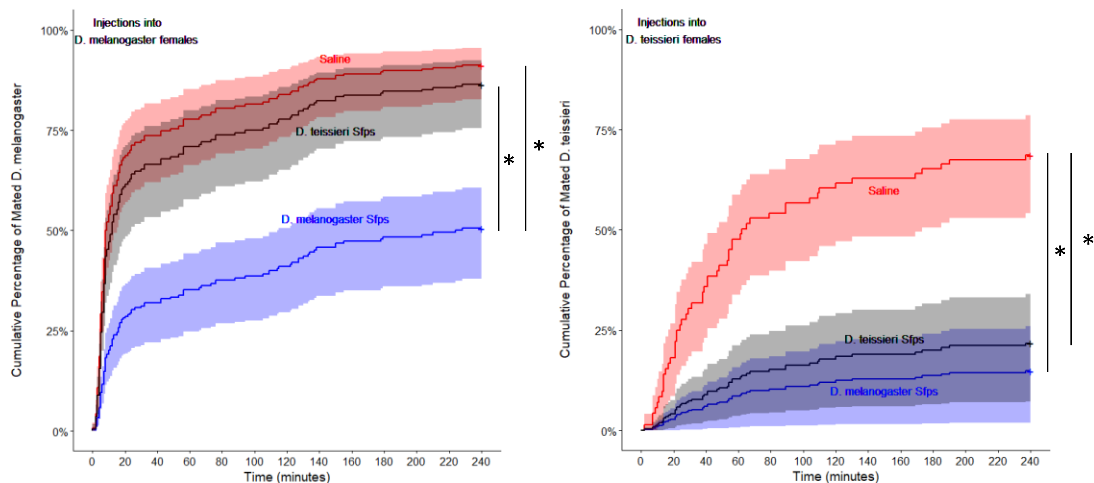


Figure 3.4: Percentage of females that mated over the 3h remating assay period 24h following injection with either saline (red), *D. melanogaster* Sfps (blue) or *D. teissieri* Sfps (black). Injections were into *D. melanogaster* females (left) and *D. teissieri* females (right). Shown in the shaded areas are the 95% confidence intervals for each treatment, asterisks indicate significant differences (p<0.05).

D. melanogaster* x *D. yakuba

No asymmetries were observed between *D. melanogaster* and *D. yakuba*. *D. melanogaster* female mating receptivity was significantly reduced by injections of *D. melanogaster* Sfps (HR: 0.23; 95% CI [0.15, 0.35]; p<0.001) and *D. yakuba* Sfps (HR: 0.16; 95% CI [0.1, 0.26]; p<0.001) compared to the saline control. Similarly, *D. yakuba* female mating receptivity was significantly reduced from injections of *D. melanogaster* Sfps (HR: 0.22; 95% CI [0.09, 0.52]; p<0.001) and *D. yakuba* Sfps (HR: 0.15; 95% CI [0.07, 0.34]; p<0.001) compared to the saline control (Figure 3.5).

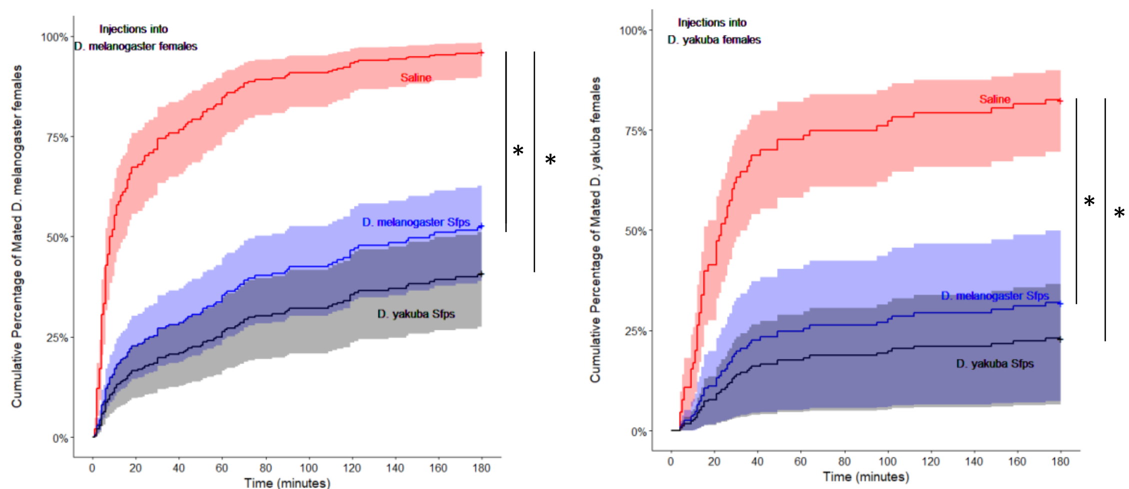


Figure 3.5: Percentage of females that mated over the 3h remating assay period 24h following injection with either saline (red), *D. melanogaster* Sfps (blue) or *D. yakuba* Sfps (black). Injections were into *D. melanogaster* females (left) and *D. yakuba* females (right). Shown in the shaded areas are the 95% confidence intervals for each treatment, asterisks indicate significant differences (p<0.05).

D. melanogaster* x *D. erecta

D. erecta females significantly reduced their mating receptivity when injected with *D. erecta* Sfps (HR: 0.33, 95% CI [0.16, 0.67]; p=0.002) and *D. melanogaster* Sfps (HR: 0.1; 95% CI [0.02, 0.41]; p=0.002) compared to the saline control. *D. melanogaster* females similarly significantly reduced mating receptivity when injected with *D. erecta* Sfps (HR: 0.2; 95% CI [0.13, 0.31]; p<0.001) and *D. melanogaster* Sfps (HR: 0.18; 95% CI [0.11, 0.28]; p<0.001). As for the test with *D. yakuba*, there was no evidence for asymmetry in post-mating receptivity responses (Figure 3.6).

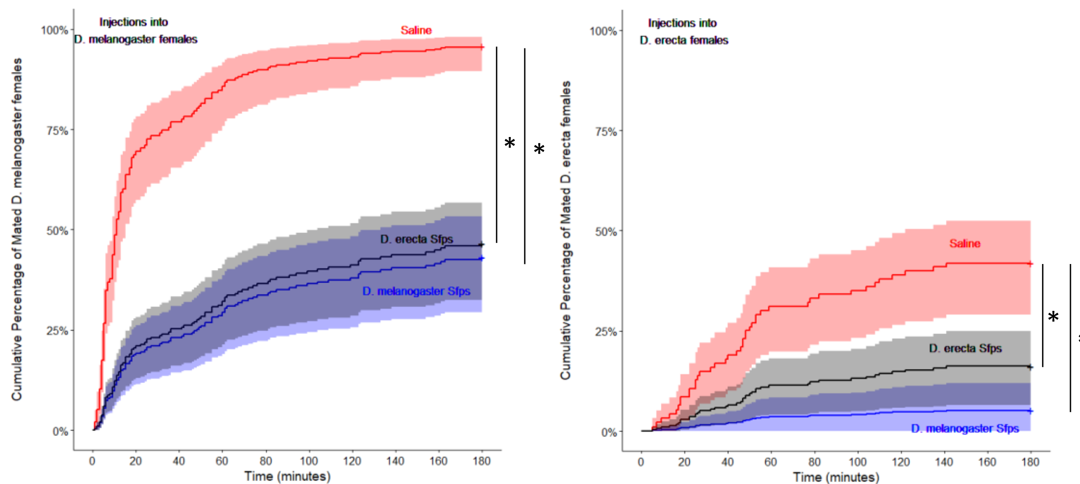


Figure 3.6: Percentage of females that mated over the 3h remating assay period 24h following injection with either saline (red), *D. melanogaster* Sfps (blue) or *D. erecta* Sfps (black). Injections were into *D. melanogaster* females (left) and *D. erecta* females (right). Shown in the shaded areas are the 95% confidence intervals for each treatment, asterisks indicate significant differences (p<0.05).

3.5 **Discussion**

Tests for Effects of Sfps on Post-Mating Receptivity across Species Pairs Revealed Asymmetries

These results showed that post-mating asymmetries, symptomatic of satyrization, were unexpectedly prevalent within the species tested. Members of the *D. melanogaster* species subgroup have been studied for over a century and yet it is only recently that evidence of satyrization between species within the subgroup has emerged (Yassin & David, 2016). This is promising for the use of satyrization as an insect control method, as it implies that many taxa may potentially be susceptible to satyrization, including many insect control targets.

Asymmetries in post-mating receptivity responses were seen between *D. melanogaster* and *D. simulans, D. sechellia* and *D. teissieri.* In each of these assays, *D. melanogaster* Sfps significantly reduced receptivity in females of the reciprocal species, but the reciprocal species Sfps produced either no significant effect or a significantly weaker effect when injected into *D. melanogaster* females. There was no asymmetry in the injections between *D. melanogaster* and *D. erecta* or *D. yakuba.* In these tests all Sfps from conspecific or heterospecific species significantly reduced mating receptivity to the same extent.

The results supported the prediction of an association between ability to hybridize and asymmetry of post-mating effects as asymmetries were found only in species that can still hybridize. Turissini et al. (2018) show that laboratory hybrid crosses are possible between *D. melanogaster* and *D. simulans, D. sechellia and D. teissieri* but not with *D. erecta* or *D. yakuba*. Taken in conjunction with my injection data here, this suggests a positive correlation between post-mating asymmetries and incomplete mating barriers i.e. those that can hybrid mate with *D. melanogaster* also show asymmetry in the post-mating response with *D. melanogaster.*

Consistent with the hybridization probability, *D. yakuba* and *D. erecta* are more evolutionarily distant to *D. melanogaster* than are *D. simulans* and *D. sechellia*. *D. teissieri* seems to lie between the branches between *D. erecta* and *D. yakuba*, though debate still remains as to its exact phylogenetic relationship (Obbard et al., 2012). This implies that asymmetries in the post-mating response are more prevalent between species that are more closely related to each other. Why this pattern exists may be due to several factors, as outlined below.

Evolution of Resistance to Costly Hybrid Matings

It has been hypothesized that Sfps can evolve under rapid selection as a failsafe to protect against interspecific mating if complete pre-mating isolation has not yet evolved (Billeter & Wolfner, 2018). Highly diverged species generally evolve complete pre-mating barriers which can take the form of behavioural or mechanical pre-mating isolation mechanisms (Ehrman, 1964).

D. melanogaster and *D. yakuba / D. erecta* are highly diverged and show strong pre-mating barriers (Turissini et al., 2018). Therefore, there is no selection for *D. melanogaster* and *D. yakuba / D. erecta* to evolve strong post-mating barriers. As such, even though there is no hybrid mating possible between *D. melanogaster* and *D. yakuba / D. erecta*, seminal fluid proteins are still compatible between these species and can cause the refractory effects seen here. However, this is a potentially puzzling result, as given there is no selection for post-mating resistance between *D. melanogaster* and *D. yakuba / D. erecta*, increasing species divergence would instead be expected to degrade interspecific Sfp functions (Orr, 1996), rather than retain them, as observed here.

D. simulans and *D. sechellia* are less evolutionarily diverged from *D. melanogaster* than other members of the *D. melanogaster* species subgroup (Obbard et al., 2012) and therefore have not yet evolved robust pre-mating barriers. Hybrid mating between *D. melanogaster* and the

most recent common ancestor of *D. sechellia* and *D. simulans* may have facilitated post-mating resistance to hybrid mating that still persists in these species.

Genes conferring resistance may be costly to evolve (Bargielowski et al., 2019) therefore post-mating barriers are likely to be stronger in species that commonly mate such as *D. melanogaster* and *D. simulans* (Chapter 2; Moulin et al., 2004; Gromko & Markow, 1993). The relaxed selection to resistance occurring due to the geographic isolation between *D. melanogaster* and *D. sechellia* until relatively recently (but not between *D. melanogaster* and *D. simulans*) may explain why *D. melanogaster* females are more susceptible to *D. sechellia* Sfps than to *D. simulans* Sfps.

D. teissieri is less closely related to *D. melanogaster* that *D. simulans* and *D. sechellia* but can hybrid mate with *D. melanogaster*. Post-mating resistance to heterospecific male-induced refractoriness to remating therefore may have evolved between populations of both species in sympatry immediately after the branching off event between their most recent common ancestor, where complete pre-mating isolation has yet to evolve. Hybrid mating between the two species is unidirectional (Turissini et al., 2018) – with *D. melanogaster* females mating with *D. teissieri* males. Therefore, selection to evolve post-mating resistance would have only been present for *D. melanogaster* females, offering an explanation as to why *D. melanogaster* Sfps can induce a post-mating response in *D. teissieri* females but not vice versa.

Consequences of Sexual Conflict in the *D. melanogaster* Species Subgroup

Sfps are under strong selection (Findlay et al., 2014) and it has been hypothesised that intraspecific sexual conflict in the *D. melanogaster* species subgroup promotes the rapid evolution of Sfps because both males and females have mechanisms to shape the extent of post-mating Sfp responses (Hollis et al., 2019; Minekawa et al., 2018; Sirot et al., 2015; Sirot et al., 2014; Findlay & Swanson, 2010; Pitnick et al., 2001). *Drosophila* have >150 Sfps, with

multiple functions, but high apparent functional redundancy. One hypothesis to explain this is that high redundancy is advantageous because the possession of a bank of functionally similar, but compositionally different, proteins may prevent females from easily evolving resistance receptivity-inhibiting compounds (Chapman, 2008).

It is possible that Sfps in *D. melanogaster* are particularly redundant, giving Sfps the capacity to retain function and induce a post-mating response in females of each of the species in the *D. melanogaster* species subgroup. However, this may not be true of the other species tested, which may have lost some flexibility in Sfp functions. Many of the Sfps in the *D. melanogaster* species subgroup are unique to this group (Tsuda & Aigaki, 2016). Hence, the first Sfps to evolve would have been specific to the common ancestor of the subgroup and may still be effective in many of the current species. This could explain why *D. melanogaster* and *D. erecta / D. yakuba* Sfps retain the ability to induce refractory effects in females in the reciprocal species despite their relatively high evolutionary divergence.

However, producing so many different types of Sfps is likely to be costly and unless there is an advantage to retaining them, there should be selection to trim the number of Sfps in *D. simulans* and *D. sechellia.* The resources required to produce a varied repertoire of Sfps might also trade off against other traits constrained by species ecology. *D. sechellia* are endemic to the Seychelles, where they have low genetic diversity and a small effective population size (David & Capy, 1982; Legrand et al., 2009). It is plausible that this reduction in genetic diversity has resulted in shedding of redundant Sfp genes, explaining why *D. melanogaster* Sfps reduce *D. sechellia* receptivity, but *D. sechellia* Sfps have a significantly weaker effect in *D. melanogaster* females. Fewer Sfps are found in *D. simulans* than *D. melanogaster* (Findlay et al., 2008) which is evidence of either *D. simulans* shedding redundant Sfps or *D. melanogaster* evolving more novel Sfps. This might explain why *D.*

melanogaster Sfps can induce a significantly stronger refractory response in *D. simulans* females than conspecific *D. simulans* Sfps.

Seminal Fluid Protein Evolution as a Driver of Speciation

The idea that sexual conflict could drive speciation (Gavrilets, 2000) has experimental support (Gay et al., 2009; Martin & Hosken, 2004; Martin & Hosken, 2003; Arnqvist et al., 2000) and gives rise to the idea that fast evolving seminal fluid proteins could be a driving force in speciation and contribute to the pattern of results seen here. This idea was modelled by van Doorn et al. (2009). The model found that divergent selection reinforced by sexual selection facilitates speciation. In *Drosophila*, sexual conflict could have promoted speciation between already diverging natural populations of *Drosophila.* Divergent selection could have created different Sfp compositions. Females in one population may have been more receptive to intrapopulation than interpopulation Sfps, therefore showing differential stimulation of oogenesis. This would result in more offspring from intrapopulation matings compared to interpopulation matings and fitness would be enhanced. This in turn could gradually reinforce population divergence.

The initial introduction of the ancestral *Drosophila* species which speciated into *D. sechellia* is not well understood but may have come from ships of human settlers or blown to remote islands through strong winds (Jones, 2005). The resulting speciation event from this new allopatric population that would evolve into *D. sechellia* may have in part been due to fast evolving Sfps as a consequence of sexual conflict and consequently *D. sechellia* Sfps would evolve specificity towards *D. sechellia* females. A similar scenario seems likely in *D. simulans* which originated in Madagascar, an island where *D. melanogaster* are rare and thus initial populations of the two species were allopatric (Lachaise et al., 1988), facilitating Sfp specificity within *D. simulans* populations. These species-specific Sfps are unlikely to have an effect in *D. melanogaster* females but as *D. melanogaster* males produce a larger number of

‘more ancestral’ Sfps that are less species-specific, these may retain the ability to induce a post-mating response in *D. simulans* and *D. sechellia* females. Unfortunately, this does not work as a general explanation for all patterns seen across the *D. melanogaster* species subgroup. For example, *D. yakuba,* another relatively young species in the *D. melanogaster* species subgroup, and one closely related to *D. teissieri*, did not show any asymmetry with *D. melanogaster.*

Each factor described above is not by itself able to explain the entire observed pattern of results. The close relationship between *D. simulans*, *D. sechellia* and *D. melanogaster* is consistent with all the hypotheses. However, the pattern of asymmetry between *D. melanogaster* and *D. yakuba / D. erecta / D. teissieri* shows there is no simple relationship between evolutionary divergence and asymmetry. *D. teissieri* shows asymmetry with *D. melanogaster* and yet is of intermediate relatedness to *D. melanogaster* between *D. yakuba* and *D. erecta*, neither of which show asymmetry with *D. melanogaster.* This lack of a simple relationship between evolutionary divergence and asymmetries suggests that additional factors may be responsible.

Of course it is also important to note that these results are a product of testing a single population in each species. Populations were sourced from multiple labs that have been reared for many years in laboratory conditions and the degree of genetic variation in each population that I tested here is variable between species. These results present an interesting snapshot of satyrization between different species in the *D. melanogaster* species subgroup that should be subject to confirmatory tests on other populations to fully establish the degree of satyrization between these species.

Reproductive Interference

These results shed light into the potential drivers of reproductive interference such as protection against costly hybrid matings and the consequences of sexual conflict. In the

context of pest control, to identify satyrization requires two parts - identification of hybrid mating species and quantification of the consequences of hybrid mating. While there are many documented examples of hybrid mating species (Shuker & Burdfield-Steel, 2017; de Bruyn et al., 2008; Gröning & Hochkirch, 2008; Seehausen et al., 1997; Landolt & Heath, 1987), there is little work on the consequences of hybrid mating and there are likely to be many examples of satyrization in nature that have been overlooked. Hence, the potential for satyrization as a form of control is almost certainly more widespread than is currently appreciated. Here I found a positive correlation between hybrid mating species and asymmetric post-mating costs indicative of satyrization. If future work confirms this correlation as significant across different insect taxa, then incomplete pre-mating isolation could become an effective indicator for identifying satyrizing species. Insect species are prime taxa for satyrization to develop as the short generation times exhibited by most insect species facilitate fast evolution and speciation leading to incomplete pre-mating isolation and post-mating incompatibilities. Satyrization is therefore likely to be present and potentially applicable to develop as a form of control in many target insect pest species.

3.6 Conclusions

Asymmetric post-mating effects due to reciprocal cross injections of Sfps occurred between *D. melanogaster* and *D. sechellia*, *D. simulans*, and *D. teissieri.* In these tests, *D. melanogaster* induced a significant refractory effect in females of the other species but the reciprocal effect was significantly weaker or non-existent. No asymmetric effects were seen in the tests of conspecific and heterospecific Sfps between *D. melanogaster* and *D. yakuba* & *D. erecta*, in which the refractory responses of females of each species were equivalent. A key finding was a positive correlation between the ability to hybrid mate in one or both directions and asymmetry in the post-mating response. Asymmetric post-mating effects were also seen between more closely related species. This may be due to evolved resistance to costly hybrid

mating or the consequences of fast evolution of Sfps due to sexual conflict. These results show that satyrization is probably more widespread in nature than is currently realised. Identifying satyrization is difficult as it requires a knowledge of not only the ability to hybrid mate but also quantification of asymmetric post-mating responses. Future work should focus on investigating how general is the correlation between hybrid mating and asymmetric fitness costs, to develop an easy indicator for identifying satyrization control targets.

Chapter 4

Testing for Pre-Mating and Post-Mating Resistance to Satyrization via Experimental Evolution

4.1 **Abstract**

Satyrization, or asymmetric reproductive interference, is present in reciprocal matings between *D. melanogaster* and *D. simulans* (Chapter 2). Such satyrization has potential as a complementary tool for insect control, because it can result in the competitive exclusion of one species by another. However, the likelihood and speed of resistance to the costs of satyrization needs to be investigated in order to produce strategies of application that will ensure its long term viability. In this chapter I addressed this question in an experimental evolution study. I set up replicated populations of *D. melanogaster* and *D. simulans* in allopatry and sympatry for 12 generations. At regular intervals throughout I performed mating assays to observe the frequency of hybrid matings in each treatment. This allowed me to track the rate of hybrid mating over time. I hypothesised that the fitness costs of satyrization would select for a decreased frequency of hybridization under sympatry. After 12 generations I also tested for post-mating resistance to satyrization. To do this I injected the seminal fluid proteins (Sfps) of sympatric and allopatric populations into females of each species in all combinations. This allowed me to test whether receptivity post mating responses had changed and to test the explicit hypothesis that sympatric populations would lower costs of satyrization by showing reduced responses to heterospecific Sfps in comparison to the allopatric populations. The results showed that after 12 generations there was no significant change in hybrid mating frequency in either the sympatric or allopatric treatments. Post-mating responses to Sfps showed inconsistent responses in *D. melanogaster* females across sympatric and allopatric regimes. In contrast, sympatric *D. simulans* females showed some evidence of lowered Sfp responses and hence reduced costs of satyrization in one sympatric treatment. This result may indicate incipient resistance to satyrization, though this needs to be confirmed. The lack of pre-mating changes is in contrast to previous work in *Aedes* that shows resistance to satyrization can evolve rapidly. This indicates that the potential effectiveness of satyrization as an insect control method needs to be assessed on a case by case basis.

4.2 **Introduction**

A potential concern with most, if not all, insect control strategies centres on the evolution of resistance to the control strategy. This can, if unchecked, reduce the efficiency of control of pest insect populations. Such resistance can potentially affect the effectiveness of various methods - from chemical insecticides such as DDT and pyrethroids (Hemingway et al., 1989; Malcolm & Wood, 1982; Pimentel et al., 1951) to ecologically-based strategies such as Sterile Insect Technique (SIT) (McInnis et al., 1996; Hibino & Iwahashi, 1991).

As the development of insect control strategies incurs significant time, monetary, labour and environmental costs, it is imperative that the up-front planning for any method includes significant consideration of resistance mitigation. Key to this is to anticipate and mitigate the speed and mechanisms of resistance evolution. This can aid in ensuring effective and persistent insect control strategies (Hackett & Bonsall, 2019; Burt, 2014). Resistance to pest control has prompted the discussion of evolutionary counter strategies, including deployment of multiple different approaches in combination, or in alternating cycles (Bourguet et al., 2013). This has the advantage of weakening selection pressure against any single method while having alternative mechanisms available to kill individuals that evolve resistance to at least one. One insect control technique that may be involved in this type of release strategy and that has been studied in the context of resistance evolution (Bargielowski & Lounibos, 2014) is satyrization.

Satyrization, or asymmetric reproductive interference, has two main components - incomplete mate recognition and variation in post-mating responses. Incomplete mate recognition can result from relatively recent divergence between sister species. In this, females of one species may evolve post-zygotic sexual isolation faster than the mate recognition mechanisms required to differentiate between conspecific and heterospecific males. This can result in hybrid matings (Mair et al., 2018; Gröning & Hochkirch, 2008) that

have zero fitness because a satyrized female will often produce no viable offspring. This situation is likely to create strong selection for increased mate recognition systems, as observed in wild populations of *D. arizonae* and *D. mojavensis* (Markow, 1981) and will facilitate the evolution of resistance to hybrid matings.

Though expected to be slower to evolve in *Drosophila* than pre-mating barriers (Turissini et al., 2018), post-mating responses can also potentially evolve resistance to satyrization, via the responses to seminal fluid proteins (Sfps). Sfps have a wide range of fitness effects – for example, they stimulate females to produce significantly more eggs and become significantly less receptive to mating (Chapman, 2001; Heifetz et al., 2000). Within species, these responses increase the number of viable offspring produced. However, in hybrid matings, females that respond to the Sfps of heterospecific males lose fitness - they produce elevated numbers of sterile or inviable offspring and are removed from the mating pool due to their sexually unreceptive state. Thus, they suffer energy costs through the production of zero fitness offspring and opportunity costs of lost opportunities to remate with conspecific males. This situation should select for the evolution of altered Sfp responses in satyrized females, e.g. to prevent seminal fluid proteins binding, or to increase the species specificity in the seminal fluid proteins that do (i.e. by only responding to conspecific Sfps).

There are many examples of pre-mating isolation barriers not evolving at the same rate in hybrid mating species (Kodric-Brown & West, 2014; Polačik & Reichard, 2011; Moulin et al., 2004; Mendelson, 2003; Lee, 1983). This can be partly due to post-mating costs to hybrid mating also being asymmetric, driving faster pre-mating isolation in the species that suffers higher fitness costs from hybridization (Arthur & Dyer, 2015). This asymmetry underlies the strategy behind satyrization, in which asymmetric hybrid mating frequency and hybrid fitness costs factor into competition between two species that eventually results in a satyrized species becoming replaced by the other. Using satyrization as a method of insect

control involves introducing a less harmful target species into sympatry with a target species. If the introduced species can hybrid mate with the target and cause asymmetric costs by doing so, the target can be outcompeted by the introduced species and may even eventually be replaced (Tripet et al., 2011).

Investigations into the applied use of satyrization in *Aedes* mosquitoes found evidence for rapid evolved resistance to hybrid matings, in the form of increased mating recognition, which resulted in a more robust pre-mating barrier. Using population cage experiments of *Ae. aegypti* and *Ae. albopictus* in sympatry, Bargielowski et al. (2013) found that satyrization was strongly selected against due to the detrimental effects of hybrid mating. *Ae. albopictus* Sfps causes heavy fitness costs in *Ae. aegypti* females such as infertility, reduced willingness to remate, and infertile and inviable offspring. Because of this, *Ae. aegypti* females kept in sympatry with *Ae. albopictus* became significantly less willing to mate with the *Ae. albopictus* males after only a couple of generations.

Satyrization has been identified between *Drosophila melanogaster* and *D. simulans* (Chapters 2 and 3). In this, asymmetric fitness costs from hybrid matings were identified that put *D. simulans* at a competitive disadvantage when in sympatry with *D. melanogaster.* Incomplete pre-mating barriers in *D. simulans* females meant that hybrid mating was unidirectional - *D. melanogaster* males mated with *D. simulans* females but reciprocal matings were rarely observed. *D. simulans* females then suffered a high cost of hybrid mating due to *D. melanogaster* Sfps producing a detrimental post-mating response. *D. simulans* females suffered a significant reduction to receptivity from hybrid mating and they produced offspring that were either inviable or infertile, representing a large waste of energy. In sympatry this could contribute to the competitive exclusion of *D. simulans* by *D. melanogaster*. Previous work shows that competitive exclusion is temperature dependent, but that *D. melanogaster* does dominate and competitively exclude *D. simulans* at 25°C

which is the primary temperature at which I ran the satyrization experiments in Chapters 2 and 3 (Montchamp-Moreau, 1983). This is predicted to facilitate the evolution of improved pre-mating barriers in the form of increased mate recognition by *D. simulans* females to avoid hybrid mating, or improved post-mating barriers via reduced sensitivity to the post-mating effects of *D. melanogaster* Sfps.

I addressed this prediction in an experimental evolution study. I set up replicated populations of *D. melanogaster* and *D. simulans* in allopatry and sympatry for 12 generations. This allowed me to track the rate of hybrid mating over time. I hypothesised that the fitness costs of hybrid mating would select for an increased resistance to satyrization.

4.3 **Methods**

Experiment Rationale

Pre-mating resistance was tested by conducting free choice mating assays throughout to measure the frequency of hybrid mating within these treatments every few generations. Post-mating resistance to Sfps was measured by experimental injections of Sfps into females of both species in each treatment. *D. melanogaster* and *D. simulans* females were injected with either saline, conspecific Sfps or heterospecific Sfps. After 24 hours, receptivity was tested by placing each injected female into a vial with a conspecific male of the same treatment and assaying copulation latency and frequency. If pre-mating satyrization had evolved in my populations, I expected the frequency of hybrid matings to decrease under sympatry over the course of the twelve generations. If post-mating resistance to satyrization had evolved, I would expect that *D. melanogaster* Sfps would become significantly less effective at reducing *D. simulans* female receptivity in the sympatric treatments, compared to the allopatric treatments.

Baseline Hybrid Mating Frequency in Sympatry (Cage Conditions)

I first investigated the frequency of hybrid matings in sympatry between *D. simulans* and *D. melanogaster*. This was to test if hybrid matings would occur at sufficient frequency in sympatry, under free choice of conspecific and heterospecific mates (previous experiments in Chapter 2 were single pair, no choice assays). This also served to produce a baseline rate of hybrid mating, from which to compare any subsequent changes.

All experiments and culturing were conducted at 25ºC 60%RH 12h:12h light:darkness in a constant temperature (CT) room. *D. melanogaster* (Dahomey) and *D. simulans* (San Diego Stock Center) were used. Eggs were collected by placing a purple agar plate (550ml H_2O, 25g agar, 300ml red grape juice, 21ml 10% w/v Nipagin solution per batch of medium) with added yeast into a population cage containing either *D. melanogaster* or *D. simulans* for 3h. The plates containing eggs were then removed and left to incubate in the CT room for 24h. First instar larvae were picked from these plates and placed 100 per vial, each containing 7ml SYA (Standard Yeast Agar: 30ml 10% w/v Nipagin solution, 3ml propionic acid, 15g agar, 50g sugar and 100g brewer's yeast per litre of medium). Virgins were collected upon eclosion, separated into separate sexes and placed 10 per vial.

To identify each species when in sympatry, one species was chosen at random for wing clip identification. This was counterbalanced so that the clipping, sex and species were different in each treatment to ensure that wing clipping could not bias the results towards the mating success of either species. There is also no evidence that wing clipping has any effect on mating success (Dodd & Powell, 1985).

After 4-6 days to allow all adults to reach sexual maturity, three treatments were set up. In each treatment 50 males and 50 virgin females from both species were placed into a plastic mating cage with a gauze lid (8cm x 15.5cm x 8.5cm) containing three SYA vials. Spot checks were carried out by observing each mating and recording the sex and species involved. Spot

checks were continued every 20 minutes over a 3h morning period and repeated over three days. After the final spot check on the third day the flies from each treatment were discarded.

Experimental Evolution of Hybrid Mating Frequency and Sfp Responses in Sympatry (Cage Conditions)

Experimental Evolution Setup: Eggs were collected by placing petri dishes containing purple grape juice agar with added yeast into mating cages containing either *D. melanogaster* or *D. simulans* adults for 3h. The dishes were then removed and incubated in the CT room for 24h. First instar larvae were picked from these plates and placed 100 larvae per vial. Upon eclosion males and virgin females were placed into small (8cm x 15.5cm x 8.5cm) or large (8cm x 15.5cm x 17cm) plastic mating cages with gauze lids containing 3 or 6 SYA vials, respectively, as described further, below.

Experimental Evolution Treatments: I set up three replicates of allopatric treatments composed of 50 males and 50 females for each species in the small cages. I created an additional single allopatric treatment of 100 males and 100 females for each species in large mating cages. This was to serve as an additional density control. Two replicates of sympatric treatments were set up in large cages each comprising 50 males and 50 females of *D. melanogaster* and 50 males and 56 females of *D. simulans.* 11% of matings occurred between *D. melanogaster* (♂) x *D. simulans* (♀) in the baseline mating experiment, which represents 6/50 *D. simulans* females. These females will be experiencing null fitness as a result of satyrization and therefore likely not to be contributing offspring to the next generation. To account for this, six extra *D. simulans* females were added in each sympatric treatment each generation to maintain a rough effective population size of 50. The experimental evolution experiment thus comprised:

- *D. melanogaster* allopatry x3 replicates
- *D. simulans* allopatry x3 replicates

- *D. simulans / D. melanogaster* sympatry x2 replicates
- *D. simulans* allopatry x1 density control cage
- *D. melanogaster* allopatry x1 density control cage

Experimental Evolution Maintenance: Food was changed in the experimental cages every two or three days by anaesthetising adults with CO_2 and replacing the SYA vials with fresh vials. Old vials were retained as backup and placed into the CT room. For the first eight generations, adults were maintained in their treatments for 16 days before egg collection. However, due to the observation that mortality was elevated during the latter part of this 16d period, this was reduced to 9d for the final four generations.

For egg collection of the next generation, SYA vials were removed and purple grape agar plates placed into the cages. An initial pre-purp (purple) agar plate was placed into each cage for 24h, removed and replaced with a purp for egg collection for another 24h. Larvae were picked from the plates and placed 100 per SYA vial. If insufficient larvae were obtained, larvae were taken from the pre-purp plates as well.

Upon eclosion, flies were collected and set up in their respective treatments. If insufficient adults were collected from the purp and pre-purp plates, extra adults from the backup vials were taken and placed into the required mating cages to make up the required number of males and females from each species. This procedure was repeated for 12 generations.

Pre-Mating Isolation: Assays of Mating Frequencies in Allopatric and Sympatric Populations

Mating Assays: After generations 3, 6, 9, 10 and 12, a mating assay was carried out to test the receptivity of females to hybrid matings. Larvae to be used in the mating assay were collected from the same purps as the larvae collected for treatment maintenance. Males and females from one species in each treatment were wing clipped and this was counterbalanced

across treatments. 50 males and 50 females from the same species and treatment were placed together into an SYA vial a day before the assay in order to mate so that non-virgins could be used in the assay. Virgin females were initially used in assays for generations 3 and 6. However this was altered in assays for generations 9, 10 and 12 where non-virgin females were used to simulate a real-world sympatric setting where non-virgins would be more numerous.

On the morning of the assay, small mating cages (8cm x 15.5cm x 8.5cm) with three SYA vials were set up containing 50 male and 50 female *D. melanogaster* and *D. simulans* from the same replicate of each treatment and placed into the CT room. Spot checks were carried out every 20 minutes over 3h to record the species of any mating pairs. This was repeated over the following two mornings after which the flies were discarded.

Post-mating Isolation: Assays of Responses to Conspecific and Heterospecific Sfps in Allopatric and Sympatric Populations

After the 12th generation of the experiment, species were separated and placed into glass bottles containing SYA. Stocks in glass bottles were reared at low density until enough adults could be obtained for egg collections of the injection assays to test for the post-mating resistance to satyrization.

Seminal Fluid Protein Collection: Eggs were collected over 3h on a purple agar plate placed into cages containing *D. melanogaster* and *D. simulans* from each allopatric and sympatric treatment. Larvae were picked 24h later and placed 100 per SYA vial. Males were collected and placed 10 per vial for 3-6 days to reach sexual maturity. 90-120 Pairs of accessory glands from each species in each treatment were dissected into sterile 1xPBS and collected at a concentration of three accessory gland pairs/µl. These were stored in 0.5ml centrifuge tubes at -20°C until use in the injection experiments. The day before the injection assay, the accessory gland pairs were sonicated with 5 x 1 second pulses and centrifuged at 12,000g for

15 minutes at 4°C. The supernatant was placed into a new 0.5ml centrifuge tube and returned to the -20°C freezer.

Sfp Response Assays: Males and virgin females for use in the injection assays were collected as described in the "Seminal Fluid Protein Collection" section. Males and virgin females were given 3-6 days to reach sexual maturity before use. On the day of the injection assay, virgin females of both species were injected with 0.1µl of either 1xPBS saline, 0.1µl conspecific Sfps or 0.1µl heterospecific Sfps. After injection, each injected female was placed into a separate SYA vial, yeasted to promote mating, for 24h.

24h post-injection, one male from the same treatment as the injected female was placed into each vial containing a female and watched for 3h. The time placed into the vial, time to start mating and time to stop mating was recorded and the adults were discarded after 3h had passed. Because of time constraints, the experiment was done in blocks with one set of matched replicates in each assay. To control for any uncontrolled block variation, there was always a saline control (negative control) and conspecific Sfp treatment (positive control) in each assay.

Statistical Analysis

Pre-mating resistance was analysed using a linear mixed model to test for differences in the frequency of hybrid mating across all time points. Post-mating receptivity was analysed using a Cox-proportional hazards model to test for differences in the number and speed of matings within each treatment and a linear mixed model to test for significant differences in the total number of females who mated between treatments. All analysis was performed using R v3.2.2. (R Core Team, 2012).

4.4 <u>Results</u>

Baseline Hybrid Mating Frequency in Sympatry (Cage Conditions)

The free choice assay revealed that unidirectional hybrid matings did occur in sympatric populations between *D. melanogaster* (♂) and *D. simulans* (♀), consistent with the results from single pair no choice assays (Chapter 2). The majority of matings in the assay overall were conspecific (Table 4.1). However, there was a percentage (11.11%) of hybrid matings between *D. melanogaster* (♂) x *D. simulans* (♀). This counted for 30.4% of all matings by *D. simulans* females. No hybrid matings were seen between *D. simulans* (♂) x *D. melanogaster* (♀).

Table 4.1: Number of hybrid matings in the initial baseline mating tests. 50 virgin males and females from each species were placed into each of the four conspecific and heterospecific treatments listed below. Wing clipping was used to identify individuals in each treatment. Spot-checks of mating behaviour were performed every 20 minutes for 3h over three consecutive days and the total frequency of matings presented is the total for this whole assay period. Also shown are the relative proportion of matings observed within each treatment in relation to the total number of matings.

	***D. melanogaster* (♂) x *D. melanogaster* (♀)**	***D. melanogaster* (♂) x *D. simulans* (♀)**	***D. simulans* (♂) x *D. melanogaster* (♀)**	***D. simulans* (♂) x *D. simulans* (♀)**
Number of Matings	*137*	*24*	*0*	*55*
Proportion of Matings in Relation to the Total	*0.63*	*0.11*	*0*	*0.26*

Pre-mating Isolation: Assays of Mating Frequencies in Allopatric and Sympatric Populations during Experimental Evolution

Allopatric treatments on average exhibited a higher frequency of heterospecific matings in the mating assay tests than did sympatric treatments across all 12 generations (Table 4.2, Figure 4.1), however this was not significant ($F_{(2)}=3.13$; $p=0.059$). There was no change over time in the frequency of heterospecific matings frequency over time in allopatric or

sympatric treatments ($F_{(1)}$=0.38; p=0.54), with the mean number of heterospecific matings in the sympatric treatments even slightly increasing over the course of the experiment.

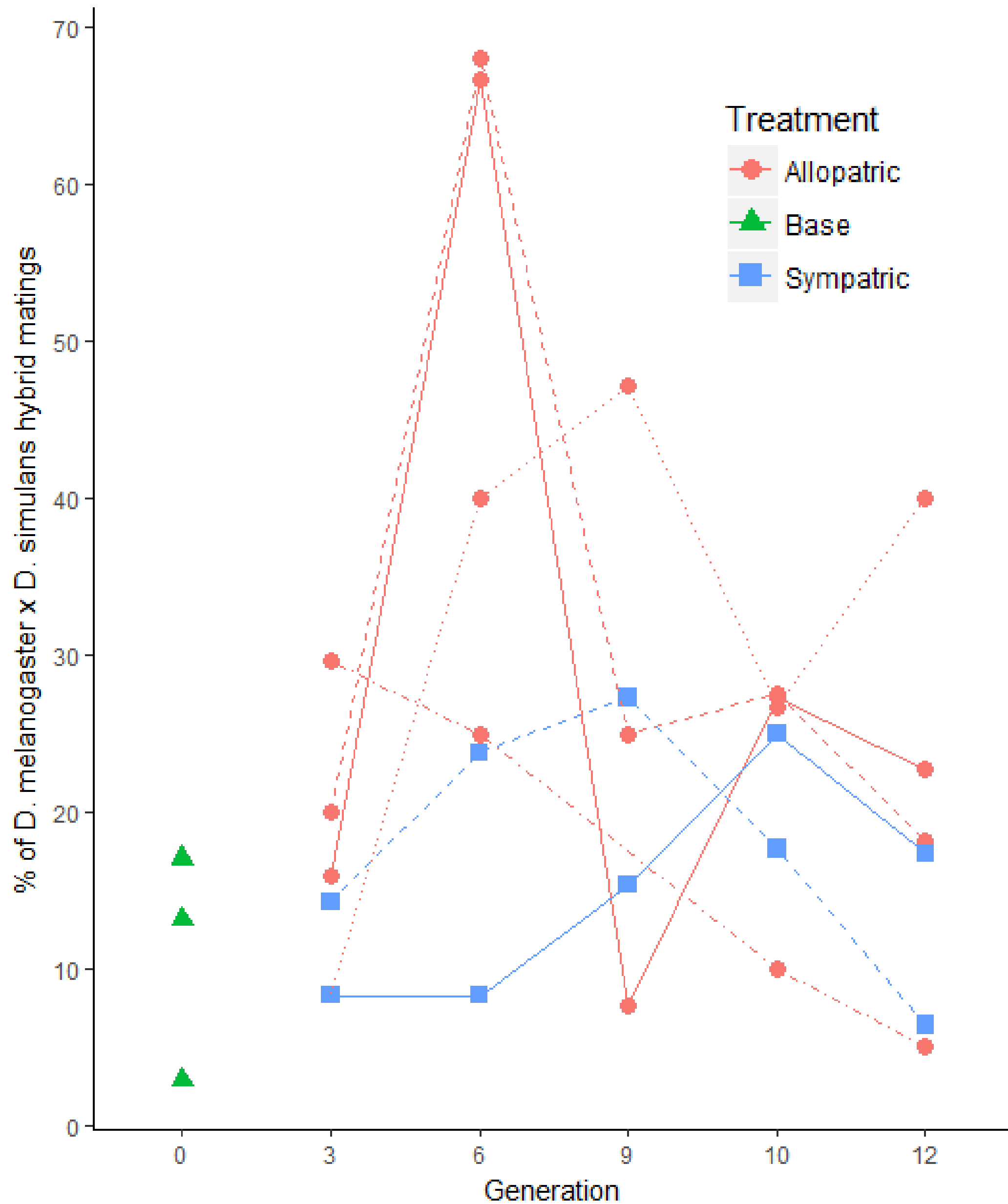


Figure 4.1: The percentage of heterospecific matings between *D. melanogaster* and *D. simulans* in sympatric and allopatric populations over 12 generations of experimental evolution. Red circles refer to populations that were maintained in allopatry, blue squares to populations maintained in sympatry, Green "base" triangles refer to the results of the initial baseline mating assay. Connected points show the same replicate line tested in each experiment throughout the study.

Table 4.2: The frequency of heterospecific matings in allopatric and sympatric populations in the experimental evolution regimes. Assay (generation) refers to assay numbers 1-5 conducted in generations 3, 6, 9, 10 and 12, respectively. The number and % (of total matings) of heterospecific matings (*D. melanogaster* (♂) x *D. simulans* (♀) or the reciprocal) are shown. One sympatric treatment was lost early in the experiment (sympatric 1).

	Assay (Generation)	Number of Heterospecific Matings	% (of Total Matings) of Heterospecific Matings
Allopatric 1	1 (3)	10	20
	2 (6)	17	68
	3 (9)	4	25
	4 (10)	11	27.5
	5 (12)	6	18.18
Allopatric 2	1 (3)	6	8.45
	2 (6)	2	40
	3 (9)	17	47.22
	4 (10)	8	26.67
	5 (12)	12	40
Allopatric 3	1 (3)	16	29.63
	2 (6)	2	25
	3 (9)	N/A	N/A
	4 (10)	2	10
	5 (12)	2	5.13
Allopatric (Density Control)	1 (3)	8	16
	2 (6)	8	66.67
	3 (9)	1	15.38
	4 (10)	3	27.27
	5 (12)	5	22.73
Sympatric 2	1 (3)	4	8.33
	2 (6)	1	8.33
	3 (9)	2	15.38
	4 (10)	1	25
	5 (12)	4	17.39
Sympatric 3	1 (3)	4	14.29
	2 (6)	5	23.81
	3 (9)	3	27.27
	4 (10)	3	17.65
	5 (12)	2	6.45

Post-mating Isolation: Assays of Responses to Conspecific and Heterospecific Sfps in Allopatric and Sympatric Populations

Allopatry: Injections into *D. simulans* females were consistent across both allopatric treatments. *D. simulans* females had significantly reduced mating receptivity following receipt of conspecific and heterospecific Sfps in both "allopatric 1" (*D. simulans* Sfps - Hazard Ratio (HR): 0.54; 95% Confidence Intervals (CI) (0.3, 0.96); p=0.035) (*D. melanogaster* Sfps - HR: 0.62; 95% CI (0.4, 0.97); p=0.035) and "allopatric 2" (*D. simulans* Sfps - HR: 0.42; 95% CI (0.25, 0.7); p<0.001) (*D. melanogaster* Sfps - HR: 0.55; 95% CI (0.33, 0.92); p=0.023) in comparison to the saline control (Figure 4.2).

Sfp injections into *D. melanogaster* females were less consistent. In both allopatric treatments, *D. melanogaster* females significantly reduced their mating receptivity following receipt of conspecific *D. melanogaster* Sfps ("Allopatric 1" - HR: 0.53; 95% CI (0.36, 0.77); p<0.001) ("Allopatric 2" - HR: 0.28; 95% CI (0.19, 0.41); p<0.001). However, *D. simulans* Sfps significantly reduced female *D. melanogaster* receptivity in "allopatric 2" (HR: 0.30; 95% CI (0.19, 0.48); p<0.001) but not in "allopatric 1" (HR: 0.85; 95% CI (0.58, 1.25); p=0.406) compared to the saline control.

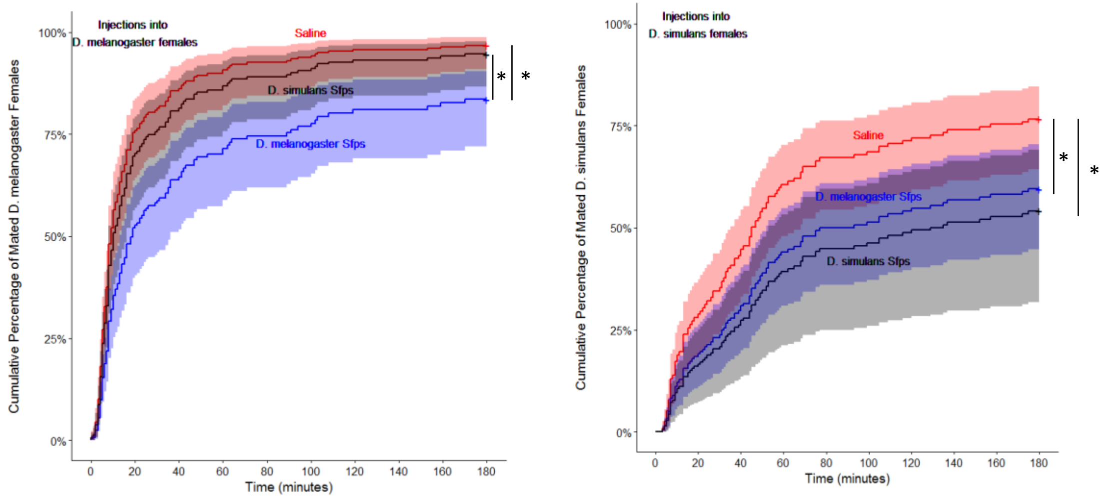
Sympatry: *D. melanogaster* females significantly reduced receptivity to conspecific *D. melanogaster* Sfps in both "sympatric 2" (HR: 0.41; 95% CI (0.27, 0.63); p<0.01) and "sympatric 3" (HR: 0.32; 95% CI (0.22, 0.48); p<0.001). However, there was again inconsistency with the receipt of *D. simulans* Sfps. *D. melanogaster* females did not reduce receptivity when injected with *D. simulans* Sfps in "sympatric 2" (HR: 1.18; 95% CI (0.8, 1.75); p=0.409) but did significantly reduce receptivity when injected with *D. simulans* Sfps from "sympatric 3" (HR: 0.51; 95% CI (0.35, 0.74); p<0.001) compared to the saline control. Both *D. melanogaster* and *D. simulans* Sfps significantly reduced *D. melanogaster* mating

receptivity in "sympatric 3." However, *D. melanogaster* Sfps induced a significantly stronger effect than *D. simulans* Sfps (HR: 0.63; 95% CI (0.43, 0.93); p=0.019) (Figure 4.3).

Injections into *D. simulans* females were also inconsistent between sympatric treatments. *D. simulans* receptivity was significantly reduced in "sympatric 2" from Sfp injections of *D. melanogaster* (HR: 0.41; 95% CI (0.26, 0.65); p<0.001) and *D. simulans* (HR: 0.43; 95% CI (0.25, 0.74); p=0.002) consistent with injections into *D. simulans* females in the allopatric treatments. However, in "sympatric 3", neither species Sfps significantly reduced *D. simulans* receptivity compared to the saline control (*D. melanogaster* Sfps - HR: 0.74; 95% CI (0.47, 1.14); p=0.17) (*D. simulans* Sfps - HR: 0.73; 95% CI (0.46, 1.17); p=0.19).

Quantitative Analysis between Allopatric and Sympatric Treatments: The linear mixed model showed significant differences in the induced post-mating effects between injection treatments ($F_{(2)}$: 56.05; p<0.001) concurrent with the data shown in Chapter 3. However, there was no significant difference between the allopatric and sympatric treatments regarding the effect of Sfps on post-mating receptivity after 12 generations ($F_{(1)}$: 2.74; p=0.24), indicating that there was no evolution of post-mating responses occurring within this study.

Allopatric 1



Allopatric 2

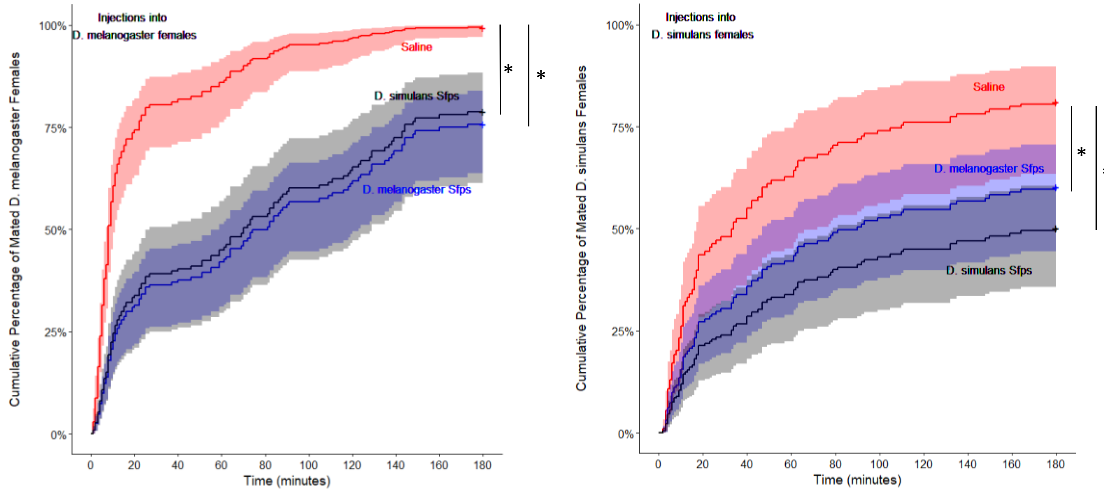
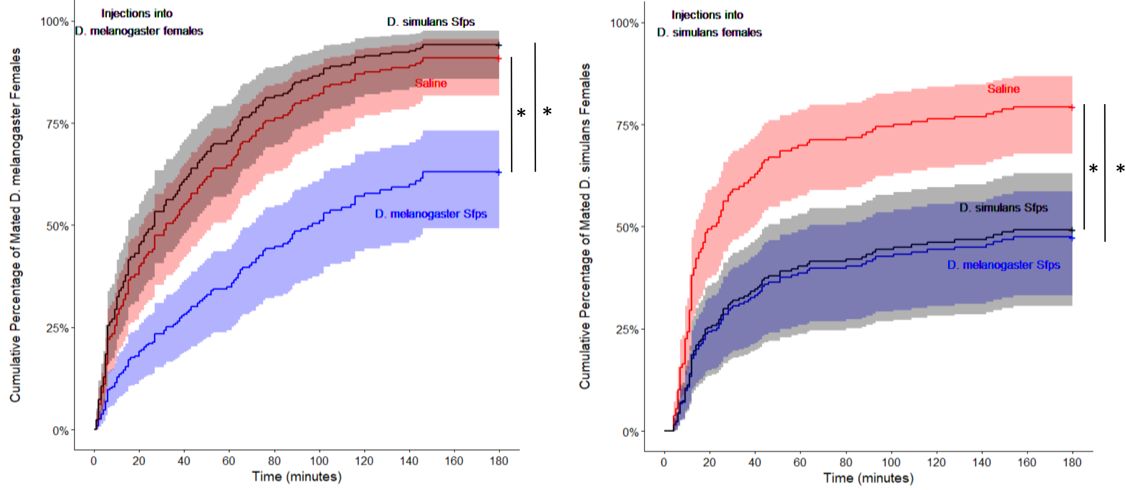


Figure 4.2: Percentage of *D. melanogaster* and *D. simulans* females from the two allopatric treatments that mated over the 3h remating assay period 24h following injection with either saline (red), *D. melanogaster* Sfps (blue) or *D. simulans* Sfps (black). Shown in the shaded areas are the 95% confidence intervals for each treatment. Asterisks indicate significant differences ($p<0.05$).

Sympatric 2



Sympatric 3

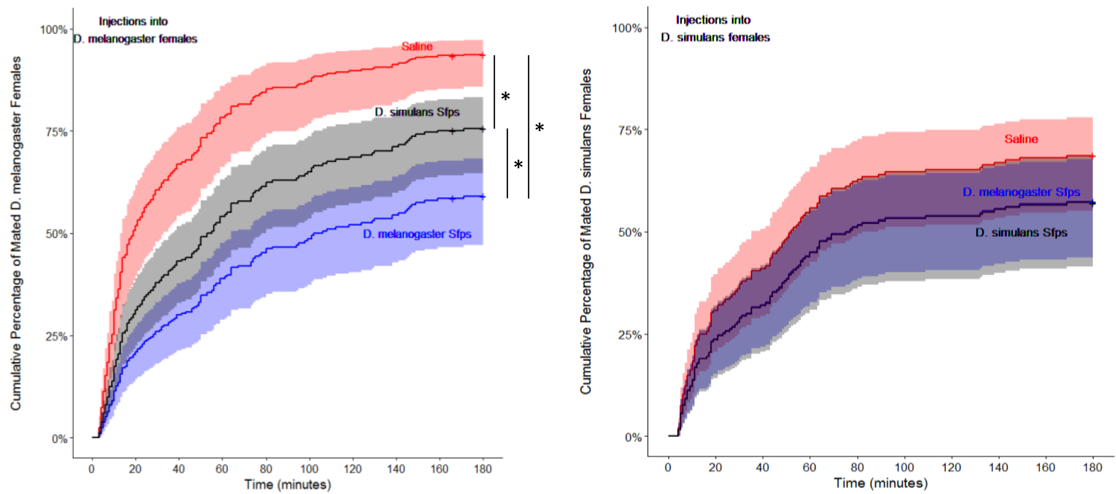


Figure 4.3: Percentage of *D. melanogaster* and *D. simulans* females from the two allopatric treatments that mated over the 3h remating assay period 24h following injection with either saline (red), *D. melanogaster* Sfps (blue) or *D. simulans* Sfps (black). Shown in the shaded areas are the 95% confidence intervals for each treatment. Asterisks indicate significant differences ($p<0.05$).

4.5 **Discussion**

This experiment demonstrated no clear pattern of resistance to hybrid mating evolving in 12 generations of sympatry between *D. melanogaster* and *D. simulans.* Pre-mating isolation assays showed no significant differences in hybrid mating frequency between allopatric and sympatric populations of *D. melanogaster* and *D. simulans*, nor did the frequency of hybrid matings within each treatment significantly change over time. Post-mating assays similarly showed no evidence of evolved resistance to Sfps as no significant differences were found between the responses of allopatric and sympatric treatments to cross species Sfp injections.

Frequency of Hybrid Mating in Sympatry

The initial base line mating assay showed that hybrid matings between *D. simulans* and *D. melanogaster* occurred in free choice environments in which individuals of both species could interact. As well as establishing a useful baseline for the experimental evolution experiment, this showed that hybrid matings were also observed under free choice assay conditions. This supports the idea that hybridizations between these two species will occur in sympatry under biologically realistic scenarios. 11% of all matings (30.4% of all *D. simulans* female matings) that occurred were between *D. melanogaster* (♂) and *D. simulans* (♀). In the no choice mating assays, the corresponding hybrid matings were more frequent and occurred ~50% of the time. That there were fewer hybrid matings in 'free choice' sympatry presumably results from the operation of conspecific choice and mate recognition mechanisms although the number of hybrid matings between *D. melanogaster* (♂) and *D. simulans* (♀) in this experiment was still relatively high. In agreement with the no choice assays (Chapter 2), hybrid crosses between *D. simulans* (♂) and *D. melanogaster* (♀) were not observed. The results suggested that satyrization between these two species is likely to be a biologically relevant occurrence. The presence of unidirectional hybrid mating over the

three days observed would have resulted in a proportion of *D. simulans* females suffering significant fitness costs via the production of inviable and infertile offspring.

Pre-Mating Resistance to Satyrization

There was no significant difference in hybrid mating frequency between the sympatric lines or allopatric lines during the 12 generations. The results therefore provided no evidence of evolved pre-mating barriers in the form of mate recognition in sympatry. These results contrast to previous research showing that resistance to satyrization can evolve in wild populations of *Aedes* mosquitoes in just a few generations (Bargielowski & Lounibos, 2014). One explanation for this discrepancy could be that differences in the strength of selection between satyrizing pairs due to differing fitness costs from hybrid mating between *Drosophila* and *Aedes* pairs. However, it is hard to establish any firm conclusions regarding *Drosophila* satyrization evolution due to the high variability and low repeatability of collected data across each mating experiment. The erratic mating behaviour of *Drosophila* species in this study means that even if some low level of satyrization resistance was occurring between *D. melanogaster* and *D. simulans*, it would be undetectable through the high stochasticity of the data shown here.

The large amount of work needed to maintain and test each line meant that I had only two or three replicates of each treatment and therefore low statistical power for this study. This made it extremely difficult to find a consistent pattern of mating success for each treatment, with each replicate varying rapidly across the timeframe of the study. If this study could be up-scaled with many more replicates per treatment, it may be possible to detect a clearer pattern of mating frequency across time and between treatments if such a pattern existed.

Though *D. simulans* (♂) x *D. melanogaster* (♀) matings were not observed in the initial base line mating assay (or in the no choice assays reported in Chapter 2), some of these hybrid matings were observed during the experimental evolution, albeit at a very low level.

However, the very low frequency of such matings was not expected to strongly select for *D. melanogaster* resistance to hybrid mating with *D. simulans* males and thus a change in hybrid mating frequency over time. It is unclear why the initial base line hybrid mating assay reported the lowest frequencies of hybrid mating over the whole experiment but it may again be a consequence of the highly stochastic hybrid mating rate of the *Drosophila* populations and the low sample size masking any patterns that may be present.

Post-Mating Resistance to Satyrization

It is difficult to establish firm conclusions from the post-mating assay again due to the inconsistency in the response of the *D. melanogaster* allopatric and sympatric treatments. *D. melanogaster* females in “allopatric 1” and “sympatric 2” did not respond to *D. simulans* Sfps. *D. melanogaster* females in “allopatric 2” by contrast were significantly less receptive to mating following receipt of *D. simulans* Sfps. “Sympatric 3” *D. melanogaster* females were responsive to Sfps of both species but conspecific Sfps significantly reduced their receptivity to a greater extent than *D. simulans* Sfps. In all *D. melanogaster* treatments, both the positive and negative controls were working, and the *D. simulans* Sfps also showed significant responses in *D. simulans* females, implying that injected Sfps were functional in both species. Why *D. simulans* Sfps injected into *D. melanogaster* females had such strikingly different effects is not clear but there are a few possibilities for further consideration.

One hypothesis is that genetic drift may have contributed to differences observed. This could be tested, in repeat experiments, by conducting additional injection experiments between replicates of the same treatment to look for variability in the strength of post-mating effects. An alternative hypothesis is that there was something that the mating cages for “allopatric 1” and “sympatric 2”, or “allopatric 2” and “sympatric 3” had in common that drove *D. melanogaster* females divergently towards resistance or susceptibility to *D. simulans* Sfps, respectively. However, all treatments were kept in the same room, had food vials changed,

and had eggs collected at the same time. Variation between treatments in the size of the cages also did not follow the divergent patterns seen.

The post-mating response assays did show more consistency in the Sfp injections into *D. simulans* females than *D. melanogaster* females. These females expressed the same receptivity pattern post-injection in three of the four treatments tested. The exception was "sympatric 3" in which *D. simulans* females were not significantly affected by *D. melanogaster* or *D. simulans* Sfps. As Sfps from both species had a significant effect in reducing receptivity in the corresponding *D. melanogaster* treatments, error in extracting the Sfps seems unlikely. Nor does a lack of statistical power within the treatment seem likely, as injection mortality in this treatment was the lowest among all treatments.

Statistically, there was no significant difference between allopatric and sympatric treatments in the post-mating effects induced by Sfp injections into females. Again, as the *Drosophila* populations displayed so much variation in this study, a much larger sample size would be beneficial to untangle any patterns that may or may not be present.

Satyrization

Satyrization resistance between species may occur if selection pressures were sufficiently high enough to select for it e.g. hybrid mating more frequently or stronger fitness costs from doing so. Hybrid matings between *Aedes* species occur between 35-55% in no choice experiments depending on the strain (Bargielowski & Lounibos, 2014), similar to the 50% no choice hybrid mating frequency between *D. melanogaster* and *D. simulans*. However, the refractory effect is larger in satyrized *Aedes* than *Drosophila.* In my injection experiments for these experimental populations, overall remating occurred at around 50% for *D. simulans* injected with *D. melanogaster* Sfps during the 3h assay 24h after injection. Only 5-10% of *Ae. aegypti* females remated during a 24h remating assay when injected with *Ae. albopictus* accessory gland proteins (Tripet et al., 2011). This stronger refractory effect leading to a

significant fitness cost for *Ae. aegypti* females may drive evolution of satyrization resistance in *Ae. aegypti* in sympatry quicker than *D. simulans* in sympatry. The initial frequency of resistance genes is important for the speed at which resistance could evolve in the absence of *de novo* mutations. Resistance genes already present in a gene pool will become beneficial when hybrid mating is present and will rapidly increase in frequency in only a few generations. However, if resistance genes are not already within a population, it may take significantly longer for resistance to evolve in other populations that is shown in studies of *Ae. aegypti*.

Further support for this comes from evidence that genes that confer resistance are expected to be costly to express or maintain (Kliot & Ghanim, 2012; David et al., 2018). Therefore, under strict allopatry, individuals that do not carry satyrization resistance alleles may be fitter, though the population as a whole would be highly susceptible to satyrization. Indeed, this is consistent with the studies investigating *Ae. aegypti* satyrization resistance. After the discovery that satyrization in *Aedes* quickly leads to resistance within just a few generations, subsequent research found that when selection was relaxed, i.e. previously sympatric populations of *Ae*. *aegypti* and *Ae. albopictus* were separated and placed into allopatry, resistance to satyrization decreased and the *Ae. aegypti* population again became largely susceptible to hybrid matings (Bargielowski et al., 2019). This further suggests that the costs of hybrid mating need to be high enough to for the evolution of costly resistance genes to be selectable and quickly increase in frequency within a population and that these costs mean that satyrization resistant alleles are unlikely to be ubiquitous across all species.

These are important considerations for the context of identifying target populations for satyrization control. The costs of resistance alleles mean that allopatric target populations are likely to be susceptible to satyrization in the absence of selective pressure from satyrization costs. This also means that some species may be unlikely to evolve satyrization

resistance in the first place as any *de novo* mutations leading the satyrization resistance may be costly and fall out of the gene pool immediately in the absence of satyrization driven selective pressure. However, for species that do contain satyrization resistance alleles, the ability for a species to quickly evolve resistance to hybrid mating may make it difficult for satyrization to contribute to competitive exclusion of the target species by the satyr species after a few generations in sympatry.

My results here demonstrate the difficulty of assessing such crucial information. Previous work on *Aedes* saw significant differences in hybrid mating incidence over just a few generations with three population cages of 150 members of each species (Bargielowski & Lounibos, 2014), a comparable scale to my work here in *Drosophila*. The higher variability in *D. melanogaster* and *D. simulans* hybrid mating points therefore to a lack of suitability for using *Drosophila* in this type of study, instead requiring a much higher sample size than *Aedes* to establish firm conclusions on satyrization resistance speeds.

This negative result, using *Drosophila* to quantify satyrization resistance speeds, highlights the varied and dynamic mating behaviour and evolutionary potential between different species, and the trouble with applying a catch all method of insect control to any and all pest species. As such, there are many considerations when using satyrization, and an assessment of satyrization resistance speeds on a population by population basis may be beneficial to predict how well satyrization will work in a target population and how best it can be applied in conjunction with other insect control strategies.

4.6 **Conclusions**

I did not find any evidence of the evolution of pre or post-mating resistance to satyrization by *D. simulans* females occurring within populations of *D. melanogaster* and *D. simulans* over 12 generations in sympatry. This negative result highlights the issues of varying evolutionary responses to satyrization between different pest species and populations, making resistance

speeds difficult to accurately test for all species and the results of one species unsuitable to apply to another. Therefore the application of satyrization in the field should take potential resistance into account on a case by case basis in order to deliver the most effective pest control strategy for each particular species.

Chapter 5

Design and Construction of an Underdominant Killer-Rescue Insect Control Strategy in *D. melanogaster*

5.1 **Abstract**

Genetic underdominance, whereby heterozygotes are less fit than homozygotes, can be artificially engineered as an insect control strategy. The strategy I discuss here used artificially-induced underdominance to create a killer-rescue system, based on the *Minute* phenotype. My aim was to build a test case in *Drosophila melanogaster*, aiming for eventual targeting of *Aedes* mosquitoes. *Minutes* are dominant phenotypes induced from knock-outs of specific Ribosomal Protein (*Rp*) genes and are characterised by low fertility, embryonic developmental delay, and short, slender and brittle scutellar bristles. As such, *Minute* individuals are poor competitors and have low fitness. The underdominant killer-rescue strategy of insect control exploits the *Minute* phenotype by creating a strain with knockouts of one or more of the *RpS19a*, *RpS21*, and *RpS26* genes and an unlinked recoded *Rescue* gene containing modified but functional versions of those same *Rp* genes. When underdominant males are introduced into WT populations and mate with WT females, a proportion of offspring will inherit just the knockout *Rp* (*Rp-Killer*) genes leading to the *Minute* phenotype and cause a reduction in pest population size. Other offspring will inherit the *Rp-Killer* genes along with the *Rescue* construct, preventing *Minute* expression and allowing the spread and persistence of both the *Rp-Killer* and *Rescue* genes (along with any attached cargo genes) through multiple generations. The *Rescue* gene will initially increase in frequency as it is advantageous to carry in a population that also contains lethal genes but will decrease in frequency when the *Rp-Killer* genes drop below a certain frequency threshold, allowing this system to be self-limiting. I created the requisite transgenic lines for proof of principle work. After successfully preparing the transgenic *Rescue* plasmid and transforming it into populations of *D. melanogaster* I found that carriers would not express the *Rescue* gene, as evidenced by a lack of fluorescent individuals in fluorescent screening and from qRT-PCR analysis. Additionally, PCR screening revealed that the *Rescue* plasmid was almost exclusively inherited by females and that transgenic males were rare. The results showed that potential issues with the target *Rp* genes, or the design of the *Rescue* construct need to be resolved before underdominance strategies targeting these loci can be fully evaluated.

5.2 **Introduction**

Genetic Strategies for Control of Insect Vectors

Genetic strategies to control insect vectors are at the forefront of many attempts to reduce disease in countries where arboviruses are endemic, especially diseases such as dengue (Rozera et al., 2019) that do not have a widely-available vaccine or cure. Traditional insect control methods such as insecticide fogging may lack long-term efficacy, may select strongly for resistance and are often indiscriminate, leading to harmful impacts upon beneficial pollinator species (Pryce et al., 2018; Abeyasuriya et al., 2017). Species-specific methods such as the Sterile Insect Technique (SIT) can offer more effective targeting and fewer side-effects and can be very effective in controlling insect populations (Bellini et al., 2013; Lacroix et al., 2012; Harris et al., 2011; Bellini et al., 2007). However, SIT is an intensive strategy that requires sufficient infrastructure for new cohorts of males to be irradiated and released every generation. This can be costly and laborious (Honma et al., 2019). Irradiated males are also usually less fit and less competitive than wild males due to the somatic damage that occurs due to irradiation, which can lower the effectiveness of SIT (Alphey et al., 2013). Genetic methods for insect control are therefore at the forefront of efforts to control disease vectors (Bouyer & Marois, 2018).

Release of Insect with a Dominant Lethal (RIDL) is a genetic strategy that is an evolution of SIT. It aims to overcome shortcomings, namely the low competitive performance associated with irradiating males, and the need to irradiate and release males every generation. RIDL works by rearing target insects in a lab that have been transformed with a tetracycline transactivator gene (tTAV) that constitutively expresses a toxic protein in the absence of tetracycline (Phuc et al., 2007). RIDL insects in the laboratory or factory are reared on diets containing tetracycline, which suppresses the expression of the tTAV lethal effector and thus allows the RIDL lines to be maintained. RIDL males are then released to the wild, passing on

the tTAV gene to their offspring. Offspring that express tTAV die due to the absence of tetracycline in the wild. Female-specific versions of RIDL are also available (Labbé et al., 2012) in which the lethality is expressed only in females. This allows some persistence of the RIDL genes in the wildtype (WT) population beyond the initial release generation. RIDL has been used successfully in field trials, with the performance of RIDL males being sufficient to affect control (Carvalho et al., 2015; Patil et al., 2015; Massonnet-Bruneel et al., 2013). Though not as frequently as for SIT, it too requires multiple releases, and thus could be complimented by additional, more persistent but self-limiting drive methods.

There is huge current interest in using gene editing and gene drive techniques to produce transgenic males with control potential that have a similar fitness to their WT counterparts and that can persist in the wild through multiple generations, drastically reducing monetary and labour costs. Gene drives can come in two forms, those that are self-limiting and eventually drive themselves locally extinct, and those that cause permanent population or trait replacement. Theoretically, replacement gene drive systems can become fixed within a species, although the resulting fitness costs could contribute to extinction of the species (Oye et al., 2014). For this reason, global gene drives and non-reversible gene drives have garnered some controversy due to the ecological consequences of altering population dynamics, and potential off target effects such as horizontal gene transfer to non-target species (Collins, 2018; National Academies of Sciences, Engineering and Medicine, 2016).

Here I focus on the development of a two-locus underdominant killer-rescue gene drive that is designed to be either self-limiting, reversible, or both. Self-limiting drives sustain themselves in the population for a number of generations before falling out of the gene pool. Reversible drives have several built in safety mechanisms that allow the gene drive to be easily stopped and removed from a population if desired (Noble et al., 2019; DiCarlo et al., 2015). Two-locus underdominance broadly describes heterozygous offspring from hybrid

mating between two different parental strains that are homozygous for different alleles at two unlinked loci being less fit than each parental strain. It can be exploited for use as a genetic method of insect control (Edginton & Alphey, 2018; Reed et al., 2013). Two-locus underdominance can be artificially induced in target insect vectors to render a proportion of a population inviable every generation (Reeves et al., 2014). The ultimate strategy I explore here aims to exploit an underdominant killer-rescue system by releasing transgenic cohorts of *Aedes aegypti* that contain unlinked lethal and *Rescue* genes, into WT populations. Transgenic and WT alleles segregate independently and individuals that inherit only the lethal gene die, reducing *Aedes* numbers. However, the *Rescue* gene can mask the lethality, allowing those that inherit the *Rescue* gene and lethal alleles to mate and pass these genes off to their offspring. This results in the lethal alleles spreading through a population and persisting for several generations. In addition to killing a proportion of offspring each generation, a cargo gene conferring a desired effect could be linked to the *Rescue* gene and driven through the population to be inherited by viable offspring that inherit the *Rescue* gene. This could be a refractory gene, for example, that prevents carriers from being able to transmit disease (Gould et al., 2008; Alphey et al., 2002)

Here I describe an underdominant killer-rescue system in *D. melanogaster* based on inducing the *Minute* phenotype, which causes delayed development, reduced female fertility and slender scutellar bristles, resulting in reduced viability and fitness (Marygold et al., 2007). There are many *Minute* loci, with the mutant phenotype being caused by knockouts of Ribosomal protein (*Rp*) genes that are key for protein production. I describe the design and construction of the underdominant killer-rescue strategy developed together with my CASE partner, Luke Alphey at The Pirbright Institute. I develop this underdominant killer-rescue system in *Drosophila melanogaster* as a test case to provide proof of concept for its effectiveness as a method of insect control.

Underdominant Killer-Rescue Strategy

The *Minute* phenotype can be induced with the knockout of just one *Rp* gene. However, three genes were targeted (*RpS19a*, *RpS21*, and *RpS26*) in this study, to ensure that the *Minute* phenotype would be expressed. In *D. melanogaster* these genes are located on chromosomes X, 2L and 2L respectively (Thurmond et al., 2019). The underdominant killer-rescue system relies on a *Rescue* transgene, a recoded gene cassette containing functional copies of *RpS19a*, *RpS21*, and *RpS26* to be inserted into the genome. This *Rescue* transgene nullifies the costly effect of the *Rp* knock-outs (*Rp-Killer*) and prevents the *Minute* phenotype from being expressed in individuals that carry it.

Figure 5.1 explains how this two-locus system would work in field releases. Transgenic individuals that carry two copies of the *Rescue* transgene and two copies of the *Rp-Killer* genes mate with WT individuals that have two functional *Rp* copies but no copies of the *Rescue* transgene. The G_1 offspring produced are double heterozygotes, each being heterozygous carriers of one functional copy of each *Rp* gene, one copy of the *Rp-Killer* genes and one copy of the *Rescue* transgene to counter the effect of the *Rp-Killer* genes. These G_1 offspring will be viable and perform competitively almost as well as wildtype individuals, as the *Rescue* transgene prevents the expression of the *Minute* phenotype. A quarter of the gametes produced by the double heterozygote G_1 individuals will contain *Rp-Killer* genes and no *Rescue* transgene. Hence 5/16 of the offspring will have very low fitness. However, 11/16 of the offspring will be viable, either because they are homozygous for functional endogenous *Rp* genes, homozygous for the *Rescue* transgene, or heterozygous for functional endogenous *Rp* genes and the *Rescue* transgene. This sustains the *Rp-Killer* gene, *Rescue* genes, and any cargo genes attached to the *Rescue* genes in the population and allows them to spread throughout the population for multiple generations. G_2 offspring will continue to mate, with proportions of offspring each generation being inviable or competitively unfit due

to inheritance of the *Rp-Killer* genes without the *Rescue* gene, while other offspring inherit the *Rescue* transgene to sustain the *Rp-Killer* genes in the population and spread desired traits conferred by the cargo gene.

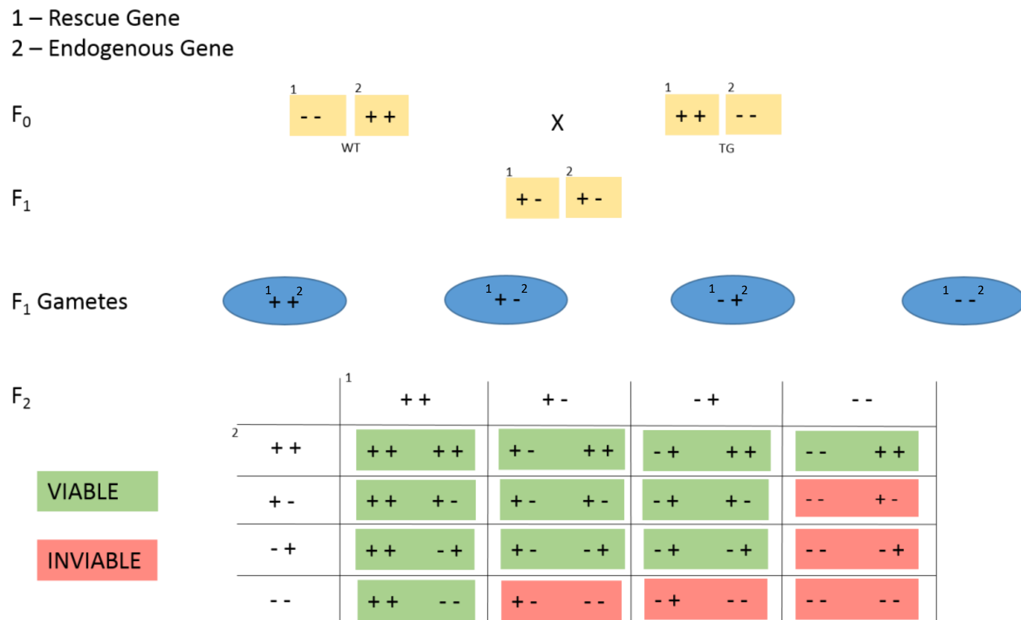


Figure 5.1: The mechanism of the two-locus killer-rescue underdominance strategy for insect control. WT individuals that are homozygous carriers of functional endogenous *Rp* genes mate with individuals homozygous for the *Rp-Killer* and *Rescue* transgene. G_1 offspring are double heterozygotes, all carrying only one functional copy of the *Rp* genes, *Rp-Killer* genes and *Rescue* transgene which nullifies the *Minute* phenotype. G_1 gametes containing *Rp-Killer* genes and no *Rescue* transgene will create inviable or competitively unfit offspring when paired with most other gamete combinations, as shown in the Punnett square. On average, 5/16 of the progeny in this population will die, but 11/16 will suffer no serious deleterious effects, allowing the *Rescue* construct and *Rp-Killer* genes to persist in the population. 10/16 will be viable and will inherit at least one copy of the *Rescue* gene as well as any cargo genes attached to the *Rescue* gene.

This strategy works to reduce insect populations and prevent the transmission of disease while saving money, time and effort by reducing the frequency of transgenic insect releases required. The *Rescue* transgene itself also confers a slight fitness cost, either due to increased genetic load or a designed fitness cost to prevent the *Rescue* gene reaching fixation. Therefore, this is a self-limiting strategy in which all transgenes in the population eventually drive themselves extinct.

5.3 Development of the Underdominant Killer-Rescue Strategy in *D. melanogaster*

The *Rescue* transgene design developed by the Alphey laboratory for *Aedes aegypti* was designed to contain recoded versions of the ribosomal protein genes *RpS19a*, *RpS21* and *RpS26*. I used this same strategy for the test case in *D. melanogaster* with the aim of conducting eventual proof of principle tests with direct comparability. I identified *Rp* homologues in *D. melanogaster* using Flybase (Thurmond et al., 2019) and designed edits to the genes according to three main guidelines to ensure maximum efficacy and safety:

(i) Edits made to the *Rescue Rp* genes had to be synonymous with the endogenous *Rp* genes in order to be functionally identical to the endogenous *Rp* genes.

(ii) Synonymous mutations in the *Rescue* genes had to be induced at every 14bp or fewer. This would allow the *Rescue* genes to be unique for specific targeting and inactivation using RNAi both as a safety measure in case the *Rescue* gene needed to be removed from the population to facilitate the development of future gene drive designs (Reeves et al., 2014).

(iii) "NGG" codons had to be synonymously mutated in the *Rescue* genes. NGG codons represent the Protospacer Adjacent Motif (PAM) site needed for the

Cas9 enzyme to attach and modify a gene using CRISPR. Removing the NGG sites allows the endogenous gene to be targeted and modified using CRISPR without also modifying the *Rescue* genes. TGG is an exception to this as it is the only codon that encode Tryptophan and so was not modified.

A region of 200-250bp in the coding sequence (CDS) of each target gene was chosen for editing according to these guidelines. Each gene in the *Rescue* transgene consists of the full promoter and 3' UTR for that gene which was to be inserted into a piggyBac plasmid containing an HR5-IE1 dsRed2 fluorescent marker and an attP and loxP site for further site directed modifications (e.g. adding cargo genes) if desired (Figure 5.2).

Figure 5.2: The ribosomal protein gene *Rescue* construct for *D. melanogaster*. It is designed to contain functional but synonymously modified versions of all three target *Rp* genes, including the promoter, CDS and 3'UTR regions (arrows represent 5' & 3' direction of the sense strand for each gene). The construct also contains an attP and a LoxP site for further integration of genetic material such as cargo genes via site-specific recombinase and a HR5-IE1 dsRed2 fluorescent marker for identifying transgenics. The construct was synthesised at Genewiz and cloned into a piggyBac vector for injection into *D. melanogaster* embryos. Marked with black arrows are the location and orientation of the primers used to screen the dsRed2 gene in identifying carriers of the transgene. Also marked are unique restrictions between each gene of interest for site directed excisions and insertions.

Transforming *D. melanogaster* with the *Rescue* Plasmid

A 6292bp genetic fragment containing the three target *Rp* genes and the SV40 3'UTR region was synthesised by Genewiz. The fragment was eluted in resuspension buffer and

transformed into XL10-Gold Ultracompetent cells to produce an abundant stock which was stored at -20°C. The pBac-HIS-attP-HR5IE1-dsRed-LoxP vector was digested with NotI and AscI and ligated to the *Rp* fragment. The newly formed ligated product containing the *Rp* fragment in the pBac-HIS-attP-HR5IE1-dsRed-LoxP *Rescue* plasmid was transformed into XL10-Gold Ultracompetent cells and screened with a colony PCR.

Positive colonies (Figure 5.3) were grown up in lysogeny broth (LB) at 37°C overnight. The desired plasmid was extracted using a Nucleospin Midiprep kit using the manufacturer's protocol. The full *Rescue* plasmid was quantified using a Nanodrop at 6.03µg/µl and stored at -20°C.

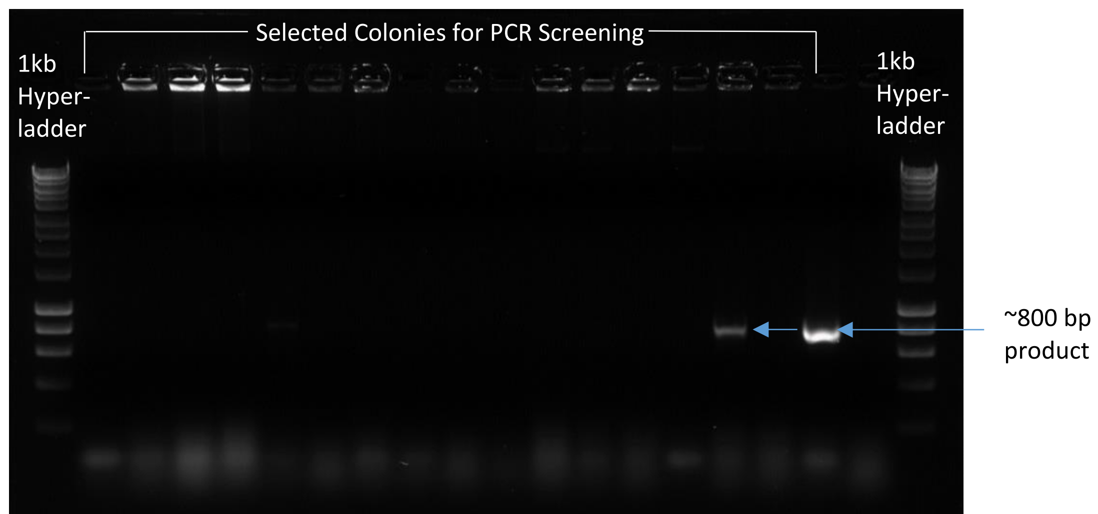


Figure 5.3: Colony PCR of the synthesised *Rescue* construct ligated into the pBac-HIS-attP-HR5IE1-dsRed-LoxP vector. Product size was ~800bp, positive samples that were incubated in LB broth are highlighted by the blue arrows.

Injecting *D. melanogaster* embryos with the *Rescue* plasmid

Two sets of injections of the *Rescue* plasmid into the *D. melanogaster* embryos were carried out. Round 1 of injections were performed by myself at The Pirbright Institute and round 2

was carried out by an external provider (Fly Facility, Department of Genetics, University of Cambridge).

Round 1: For injections into *D. melanogaster* embryos, an injection mix was made up to 30µl as follows (Table 5.1):

Table 5.1: Components of the injection mix used to create the transgenic rescue lines of *D. melanogaster* with an insertion of the *Rescue* plasmid. The final volume was made up to 30µl.

Component	Concentration (ng/µl)	Recipe (µl)
***Rescue* Construct Plasmid DNA**	500	2.5
piggyBac Transposase	300	3.3
Injection Buffer	-	3
Endotoxin Free H_2O	-	21.2

D. melanogaster embryos were collected by allowing Dahomey WT stocks to lay on apple juice agar plates (600ml H_2O, 18g agar, 20g sugar, 200ml apple juice, 20ml 20% w/v Nipagin per batch of medium) for 20-30 minutes. Embryos were individually picked from the agar using forceps and stuck on a microscope slide using double sided tape. 2µl injection mix was backloaded onto a capillary needle attached to an Eppendorf Femtojet and the air pressure was manipulated until a steady stream of injection mix was flowing from the needle. Embryos attached to the slide were covered with a thin layer of halocarbon oil and injected individually with the chorion still attached. After the entire slide of embryos was injected, the slide was placed into a dish of H_2O for 5 minutes to wash off the surface oil with the embryos still attached to the slide. Washed embryos were placed into a 7ml SYA vial (Sugar Yeast Agar: 30 ml 10% w/v Nipagin solution, 3ml propionic acid, 15g agar, 50g sugar and 100g brewer's yeast per litre of medium) and placed into a 26°C 12:12 light:dark CT incubator. The dates, number of embryos injected and survivorship of the embryos are given in Table 5.2.

Round 2: The Fly Facility created alternative TG *D. melanogaster* lines with an injection mix composed of 0.6µg/µl *Rescue* plasmid and 0.4µg/µl piggyBac transposase helper plasmid (atub-pBac-K10) into their w^{1118} line conferring a white eye marker (Table 5.2). 70 injected G_0s survived and were each given a unique number to identify them for screening.

Table 5.2: Two rounds of *D. melanogaster* microinjection. The first at the Pirbright Institute and the second by the Fly Facility. Date, number of injected *D. melanogaster*, number and % of the G_0 survivors and the number and % of G_0 lines positive for the transgene are shown.

Date	Number of Embryos Injected	Number of G_0 Survivors	% of G_0 Survivors	Number of TG G_0 Survivors	% of TG G_0 Survivors
Round 1 of Injections (Pirbright)					
03/05/2017	5	0	0	12	16.2
17/05/2017	56	1	1.8		
19/05/2017	30	4	13.3		
31/05/2017	152	21	13.3		
09/06/2017	189	29	15.3		
23/06/2017	358	19	5.3		
Round 2 of Injections (Cambridge)					
31/10/2017	200	70	35	8	11.4

Screening

Round 1: Injected *D. melanogaster* embryos were kept in the CT incubator until eclosion. Eclosed individuals were screened under a microscope to look for any transient expression of fluorescence, and then placed into a 7ml SYA vial with a male or virgin female WT *D. melanogaster* of the opposite sex in order to produce G_1 offspring. All G_0 crosses were treated as an individual line and given an identifying number. G_1 offspring were screened upon eclosion for fluorescence at The Pirbright Institute and positive G_1 individuals from each G_0 pair were placed together into their own vial to establish a transgenic stock originating

from that G_0. I attempted to make homozygous derivatives from the positive *Rescue* lines in order to cross to lines of *D. melanogaster* with knockouts of the *RpS21* and *RpS26* genes. However, once the stocks were moved to the University of East Anglia, the fluorescence was no longer easily visible and the lines from round 1 of injections were not maintained. Lines from round 2 were identified and maintained using PCR screening as an alternative screening method.

Round 2: G_1 offspring from each of the G_0 lines were PCR screened for the presence of the *Rescue* gene with primers designed around the dsRed2 gene (FWD primer: ACAACACCGTGAAGCTGAAG. REV Primer: TGTAGTCCTCGTTGTGGGAG) (Figure 5.2). Of the 70 lines originating from G_0 injections, eight of them were positively identified as carrying the *Rescue* transgene (11.4%) (Table 5.2) and four of these independent integration lines were kept and established as transgenic stocks. These lines were denoted as C4, C6, C55 and C62.

As well as PCR screening, G_1 offspring from each line were screened using a Zeiss Stereo Lumar.V2 fluorescent microscope under a dsRed filter (excitation 563 nm, emission 582 nm) to detect expression of the transgene. No expression from the fluorophore in any of the four lines could be seen. This is the same issue as occurred in round 1 of screening, where individuals positive for carrying the *Rescue* gene did not fluoresce under the fluorescent microscopes at University of East Anglia. This indicated that there may have been an issue with the expression of the *Rescue* gene expressing at either low levels or not at all. The transgenes were therefore maintained using PCR screening each generation.

No docking system was used and hence transgene insertions were random. I attempted to map the location of the insertion for each line by carrying out both a 5' and 3' inverse PCR with primers designed inside the piggyBac flanks to amplify the unknown sequence (5' IPCR FWD Primer: TGTTCTACTTACGTGATAACT. 5' IPCR REV Primer: CCAAGCGGCGACTGAGATG. 3' IPCR FWD primer: CGGTCTGTATATCGAGGTTTA. 3' IPCR REV Primer:

GATTATCTTTCTAGGGTTAA). The amplified 3' product of ~200bp was Sanger sequenced by Eurofins. However, the sequencing data was unresolved and no firm conclusions could be made about where the *Rescue* gene was within the genome of each line.

I therefore attempted to create a homozygous stock of each line with which to combine with the *RpS21* and *RpS26* knock out (KO) mutants. Homozygosity of each line was attempted by establishing single pair lines of transgenic flies, and genotyping each of the parents retrospectively for the presence of the transgene. Large scale screening of individual males and females from each line revealed that a very small number of males carried the *Rescue* transgene (9/414, 2.13%). This is in contrast to females, in which the frequency was ~50% positive. Table 5.3 shows the number of individuals positive and negative for the *Rescue* transgene in each line. I also screened pools of DNA comprised of 10 males per sample from the transgenic lines. I screened eight samples per line (80 males) and again found few positives (4 samples out of 24 samples positive for the transgene). The lack of males scoring positive for the transgene prevented the establishment of a stock of homozygous transgenic (TG) killer-rescue *D. melanogaster*.

Table 5.3: Number of individual *Drosophila* PCR screened for the presence of the transgene. Males and females were screened separately, as denoted below. The plus symbol indicates individuals positive for the transgene, the minus symbol those that were negative for the transgene. The percentage of positives and negative individuals in each sex from each line is shown.

	C55				C4				C62				C6			
	♀+	♀-	♂+	♂-	♀+	♀-	♂+	♂-	♀+	♀-	♂+	♂-	♀+	♀-	♂+	♂-
Total	75	66	0	126	41	50	0	83	91	71	4	145	27	34	5	53
%	53	47	0	100	45	55	0	100	56	44	3	97	44	56	9	91

The low frequency of males was initially hypothesised to be due to male lethality. This could be due to a potentially toxic, sex-specific effect of the expression of elevated levels of *Rp* gene product, killing the males before they could hatch as larvae or eclose as adults. Male inviability could also be caused by the *Rescue* transgene inserting into essential genes on the X chromosome, causing hemizygous lethality in males. Three strategies were employed to test this hypothesis.

(1) Sex Ratio Bias: First, I tested for sex ratio biases in the sex ratio of the transgenic lines. If the *Rescue* gene was killing males before eclosion, there should be significantly more females than males in the stocks. To test this, I set up single pair crosses from two of the transgenic lines, mating one male and one female, both originating from a transgenic mother from the same line, in each pair. I left each pair in a vial for three days to lay eggs. I screened the combined DNA from each pair for the presence of the transgene and compared the sex ratio of the offspring from positive pairs compared to negative pairs. This test showed no difference in offspring sex ratio between the positive and negative pairs which strongly indicated that the lack of males inheriting the *Rescue* gene was not due to male inviability as a result of toxic overexpression of the *Rescue* gene (Table 5.4).

Table 5.4: The sex ratio of offspring from parents positive for carrying the *Rescue* gene as confirmed from PCR screening, compared to offspring from parents who are negative for carrying the *Rescue* gene. Shown are the number and percentage of males and females from each parent pair. Also shown are the transgenic line from which each parental pair was derived.

TG Line	Transgenic Parents?	Male	Female	Male %	Female %
C4	No	10	10	50	50
		12	13	48	52
		10	3	77	23
		9	12	43	57
		15	12	56	44
	Yes	10	9	53	47
		22	21	51	49
		14	5	74	26
		23	22	51	49
		12	14	46	54
C62	Yes	10	9	53	47
		3	9	25	75
		21	16	57	43
		5	10	33	67
		6	4	60	40

(2) Attempting to Rescue Potential *Rp* Overexpression: Next, females carrying the *Rescue* transgene were crossed to two lines containing a knockout for endogenous *RpS21* (Kyoto Stock Center, Stock No. 103045, Genotype: $y^{1}w^{67c23}$;P{lacW}$RpS21^{k16814}$/CyO) and *RpS26* (Bloomington Stock Center, Stock No. 12048, Genotype: P{PZ}$RpS26^{04553}$/CyO;ry^{506}). Offspring from these crosses would have three functional *Rp* alleles from the *Rescue* transgene and four functional endogenous *Rp* alleles (two functional endogenous *RpS19a* alleles and one functional endogenous *RpS21* and *RpS26* allele) compared to six functional endogenous *Rp* alleles present in WT *D. melanogaster*. This could reduce any toxic effect of elevated *Rp* expression. After the eclosion of the first adult offspring from the vial of each cross between *Rescue* and KO parents, I incubated the vials for several days more to account for the developmental delay of possible transgenics before collecting males for PCR screening. Male offspring from these crosses were PCR screened for the presence of the

Rescue transgene. After the first cross to *RpS21*, 0 out of 24 PCR screened males tested were scored as positive for the *Rescue* transgene. Female offspring from this first cross that were positive for the *Rescue* transgene were then crossed to the second mutant KO strain – *RpS26*. Males from this second cross were again PCR screened but 0 out of 24 of them were positive for the *Rescue* transgene. Knocking out two of the three *Rp* alleles failed to rescue male viability and from this it seemed unlikely that toxic overexpression was causing male inviability.

(3) Detecting Expression of the *Rescue* Transgene: Finally, I measured expression of the *Rescue* transgene in transgenic females to further test the 'toxic overexpression' hypothesis and to attempt to explain the absence of transgenic dsRED fluorescence. This was performed using a quantitative reverse transcriptase PCR (qRT-PCR) using primers that flanked an area within the recoded *RpS19a* coding region (FWD: TTGGTAATCGAACCGACGCC. REV: CAAGACCGCCAAGTTCAAG) and primers that flanked an area within the endogenous *RpS19a* (FWD: CCGACTGGTTCTATGTGCGT. REV: GGTGATCGAACCGACTCCAG) and endogenous *RpS26* (FWD: AATCCACTCCAAGGTGGTGC. REV: CTTGGGGAAGGAACGCAGT) coding regions. RNA from WT and TG female *D. melanogaster* was extracted using a mirVana miRNA isolation kit, following the manufacturer's protocol. Each sample of RNA was extracted from a combination of 10 female *D. melanogaster* from wildtype lines (WT) and *Rescue* lines (TG). Nanodrop values were recorded following treatment of the extracted RNA with DNase: WT 1 – 399.7ng/µl; WT 2 – 253ng/µl; TG 1 – 347ng/µl; TG 2 – 244ng/µl. This RNA was used to assemble cDNA using Quantiscript Reverse Transcriptase and the following Nanodrop values of cDNA were recorded: WT 1 – 2.87µg/µl; WT 2 – 3.05µg/µl; TG 1 – 2.99µg/µl; TG 2 – 2.76µg/µl. Using this cDNA, the qRT-PCT was carried out using a StepOnePlus RT-PCR system with components and cycling conditions that are stated on Table 5.5.

Table 5.5: Components and cycling conditions used in the qRT-PCR. Shown are the components used in the qRT-PCR, the concentration, and the quantity of each component that was added. The temperature and time of each step and the number of cycles used in the denaturation, annealing and extension step are also shown.

qRT-PCR Components			
	Concentration	**Quantity (µl)**	
SYBR Green	-	10	
FWD Primer	10µM	1	
REV Primer	10µM	1	
Nuclease Free H_2O	-	7	
cDNA	2-4ng	1	
Cycling Conditions			
	Temperature	**Time**	
Initial Denaturation	95ºC	30s	-
Denaturation	95ºC	15s	40 Cycles
Annealing/Extension	58ºC	15s	

Primers were first tested by carrying out a PCR on the *Rescue* plasmid as a positive control. After confirmation that they were working correctly, the qRT-PCR was then performed with cDNA from WT and TG females (Figure 5.4, Figure 5.5, and Figure 5.6). The results from this showed that the endogenous *Rp* genes were expressing in both the WT and TG lines. Expression of the majority of the TG and WT cDNA samples were detected between 4-8 cycles above the background negative control when amplified with the endogenous *RpS26* primers, and between 2-5 cycles above the background negative control when amplified with the endogenous *RpS19a* primers. This was as expected, as both lines contain endogenous *Rp* genes and thus this also acts as a positive control. When amplified with the recoded *RpS19a* primers, expression of all TG and WT DNA samples was similar to the background negative control, suggesting that *Rescue* gene expression is at least 2^3 fold lower than the expression of the endogenous genes. This result was expected for the WT lines (no *Rescue* genes = negative control). However, the absence of expression of the *Rescue* genes in the TG cDNA meant that the *Rescue* gene was either not expressing at all, or expressing at very low levels,

rejecting of the toxic overexpression hypothesis and also explaining why fluorescence could not be seen in females carrying the *Rescue* gene.

	Primers amplifying endogenous *RpS19a* and *RpS26*	Primers amplifying the recoded coding region of *RpS19a*
cDNA from Wildtype *D. melanogaster*	Positive for detectable expression	No detectable expression
cDNA from transgenic lines of *D. melanogaster* carrying the rescue gene	Positive for detectable expression	No detectable expression

Figure 5.4: Summary results from the qRT-PCR showing the patterns of *Rp* expression detectable from WT *D. melanogaster* and transgenic *D. melanogaster* females containing the *Rescue* plasmid. Primers were designed within and to amplify the CDS of endogenous *RpS19a* and *RpS26* genes and the CDS of the recoded *RpS19a* gene

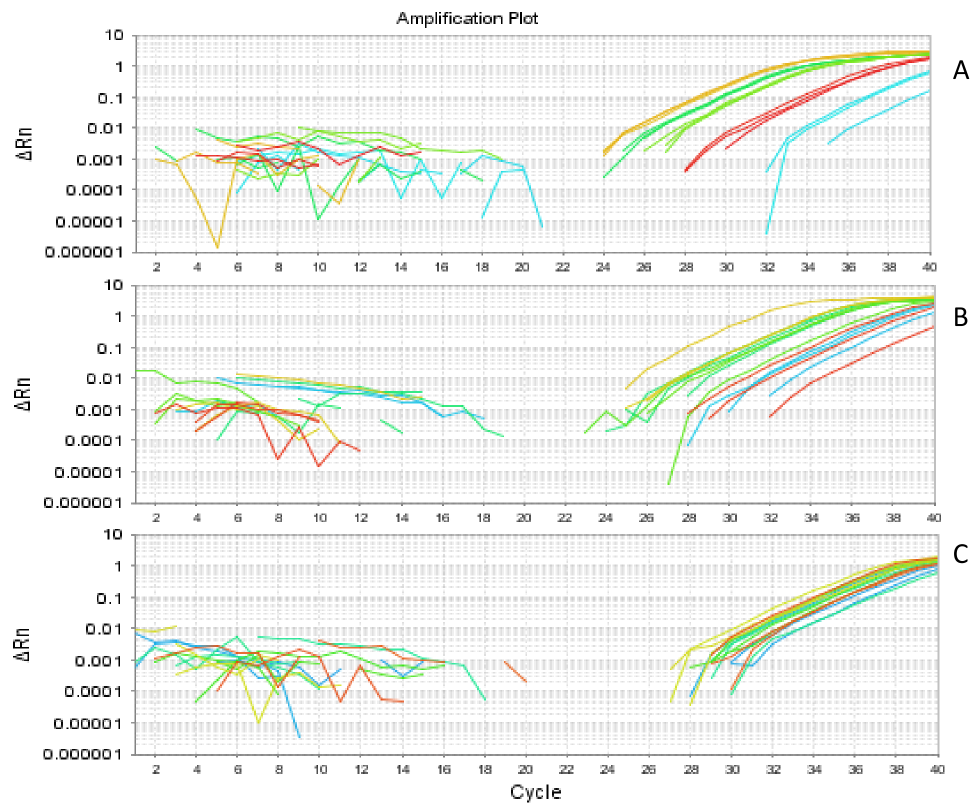


Figure 5.5: Quantitative Real Time PCR (qRT-PCR) amplification plots carried out on TG1 (red), TG2 (yellow), WT1 (light green), WT2 (dark green) cDNA, and a negative control (blue) containing no cDNA. The three graphs show the three different sets of primers used to amplify a different target gene. These primers were designed to amplify (**A**) Endogenous *RpS26* (**B**) Endogenous *RpS19a* (**C**) Transgenic *RpS19a*. WT and TG cDNA is strongly amplified with the endogenous *RpS26* and *RpS19a* (though amplification of TG1 is low when amplified with endogenous *RpS19a* primers) compared to the negative control (**A** + **B**). The recoded *RpS19a* primers did not seemingly amplify any of the WT or TG cDNA samples, presenting an amplification profile similar to the negative controls (**C**) indicating either no expression or very low expression of the *Rescue* gene.

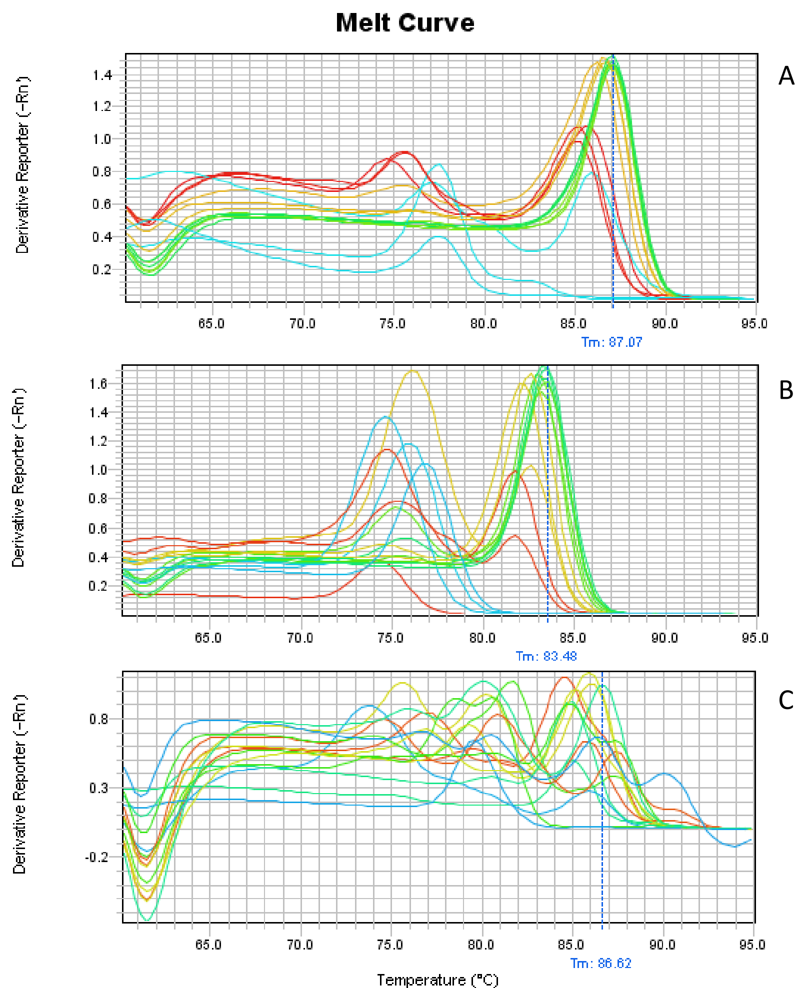


Figure 5.6: Quantitative Real Time PCR (qRT-PCR) melt curve plots carried out on TG1 (red), TG2 (yellow), WT1 (light green), WT2 (dark green) cDNA, and a negative control (blue) containing no cDNA. The three graphs show the three different sets of primers used to amplify a different target gene. These primers were designed to amplify (**A**) Endogenous *RpS26* (**B**) Endogenous *RpS19a* (**C**) Transgenic *RpS19a*. These melt curves again show strong amplification of all DNA samples from primers designed around the endogenous *RpS26* and *RpS19a* genes compared to the negative control (**A** + **B**). The recoded *RpS19a* primers did not seemingly amplify any of the WT or TG cDNA samples, presenting an amplification profile similar to the negative controls (**C**) indicating either no expression or very low expression of the Rescue gene.

5.4 **Discussion**

Here I designed and attempted to develop components for a killer-rescue underdominance system as a test case in *D. melanogaster.* I successfully produced the recoded *Rescue* transgene and introduced it in two sets of microinjection experiments. However, the results of the in depth investigation I performed on round two of the microinjected individuals and their descendants showed that only a very low % of males ever carried the *Rescue* transgene. The fluorophore of the transgenic construct was also difficult to visualise in flies of either sex in both rounds of injection, indicating an issue with expression of the transgene.

I initially hypothesised that the transgene was toxic to males, rendering male carriers of the *Rescue* genes inviable. This hypothesis is supported by studies that show that haploinsufficient genes are sensitive to overexpression, and that a single extra copy can cause growth defects (Morrill & Amon, 2019). My transgenic lines have three extra *Rp* copies, therefore it is likely that this could result in a toxic effect. However, it was unclear why this overexpression would only affect males. Several reasons for this can be proposed:

- Males may be unable to regulate expression and so even a tiny amount of overexpression of *Rp* genes renders them inviable, whereas females may have the ability to silence overexpression. This could explain why the transgene was present in females, as shown in PCR screening, but did not exhibit any dsRED expression under the fluorescent microscope.
- Endogenous *Rp* expression might be naturally lower in females – hence they may be able to tolerate elevated expression from the transgene whereas males cannot.

Alternatively, males could have been inviable due to the *Rescue* gene randomly inserting into essential genes on the X chromosome for each line, causing hemizygous lethality in males.

To test the potential reasons for the lack of transgenic males, I conducted three sets of tests: to observe sex ratio of lines from transgenic parents, to rescue viability by crossing the *Rescue* lines to *Rp* KO lines, and to quantify expression of the *Rescue* genes using qRT-PCR. These tests suggested that the rarity of males was unlikely to be due to male inviability. This then raised the question of why I did not observe males positive for the transgene in my PCR screening. It is possible that the males in my PCR screening tests were carrying the transgene but were manifesting as false negatives. However, each PCR was run with a positive control (the *Rescue* plasmid diluted in H_2O) and positive females were common in all screens. This would suggest that any false negatives would have to be male specific, which is highly unlikely.

Expression levels from the qRT-PCR also suggested that the *Rescue* genes were not being expressed in the transgenic lines, explaining why dsRED could be detected in any of the insertion lines. One explanation for this is that the *Rescue* genes may have been randomly inserted into transcriptionally repressed areas of chromatin. Alternatively, it is possible, if unlikely, that mutations could have appeared in the *Rescue* transgene, rendering it non-functional. A mutation may also have been present in the original recoded *Rescue* construct, though this again seems unlikely.

It is unclear that a gene that shows no detectable expression should have shown such a strong sex-specific effect. It is possible that *Rescue* gene expression was present but so low as to be undetectable. The sensitivity of haploinsufficient genes to overexpression may mean that even very low expression could produce these unusual effects, which would indicate that these three target *Rp* genes (*RpS19a*, *RpS21*, and *RpS26*) may not have been suitable for this type of insect control system.

Therefore, building the construct again from the ground up, using just a single *Rp-Killer* gene that could be rescued using a single *Rescue* gene would be a prudent first step. After

confirmation that this single gene system works, a second and third *Rp-Killer* and *Rescue* gene could then be added one at a time, and swapped out if shown not to work, for an effective three gene system.

Additionally, it would be beneficial to resolve the sequencing data in order to fully characterise the insertion of the *Rescue* gene. Knowing where the *Rescue* gene is within the genome may offer an explanation as to why males are not inheriting the gene.

My results show that the underdominant killer-rescue system developed here does not work in *D. melanogaster* in its current form. Despite this, the underdominant killer-rescue strategy holds many advantages as a method of insect control due to its persistence in a population and ability to be self-limiting. Future work, through simplifying the *Rescue* gene or targeting different haploinsufficient genes, should be carried out to establish a working example in *D. melanogaster*.

5.5 <u>**Conclusion**</u>

Here I attempted to develop an underdominant killer-rescue underdominant strategy in *D. melanogaster.* I was able to transform *D. melanogaster* with the *Rescue* gene, however I could not make a homozygous derivative stock for crossing because males carrying the *Rescue* transgene were hardly ever obtained. qRT-PCR analysis also showed that the transgene was not expressed in females that carried the *Rescue* transgene. This may suggest that the transgenic construct in its current form is not suitable for developing this underdominant killer-rescue system though the strategy itself still holds promise and alternative approaches could be taken to develop a working example in *D. melanogaster*.

Chapter 6

Simulating the Release of an Underdominant Killer-Rescue Strategy for Insect Control

6.1 **Abstract**

Artificially-induced genetic underdominance can be used as a method of insect control. The strategy described here and in Chapter 5 is to introduce the deleterious *Minute* genotype, and cargo genes attached to the *Rescue* gene, into an insect population (resulting in high fitness costs and population reduction of the target vector population and spread of desired traits expressed by the cargo gene). However, rather than a one-shot strategy, the design of the underdominant killer-rescue strategy allows the costly *Minute* phenotype and cargo genes to spread and to be maintained within the population for multiple generations before eventually driving itself extinct. Here I aimed to quantify fitness costs of *Minute* expression and use these data as parameters in a model to simulate the release of genetic constructs that deliver this control strategy in the wild. I first experimentally measured the fitness costs of knocking out expression of two *Minute* expression loci, *RpS21* and *RpS26*. Development time was significantly slower, and viability significantly lower than for the wildtype (WT). The actual fitness costs arising from developmental delay were then quantified experimentally, by allowing a population to establish, before introducing into it recently emerged genetically marked individuals at defined time points and measuring their competitive fitness. The results showed that a 24h developmental delay reduced offspring production by two thirds to only 33%. After 48h this dropped to 5%. True fitness costs may be even higher given other known fitness costs of *Minute* expression such as reduced fertility. Using my experimentally-determined fitness estimates, along with a range of fitness costs for the *Rescue* allele, I then used simulation modelling to predict the spread and persistence of underdominant control constructs in a 1:1 release over multiple generations. This showed that, even if each copy of the *Rescue* allele reduced fitness by 25%, the underdominant killer-rescue control system could spread and remain in the population for around 10 generations. If each copy of the *Rescue* allele reduced fitness by 10%, the underdominant killer-rescue system could spread for up to 20 generations before declining sharply and being lost. Hence the requirement for repeated releases of insects when using this strategy could be significantly reduced in comparison to existing technologies such as Sterile Insect Technique (SIT) and Release of Insects carrying a Dominant Lethal (RIDL). However, if *Rescue* fitness reduction was as low as 5% per allele, the model showed a higher risk of the *Rescue* allele reaching fixation, hence removing its self-limiting properties. Overall, the simulations show that underdominance systems based on *Minute* expression have promise as effective and long-lasting methods of insect control.

6.2 **Introduction**

My previous research attempted to develop and build a test case of underdominant killer-rescue insect control in *D. melanogaster,* based on *Minute* expression (Chapter 5). Caused by a disruption in several key Ribosomal protein (*Rp*) genes, the deleterious *Minute* phenotype is characterised by low fertility and delayed embryonic development. The fitness costs of reduced fertility are relatively easy to quantify. However, testing the actual fitness costs of developmental delay is harder and there are some conflicting data. Some studies have shown that developmental delays induced by temperature effects can reduce the absolute number of emerging offspring but may not affect their competitive performance (Fick & MacQuarrie, 2018). Other research shows a competitive disadvantage to developmental delay because slow developing individuals contain less fat body (Olcott et al., 2010). Developmental delay is predicted to reduce fitness in several ways:

(i) Larvae carrying the *Minute* phenotype will emerge into a feeding environment after others, risking nutrient limitation, as resident larvae may have already stripped the substrate of high quality nutrients.

(ii) Slower development when resources are limited may hinder the potential for an individual to sexually mature and mate, thereby severely slowing the spread of their genes through the population.

(iii) Reduced potential for adaptation due to fewer generations per unit time, which could result in a reduction of fitness in a rapidly changing environment.

A primary advantage of the underdominant killer-rescue strategy is the potential for reduced monetary and labour costs. Techniques such as Sterile Insect Technique (SIT) and Release of Insects carrying a Dominant Lethal (RIDL) have been effective and widely used to great effect (Bellini et al., 2013; Lacroix et al., 2012; Harris et al., 2011; Bellini et al., 2007). However, the

effectiveness of SIT can be negatively impacted by the costs of irradiating males every generation, which is laborious, expensive and causes potential somatic damage that puts males at a competitive disadvantage (Alphey et al., 2013). An ability to drive through a self-limiting gene that sustains itself within the population for the long term over multiple generations is also attractive as it could reduce the amount of insect rearing and releases needed to achieve effective pest control. The underdominant killer-rescue strategy has these advantages. It is composed of two genetic components that allow it to be sustained within a population for multiple generations at low fitness cost to the released individuals. The first component is the fitness-reducing mutation(s), in this case knockouts (KO) of three endogenous *Rp* genes – *RpS19a, RpS21* and *RpS26*. Heterozygous loss of function of these haploinsufficient *Rp* genes results in the expression of the *Minute* phenotype, whereas homozygous loss of function results in inviability. Secondly, it contains a *Rescue* gene that encodes functional versions of the *Rp* genes, on a separate chromosome. This *Rescue* gene nullifies the deleterious effects of the *Minute* mutations. Because the *Rescue* construct is on a separate chromosome to the endogenous KO *Rp* (*Rp-Killer*) genes, both components segregate independently and hence some offspring will inherit just the *Rp-Killer* genes without the *Rescue* gene and suffer the fitness costs associated with the expression of the *Minute* phenotype. However, a proportion of offspring will inherit the *Rp-Killer* genes along with the *Rescue*, allowing them to survive, mate, and pass on the detrimental *Rp-Killer* genes. Cargo genes can also be attached to *Rescue* genes that produce a desired trait, such as a gene that makes carriers refractory to disease and thereby preventing transmission to humans (Gould et al., 2008). As the cargo genes are attached to the *Rescue* genes, these desired traits will also spread. The presence of the *Rescue* gene therefore allows the underdominant killer-rescue system to propagate through a population for several generations, significantly reducing the labour and costs incurred by other insect control strategies

The spread of the system throughout a population is self-limiting. The *Rescue* allele itself has a slight fitness cost to carrying it due to the genetic load of carrying genetic constructs, which will eventually result in the *Rescue* allele being lost from the population. This is a designed safety feature to prevent the artificial construct from becoming a full gene drive system and from reaching fixation in a population. This reduces the risks of any unexpected health or ecological effects.

I constructed a test case underdominant killer-rescue system in *D. melanogaster* (Chapter 5). However, in this I was not able to establish homozygous stocks of *D. melanogaster* lines containing the *Rescue* gene and thus was not able to collect experimental data on its effectiveness in a controlled laboratory setting. Here I adopted an alternative and complementary approach, to use theoretical simulations to model a release of an underdominant killer-rescue system, parameterised by laboratory fitness data, to assess its effectiveness in wild populations. To provide accurate parameters of a key and hard to estimate fitness effect of *Minute* expression, I first experimentally measured the actual fitness costs of developmental delay (rather than just estimating the presence of the delay itself). I then used the fitness cost data I obtained as a parameter to theoretically simulate the release of the underdominant system in a wild release scenario. This was to test for how long the underdominant system would be sustained within a wild population. I simulated the release over a range of different fitness costs associated with the possession of the *Rescue* allele. Finally, I then compared my experimentally parameterised model with a fully theoretical one constructed for similar underdominant systems (Edgington & Alphey, 2018).

In an initial experiment I tested the developmental delay caused by the expression of the *Minute* phenotype resulting from the knockout of *RpS21* or *RpS26.* Quantification of the fitness costs associated with delayed embryonic development came from a series of experiments in which I set up replicate populations containing wildtypes (WT) into which I

introduced later-emerging, genetically-marked *Stubble* individuals over 0h to 48h. I then tested the fitness of WT and introduced individuals over the subsequent 24h. Release of the underdominant killer-rescue system was modelled using a modified version of the simulation model of Godwin et al. (2018). This model allowed me to input the fitness values attained from the developmental delay experiment and test for the frequency and persistence of *Rp-Killer* and *Rescue* alleles under biologically realistic simulation parameters.

6.3 **Methods**

Quantifying Developmental Delay

Mutant *D. melanogaster* expressing *Minute* from knockouts of *RpS26* or *RpS21* were sourced from Bloomington Stock Center (Stock No. 12048, Genotype: P{PZ}RpS26^{04553}/CyO;ry^{506}) and Kyoto Stock Center (Stock No. 103045, Genotype: y^{1}w^{67c23};P{lacW}RpS21^{k16814}/CyO) respectively. Wildtype Dahomey *D. melanogaster* were used as a control. All experiments and culturing was conducted in a 25°C constant temperature (CT) room with 12h:12h light:darkness and 60% relative humidity.

Eggs were collected by placing a purple agar plate (550ml H_2O, 25g agar, 300ml red grape juice, 21ml 10% w/v Nipagin solution per batch of medium) supplemented with live yeast paste into a mating cage containing either WT Dahomey*, RpS21* or *RpS26 D. melanogaster* for 3h. Plates were removed and incubated for 24h to allow eggs to hatch. The resulting larvae were picked, 50 larvae per 7ml SYA (Sugar Yeast Agar: 30 ml 10% w/v Nipagin solution, 3ml propionic acid, 15g agar, 50g sugar and 100g brewer's yeast per litre of medium) vial, to give a total of 10 WT, 7 *RpS26* and 4 *RpS21* vials.

To measure developmental timings, from the 5th day, third instar larvae were spot-checked every day at 9:00, 13:00 and 17:00. Upon pupation, pupae were marked with a permanent marker and the time and day they pupated was recorded. Following this, pupae were

observed for the time taken to eclose. Upon eclosion, adults were sexed and discarded with the eclosion time and date being recorded.

Experimental Simulation of the Fitness Effects of Developmental Delay

D. melanogaster eggs were collected on petri dishes containing purple grape juice agar supplemented with live yeast paste over 3h. Dishes were placed into a mating cage containing either WT or *Sb D. melanogaster* and resulting larvae were picked and placed 100 per SYA vial and incubated until eclosion. Virgins were collected upon eclosion, separated by sex, and stored 10 per vial.

Flies were maintained in these conditions for 4-6 days, to allow them to reach sexual maturity. Two mating cage treatments (denoted 24h and 48h) were then set up, each containing three SYA vials and 50 male and 50 female WT *D. melanogaster*. In the 24h treatment, WT flies were left to mate and oviposit for 24h before 50 male and 50 female *Sb* flies were introduced. Likewise, in the 48h treatment, 50 male and 50 female *Sb* flies were added 48h after the introduction of WT flies. Flies were left in each mating cage for 24h to allow for oviposition after the introduction of the *Sb* flies before being discarded. Food vials removed from both cages were placed in the 25°C CT room until the eggs hatched and eclosed into adults. Adults were segregated into sex and scored for expressing the WT or *Sb* phenotype. As *Stubble* is homozygous lethal, some offspring sired by the *Sb* adults do not show the *Sb* phenotype. To account for this, the total counted *Sb* offspring was multiplied by 1.75 and the difference between calculated and observed *Sb* offspring was subtracted from the WT total to estimate genotype frequencies and the relative abundance of resident versus introduced competitor. This allowed me to calculate the fitness costs of delayed entry into the mating pool due to developmental delay.

Modelling: This stochastic model was modified from the one used in Godwin et al. (2018) and simulated a 1:1 release of the underdominant killer-rescue system into wild populations.

The fitness of each potential genotype in the simulation was defined by the combination of WT, *Rescue* and *Rp-Killer* alleles they contain (Table 6.1). 50 female WT *Drosophila* and 50 male *Drosophila* carrying the killer-rescue system made up the initial starting population. The males and females were denoted as having two alleles per locus. Each allele of the first locus was either "A" or "a" corresponding to the presence of a functional *Rp+* gene ("A") or a loss of function, mutant *Rp-Killer* gene ("a"). Each allele of the second locus could be either "B" or "b", corresponding to whether they did not ("B") or did ("b") carry the *Rescue* allele. Each male and each female were paired randomly and 50 offspring per pair were generated with each allele from the male randomly paired up with a corresponding allele from the female. 70% (Mossman et al., 2019) of the offspring survived with the probability of survival being dependent on the allocated fitness value of each genotype. The surviving offspring themselves then randomly paired up, producing 50 offspring per pair with a 70% survival rate and this cycle continued for 200 generations with 20 repetitions. The genotypes from all survivors per generation were recorded and the frequency of the *Rescue* and *Rp-Killer* alleles in the population at each generation calculated. All modelling was carried out using R v3.2.2. (R Core Team, 2012)

Table 6.1: The relative fitness values of the alleles and genotypes in the model. Shown are the relative fitness costs of carrying each allele, the fitness categories of each genotype, the genotypes that are to be produced in each generation and the relative fitness of each genotype based on the allele fitness. The simulation was run with a range of five fitness costs for carrying the *Rescue* allele, as shown below.

Allele	Fitness Reduction of Carrying the Allele	Fitness Category	Genotypes	Fitness Reduction of the Genotypes
A, B	0%	Wildtype	AABB	0%
b	Range of Values: 5%, 10%, 15%, 20%, 25%	Heterozygous Rescue	AAbB, AabB, aAbB, AABb, AaBb, aABb	Range of Values: 5%, 10%, 15%, 20%, 25%
		Homozygous Rescue	AAbb, Aabb, aAbb, aabb	Range of Values: 10%, 20%, 30%, 40%, 50%
a	95%	*Minute*	aaBB, aaBb, aabB, aABB, AaBB	95%

6.4 **Results**

Developmental Delay in *Minute* Mutants

Pupation: Wildtype *D. melanogaster* pupated significantly quicker and had a higher viability compared to both lines containing *Rp* knockouts. This is consistent with what is known about *Minute* individuals, whose lack of *Rp* expression results in insufficient protein production for early development leading to developmental delay and high mortality. Both *Rp* mutant lines pupated at a similar speed but *RpS21* KO lines had higher viability than *RpS26* KO lines (Figure 6.1).

Eclosion: Similar to the pupation rate, WT *D. melanogaster* eclosed significantly faster and with a higher viability than was found for either of the two *Rp* knockout lines. Again, *RpS21* KO lines showed higher viability than *RpS26* KO lines, but both lines eclosed at a similar rate (Figure 6.1).

Experimentally Simulated Developmental Delay in *D. melanogaster*

24h delay: When *Sb* individuals arrived 24h later to the mating arena, their offspring made up only 33.74% of the overall offspring population. Hence approximately twice as many WT offspring than *Sb* were produced following a 24h introduction delay (Table 6.2, Figure 6.2).

48h delay: There was a dramatic change in offspring proportions in the 48h delay treatment. Under these conditions, *Sb* offspring made up just 5.11% of the total offspring (Table 6.2, Figure 6.2).

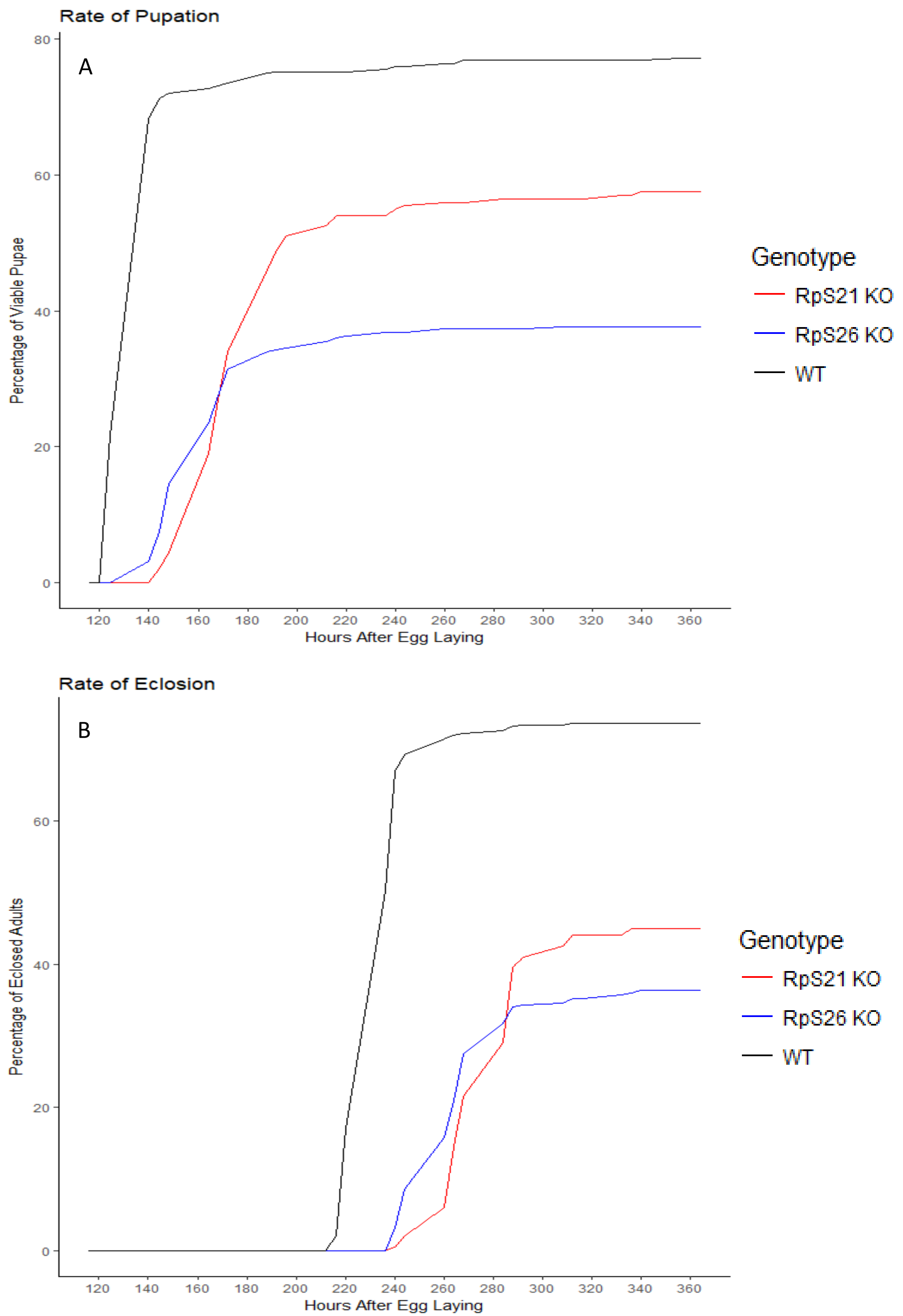
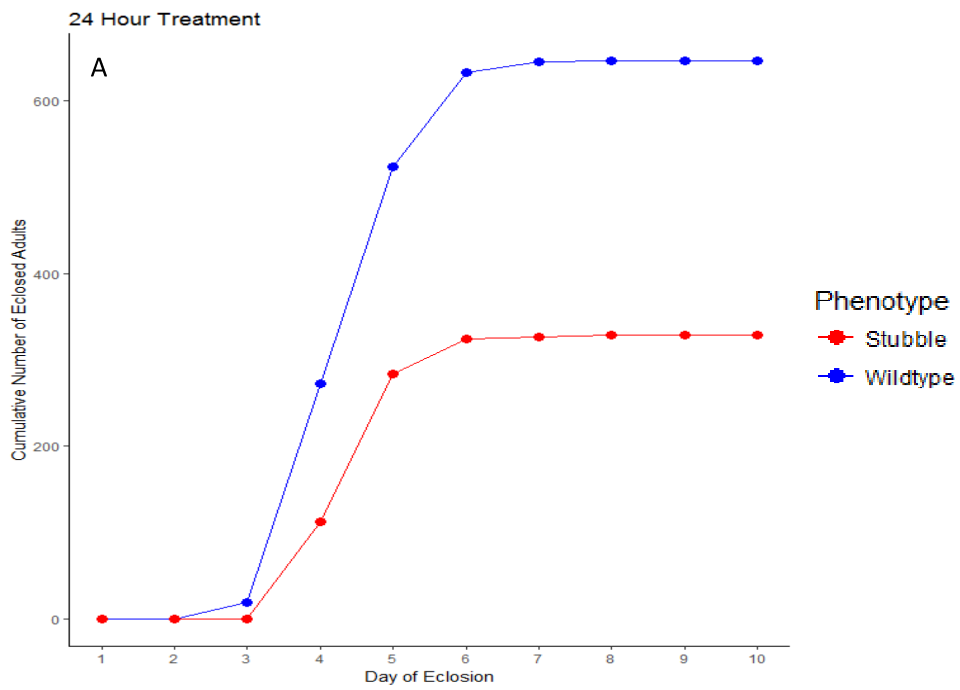


Figure 6.1: Cumulative line graphs showing the rate and percentage of individuals that pupated (A) and eclosed (B) from WT (Black), *RpS21* knockout (Red) and *RpS26* knockout (blue) *D. melanogaster* lines. Pupation and eclosion was measured at set intervals three times a day until all eclosion had ceased.



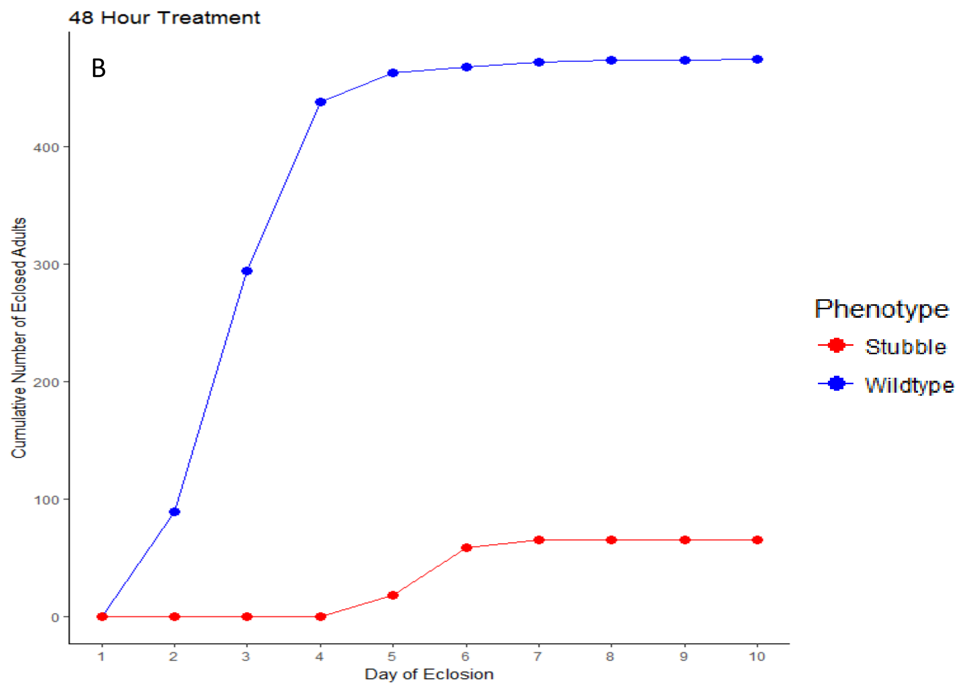


Figure 6.2: Line graph showing the cumulative number of eclosed adults expressing the *Sb* (Red) and the WT (Blue) phenotype when *Sb D. melanogaster* were placed into the population cage 24h (A) and 48h (B) after WT *D. melanogaster*. Eclosed offspring were counted every day for 10 days.

Table 6.2: The results of the 24h developmental delay experiment. Progeny production over 10 days since the introduction of *Sb* flies is shown. WT refers to offspring sired from WT parents, *Sb* refers to offspring sired from *Stubble* parents and the vial refers to the vial from which each offspring was counted. Three vials were placed into each mating cage for the adults to lay eggs into. The percentage at the bottom of each table shows the overall percentage of offspring that were from WT and *Sb* parents.

24h Delay

Vial	**1**		**2**		**3**	
Day	**WT**	***Sb***	**WT**	***Sb***	**WT**	***Sb***
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	6	0	5	0	8	0
4	57	42	53	40	143	30
5	59	39	64	9	129	124
6	30	5	12	2	68	33
7	1	0	3	0	7	2
8	0	2	0	0	1	2
9	0	0	0	0	0	0
10	0	0	0	0	0	0

% Total: WT – 66.26%

***Sb* – 33.74%**

48h Delay

Vial	**1**		**2**		**3**	
Date	**WT**	***Sb***	**WT**	***Sb***	**WT**	***Sb***
1	0	0	0	0	2	0
2	89	0	62	0	89	0
3	205	0	117	0	48	0
4	144	0	156	0	95	0
5	25	0	30	0	37	18
6	5	0	10	0	26	40
7	4	0	0	0	22	7
8	1	0	0	0	8	0
9	0	0	0	0	0	0
10	1	0	1	0	6	0

% Total: WT – 94.81%

***Sb* – 5.19%**

Modelling the Spread of *Rescue* and *Rp-Killer* Alleles in a Simulated Release of the Underdominant Killer-Rescue System

Using 5% of the WT fitness as a baseline fitness for the *Rp-Killer* allele (taken from the result of the 48h developmental delay experiment), the spread of the *Rescue* and *Rp-Killer* alleles in a 1:1 controlled release was modelled (Figure 6.3). The *Rescue* allele will suffer some unknown fitness cost due to position effects from the insertion of a genetically engineered construct or from an artificially inserted fitness cost, and thus the simulation modelled a range of potential *Rescue* allele costs: 5%, 10%, 15%, 20% and 25% per allele. Fitness costs of the alleles in this model were additive and therefore these costs were doubled in homozygous individuals. When each *Rescue* allele had a fitness reduction of 25%, the *Rp-Killer* allele was lost from the population at around 10 generations, with the *Rescue* allele being lost a few generations later. The underdominant killer-rescue system persisted for longer at a *Rescue* allele fitness reduction of 15% and 20%, with the *Rp-Killer* allele being lost at around 20 generations. When the *Rescue* fitness reduction was 10%, *Rp-Killer* persisted for approximately 30 generations. Interestingly, when the cost of *Rescue* was only 5%, the *Rp-Killer* alleles remained in the population for between 20-40 generations on average but the *Rescue* allele could become fixed within the population which occurred in 1/20 runs of the simulation. Upon *Rescue* fixation, *Rp-Killer* could remain in the population indefinitely.

At each fitness cost, the *Rescue* allele initially rose in frequency in each simulation. This is because the *Rescue* allele masks the effects of *Minute* while the *Rp-Killer* allele is common in the gene pool. As the *Rp-Killer* allele becomes rarer, the *Rescue* allele stops being advantageous to carry and the fitness cost associated with the *Rescue* allele causes it to fall out of the gene pool some generations after the *Rp-Killer* allele.

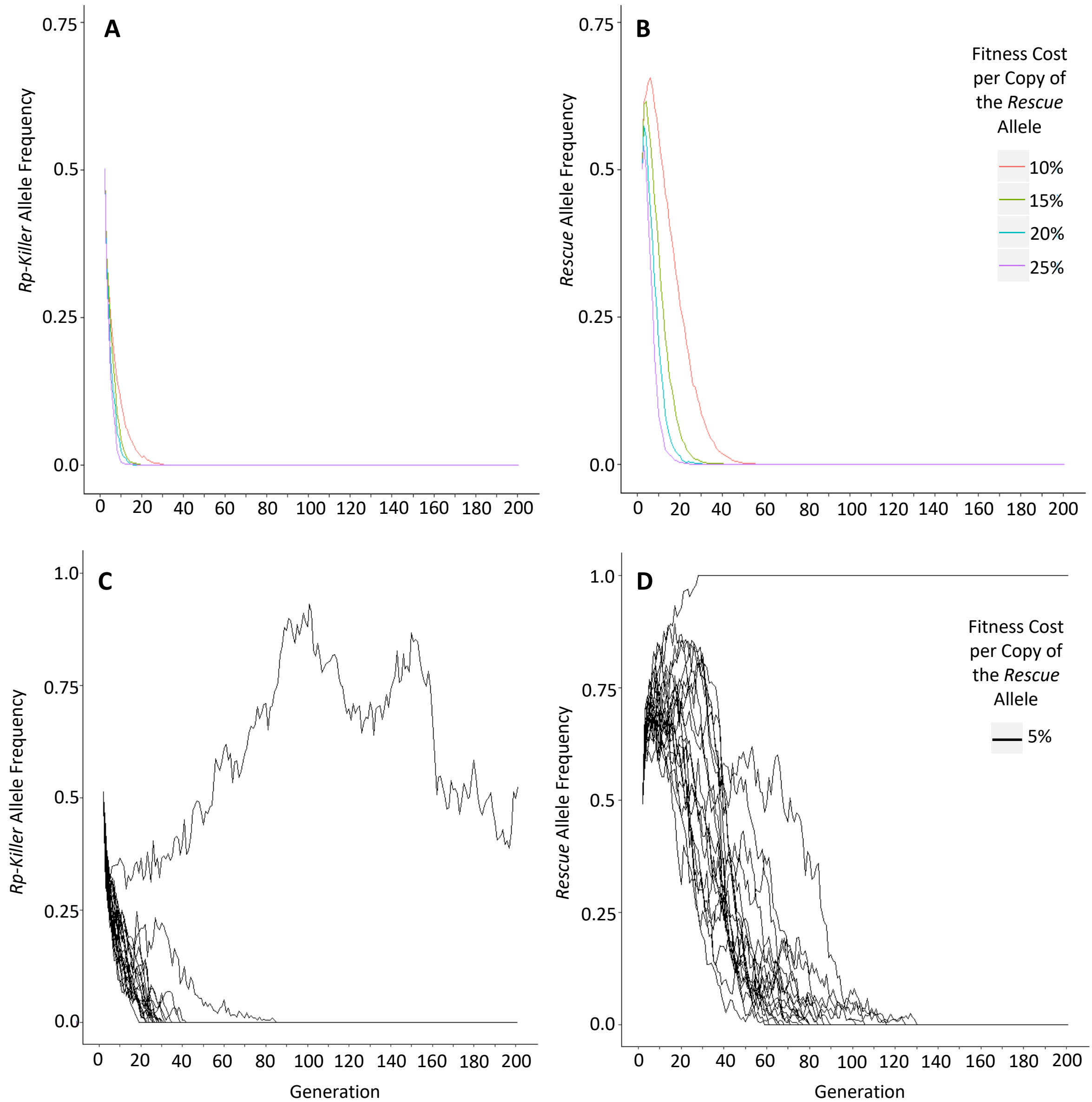


Figure 6.3: Simulation of the spread of the *Rp-Killer* (**A**) and the *Rescue* (**B**) allele in a controlled 1:1 killer-rescue:WT release (i.e. 50 killer-rescue males and 50 wildtype females). The *Minute* genotypes confer a fitness reduction of 95% compared to WT fitness. The *Rescue* allele has been altered in each simulation to confer a relative fitness reduction of 25% (purple), 20% (blue), 15% (green), 10% (orange) per copy. Each line in the graph is a mean of 20 runs of the simulation. Simulation of the spread of the *Rp-Killer* (**C**) and the *Rescue* (**D**) allele in a controlled 1:1 killer-rescue:WT release (i.e. 50 killer-rescue males and 50 wildtype females. The *Minute* genotypes confer a fitness reduction of 95% compared to WT fitness. The *Rescue* allele confers a fitness reduction of 5% (black) per copy. All 20 runs of the simulation are displayed.

6.5 **Discussion**

The results here suggest that the underdominant killer-rescue system holds promise for use as a method of insect control. I found high fitness costs associated with one aspect of the *Minute* phenotype, showing that inducible *Minute* is an effective method of reducing wild insect populations. Using *Minute* costs as parameters in a simulation demonstrated that the underdominant killer-rescue system can remain in a population for up to 30 generations and be at a high enough frequency to be effective for 20 generations while still remaining self-limiting. Even at a high cost of carrying the *Rescue* allele, the *Rp-Killer* genes can remain in the population for up to 10 generations, a marked increase on other currently-used insect control methods such as classical SIT. This model has also shown that in small populations with limited migration, the *Rescue* allele can reach fixation. Therefore the *Rescue* allele requires some sort of engineered fitness cost to avoid possible fixation if the *Rescue* allele is not sufficiently costly to carry.

Determination of Developmental Delay of *Minute* Expression

The initial experiment to determine the length of developmental delay in *Rp* KO lines showed that a knockout of the target *Rp* genes *RpS21* and *RpS26* produced a mean developmental delay of 40h and 22h respectively. This is likely to be a conservative estimate of the delay of *Minute* mutants as the *Rp* KO lines tested have only a single gene knocked out, whereas the finished killer-rescue construct will have three *Rp* gene knockouts. As each functional *Rp* gene is important for protein production it is possible that there could be a stacking effect, and that individuals with knockouts of three *Rp* genes would suffer stronger effects of *Minute* in comparison to those with just a single one. In addition, the developmental delay experiments do not take into account reduced fertility and viability of *Minute* individuals, which are likely to magnify fitness costs even further.

The results suggest that developmental delay can incur multiple costs to the individual. Firstly, a delayed individual has to spend longer in substrate as a larva in competition with others. By the time this larva hatches, the substrate is less than optimal as earlier larvae are present and may have depleted or spoiled the food source. The introduced competitor larvae may then have to spend longer in this suboptimal substrate because of the developmental delay. The resources may not be adequate to sustain development, especially as the competitor larvae near pupation. Secondly, there is a decreased potential for delayed populations to expand compared to WT populations. Delayed individuals grow and reproduce slower than their WT peers resulting in fewer generations per unit time period compared to a WT population. Additionally, fewer offspring increases the risk of *Minute* extinction through intraspecific competition as *Minute* individuals may be competing with a proportionally higher number of WT individuals.

Quantifying the Fitness Costs of Developmental Delay

Individuals placed into a population cage with a 24h delay were 33% as fit as WT flies and those introduced with a 48h delay were only 5% as fit as WT populations in terms of offspring production. This represents a substantial fitness cost for developmental delay and demonstrates that the majority of delayed larvae are inviable due to hatching into low quality food, or that delayed adults struggle to lay eggs in food already saturated with larvae.

Sb flies were placed into the mating cages as adults, which may have suppressed the full fitness cost of developmental delay. Had the parental flies been placed into the treatment as larvae, developmental delay costs would be twofold across both generations. Parental flies may have suffered fitness costs because of malnutrition due to being placed late into the substrate as larvae. This malnutrition may have made them eclose even later than the 48h that was designated for this test. Thus, their offspring may have been laid even later into worse quality food and been even less viable than what was seen here.

Additionally, the 48h developmental delay experiment resulted in the *Sb* population being only ~5% as fit as their WT counterparts in terms of offspring production due to simulations of delay from a single *Rp* gene knockout. The effect of three *Rp* knockouts combined with the other fitness costs associated with *Minute* i.e. low fertility and viability is likely to even further reduce fitness of *Minute* individuals with this construct. Therefore, the fitness costs presented by the data are likely to be extremely conservative, with actual *Minute* individuals being even less fit than their WT counterpoints than is shown here. This indicates that the use of *Minute* as an inducible method of insect control holds promise to reduce populations of target insect vectors.

Efficacy of the Underdominant Killer-Rescue System and the Spread of *Rp-Killer* and *Rescue* Genes

The simulations showed that the underdominance strategy could persist in the population for multiple generations, while simultaneously being self-limiting due to fitness costs associated with the *Rescue* allele. Even when the *Rescue* allele has a high cost to carrying it (25% fitness reduction per copy), the *Rp-Killer* allele will remain in the population for almost 10 generations (Figure 5.3). When the *Rescue* allele costs are relatively small (10% per copy), the *Rp-Killer* alleles remain in the population for almost 30 generations, though the *Rp-Killer* alleles sharply reduce in frequency at around generation 20 with the *Rescue* alleles reducing shortly after, whereby only a small proportion of the offspring will carry lethal combinations. As the *Rescue* gene declines in frequency, any attached cargo genes would also sharply decline. Therefore, a release every 20 generations would be advantageous for effective population reduction and for spreading the cargo gene through the target population over a sustained period of time. A release of 20 generations is a marked increase on traditional pest control methods and would mean that releases would have to be conducted less than twice a year for insects such as *Drosophila* that go from egg to adult in 10 days, or up to once every

two years with tropical mosquito species that exhibit a four week generation time (Sowilem et al., 2013) rather than every generation as is currently the case with classical SIT methods.

The severity of the *Rescue* allele fitness cost will depend on two factors:

(i) piggyBac was used here to insert the transgene randomly into the genome. Expression levels of the *Rescue* transgene will be dependent on where it inserts within the genome due to position effects (Namciu et al., 1998). Random insertions may also end up disrupting important genes which can lower fitness.

(ii) Haploinsufficient genes are likely to be unusually sensitive to changes in expression level (Morrill & Amon, 2019). There are potential side effects of *Rp* gene overexpression from the addition of *Rescue Rp* genes on top of endogenous *Rp* genes in individuals that do not carry any *Rp-Killer* alleles.

A fitness reduction of 25% per *Rescue* allele copy is likely to be a very high estimate and realistically the fitness cost of carrying the *Rescue* allele is expected to be substantially lower. However, even at this high fitness reduction of 25% per *Rescue* allele copy, this model shows that the *Rp-Killer* gene will remain within the population for between 5-10 generations.

However, even when exerting a high fitness cost, the *Rp-Killer* genes persist because of the masking effects of the *Rescue* allele, which is advantageous to carry while the *Rp-Killer* genes are still in the gene pool. This was observable, as the *Rescue* allele initially increased in frequency when the chance of inheriting the *Rp-Killer* genes was frequent in the population. The *Rescue* allele itself incurs a cost to carrying it through either genetic load or an artificially engineered cost. Therefore, when the *Rp-Killer* genes are removed from the gene pool, the *Rescue* allele is no longer beneficial and quickly declines in frequency. This again is seen in the model. In almost all simulations, after the *Rp-Killer* genes decline in frequency and are removed from the population, there is a directly observable effect of the *Rescue* allele

declining and being lost from the population several generations after the *Rp-Killer* allele, demonstrating the ability of this system to be self-limiting.

When the *Rescue* allele has a fitness reduction of just 5% per copy, I found that the *Rescue* alleles could reach fixation. Fixation is undesirable for this underdominance strategy due to potential ecological and off-target effects, or by unintentionally altering the genome of the target vector species in a way that can allow it to carry and transmit other diseases (Harvey-Samuel et al., 2017; James, 2005). Therefore, while this model has been useful in showing the results of *Rescue* allele spread of a range of *Rescue* costs, it is vital that the fitness cost of carrying the *Rescue* allele is quantified. This model did not parameterise migration which will affect the rate of fixation. Fixation is less likely in larger populations with high amounts of migration. However, in smaller populations with limited migration, fixation may become an issue as is shown here. An accurate idea of cost of carrying the *Rescue* construct at this stage would allow fine-tuning to the design of the system to produce an underdominant insect strain with an "optimal" *Rescue* fitness cost for use in smaller enclosed target populations. A fitness reduction of around 10% per copy of the *Rescue* allele would be ideal as this would maximize the spread of *Rp-Killer* and cargo genes in wild releases while retaining the strategy's ability to be self-limiting.

This work follows on with theoretical modelling work on underdominant killer-rescue control systems by Edgington & Alphey (2018). In this study, simulations were run on underdominant killer-rescue systems with the result that the *Rescue* and *Rp-Killer* alleles spread through a population in a similar manner to the results I showed here, with an initial rise in *Rescue* frequency while the frequency of the *Rp-Killer* allele is high in the population. The *Rescue* frequency then declines in frequency as the *Rp-Killer* allele is removed from the gene pool. My simulations confirm this form of allele spread in a model parameterised by experimental data, displaying the spread of *Rp-Killer* and *Rescue* alleles with various different *Rescue* allele

costs. A key point highlighted by my model is that optimal engineered fitness costs for the *Rescue* allele are necessary to maximise the efficacy of this system while allowing it to be self-limiting.

Extensions to the Simulation Model

My simulation model was not built to estimate the difference in insect numbers between a WT population and a population into which males carrying the underdominant construct have been released. As such, this model is useful for demonstrating the potential spread and self-limitation of the underdominant killer-rescue system. However, it cannot be used for quantifying the effect the underdominant killer-rescue system will have in reducing population size compared to natural WT populations. Future modelling work could take the *Minute* fitness parameters from this study and develop a simulation regarding population size and fluctuations over time to accurately show how effective a release of the underdominant system would be compared to a WT population for reducing population numbers over time.

This model also has some limitations that are omitted for simplicities sake that further modelling may wish to incorporate.

(i) It takes a simplistic view of the ability of the *Rescue* genes to rescue the *Minute* phenotype. This model assumes complete rescue of the *Minute* allele so that inheriting the *Rescue* allele is sufficient for inducing a blanket "*Rescue* fitness." In reality, this fitness may vary depending on how many copies of the *Rp-Killer* genes are inherited along with the *Rescue* gene so that an individual who inherits the *Rescue* gene along with *Rp-Killer* genes may produce a lower fitness than an individual who just inherits the *Rescue* gene.

(ii) This model produces a good baseline result for demonstrating the theoretical spread of the killer-rescue system in an enclosed 1:1 release of

killer-rescue males with WT females. However, in large scale field releases, the complexities of migration will affect allele frequencies and strongly affect the chances of the *Rescue* gene reaching fixation, as migration in a large scale release will likely prevent permanent fixation in a target population.

Future Work

Future work could model resistance for multiple releases over a long period. Many proposed and currently used insect control methods that are self-sustaining in a population are susceptible to resistance quickly evolving in the population that they are targeting (Lees et al., 2015; Bargielowski et al., 2013). This may also be a problem for the underdominance strategy. If there is a slight cost associated with the insertion of the *Rescue* construct, it may be that females become choosier, deciding to mate with only the competitively fittest males, which may flush the underdominance system out of the population after several generations or after just a few releases.

I was not currently able to fully build this underdominant construct in my model species of *D. melanogaster* (Chapter 5). However, once such an underdominance system has been successfully built, it could be used to provide further experimental data to realistically parameterise the model further and examine allele spread over the longer term. For example, populations of WT *D. melanogaster* could be set up in laboratory cages into which populations of underdominant killer-rescue *D. melanogaster* were introduced at different ratios to measure the spread of the *Rescue* and *Rp-Killer* alleles over multiple generations and test the effectiveness of the killer-rescue system under a greater range of scenarios.

Advantages of the Underdominant Killer-Rescue System

My results suggest that the type of underdominant killer-rescue system tested here would save time, effort and costs. If the *Rescue* allele fitness is low but still high enough to be self-limiting (i.e. 10% fitness reduction per copy) this underdominant killer-rescue system can last for around 20 generations as shown here and releases would only have to be carried out up to once every two years for tropical mosquito species. This a major advantage over classical SIT techniques which requires releases every single generation over a substantial amount of time (Alphey et al., 2010).

6.6 **Conclusion**

Here I quantified fitness costs associated with the *Minute* phenotype and used this information in a simulation to demonstrate the spread of an underdominant killer-rescue insect control system. The simulation suggested that the underdominant killer-rescue system can be successfully sustained within a population of *Aedes* for potentially up to two years. By simulating a range of fitness costs of carrying the *Rescue* gene, I also showed that engineered fitness costs are a necessary part of the *Rescue* gene to avoid reaching fixation in wild populations. Based on these results, I suggest that an underdominant killer-rescue system might be less laborious and cheaper to produce and maintain in comparison to other established insect control methods such as SIT.

Chapter 7

Conclusions

7.1 **Introduction**

In this thesis I investigated novel methods of insect control in the model organism *Drosophila melanogaster* to investigate the potential to ultimately apply these to the disease vector *Aedes aegypti*. The need for control methods of vectors such as *Ae. aegypti* is pressing, as 3.9 billion people globally are at risk of contracting vector-borne diseases such as Zika and dengue for which there is currently no widely available vaccine or cure (Kumar et al., 2019). Habitat expansion driven by climate change is heightening this emergency and it is predicted that by 2050, *Ae. aegypti* will have reached large parts of Europe, the Middle East, Japan, the USA and Canada (Kraemer et al., 2019). Control methods such as the Sterile Insect Technique have been used successfully to control vector populations (Bellini et al., 2013; Oliva et al., 2012). However, to combat the global increase of *Ae. aegypti* populations and to circumvent resistance to existing strategies, there is a requirement to develop new, effective and complementary control methods.

Here I explored the feasibility of two methods of insect control – satyrization and genetic underdominance. I used *D. melanogaster* to investigate the how widespread satyrization is in nature, what the mechanics are that drive satyrization, what methods can be used to easily identify satyrizing species pairs, and to model and build an underdominant killer-rescue system of genetic control.

7.2 **Main Findings and Implications**

With regards to satyrization, I have uncovered several important findings. First I have shown that satyrization is likely to be more common in nature than previously thought. This is encouraging as it demonstrates that satyrization is not a rare phenomenon found only in a few isolated cases, and it suggests that it could potentially be applied to control a number of insect pest species. Insects are good candidates to experience satyrization due to their short

generation times and high evolutionary potential. This facilitates the rapid evolution of seminal fluid proteins and of mate recognition systems, which can influence the probability and effects of hybrid matings. This is of particular interest for potential control via satyrization if there is asymmetric divergence in the evolution of recognition systems and incompatibilities between conspecific and heterospecific mates.

The second main finding was the identification of a possible correlation between the ability to hybrid mate and the presence of asymmetric post-mating effects due to the receipt of heterospecific Sfps. This supports the idea that costly hybrid mating may drive resistance in the form of altered responses to heterospecific seminal fluid proteins and in doing so create the asymmetries that are characteristic of satyrization. This will be of interest to those studying reproductive interference as the causes and drivers of reproductive interference are under much debate (Shuker & Burdfield-Steel, 2017). Future work can investigate if this correlation holds across a wider number of species from different clades and if there is a causal link between the ability to hybrid mate and asymmetric post-mating effects.

My third major finding was that resistance to satyrization did not evolve between populations of *D. melanogaster* and *D. simulans* during 12 generations in sympatry, despite the existence of significant satyrization asymmetry between the two species. This finding is in contrast to work on *Aedes* mosquitoes, in which it was found that *Ae. aegypti* evolved resistance to hybrid matings after just a few generations in sympatry with *Ae. albopictus*. The costs of satyrization may have been larger and therefore selection pressures may be higher between *Ae. aegypti* and *Ae. albopictus* than between *D. melanogaster* and *D. simulans*, contributing to the strong selection of resistance genes that were already present in the *Ae. aegypti* genome. This may not have been the case within my *Drosophila* populations, where either selection pressures were not strong enough, or satyrization resistant alleles were not present in my *D. simulans* stocks for selection to act on them. This result demonstrates that

long term satyrization efficacy is variable between target species and populations, and each individual target species should be assessed on a case by case basis.

In terms of policy, these findings will help us to more easily identify target pest species that may be susceptible to satyrization and provide us a novel method for controlling these pests and reducing the incidence of disease in areas where they are prevalent. Strategies that aim to integrate multiple different methods for control will be able to use this effectively in conjunction with other control methods for a multi-pronged strategy that will maximise effectiveness while reducing the chance of resistance.

My attempts to build and model an underdominant killer-rescue system suggested that this is a strategy that shows promise for further development. When building the underdominant killer-rescue system in *D. melanogaster*, I found that the *Rescue* gene was only inherited by females, and that females who inherited it did not express it effectively. The explanation(s) for this novel finding are as yet unclear, though an obvious route for subsequent investigation is to better understand the individual and collective phenotypic effects of the *Rp* genes that were targeted (*RpS19a*, *RpS21*, and *RpS26*).

Despite the killer-rescue system being unsuitable in its current form, modelled releases of the killer-rescue strategy demonstrated proof of principle, in that the spread of the alleles involved in the underdominant killer-rescue strategy could sustain themselves in a target population for several generations before eventual self-limitation. Advantages of this system include being able to drive target cargo genes through populations to achieve population suppression while drastically reducing the number of releases needed to sustain active control compared to other traditionally used methods, significantly reducing money and labour costs. Furthermore, the transgenic genes were demonstrated to eventually fall out of the gene pool as a failsafe in case of unintended but hazardous ecological and health effects. My work to quantify the fitness costs associated with the *Minute* phenotype also showed

that *Minute* genes are a good candidate for this underdominant killer-rescue system, as the haploinsufficient phenotype conferred significant fitness costs.

Overall the underdominance work demonstrated the feasibility of the underdominant killer-rescue system, while also identifying issues to resolve with the system in its current form, offering valuable information to modify and improve the *Rescue* gene.

7.3 **Future Research**

This research has opened various avenues for moving forward to better characterise and improve insect control strategies.

For example, with regards to satyrization, the experimental paradigm I used to identify the link between the ability to hybrid mate and asymmetric post-mating effects indicative of satyrization offers a potentially powerful route for identifying satyrizing species. However, much more work is now needed to show if this association is general and indeed exactly how extensive is satyrization itself. It would be beneficial to test the link between hybrid mating and asymmetrical post-mating effects in many species within a phylogenetically controlled comparative analysis.

For the underdominant killer-rescue system, the most obvious next step is to optimise this strategy to work effectively in *D. melanogaster*, which will enable further proof of principle work. There were two unusual outcomes from this project, very few males were found that carried the *Rescue* gene, and expression levels of the *Rescue* genes were extremely low. We discussed several reasons as to why males were not found to contain the *Rescue* gene. It is possible that there is indeed a male killing effect of the *Rescue* gene that my sex ratio tests in Chapter 5 were not sufficiently sensitive to detect. It is also possible that some as yet unidentified mechanism was causing sex specific inheritance. Resolving the sequencing data

to observe exactly where the *Rescue* gene is within the genome of each line could offer potential explanations for these issues.

Nevertheless, these two outcomes are somewhat incompatible, as if the transgene was transcriptionally silent, why would it produce such a strong sex specific effect? It is also possible that the specific *Rp* genes themselves were ideal for this control strategy. Redesigns of this construct could cut strip down the design into single *Rp* genes each with a fluorescent marker gene, cutting out the other two *Rp* genes and the LoxP and attP sites. If this proved to work, with detectable expression in both sexes, a second and third *Rp* gene could then be added. Testing each *Rp* gene individually would also uncover if any of the three target *Rp* genes are incompatible and would allow us to swap them for alternative candidates.

Minute fitness costs could also be quantified further. My experiments in this thesis demonstrated high fitness costs associated with developmental delay due to the knockout of a single *Rp* gene. However, there may well be a stacking effect of the knockout of multiple *Rp* genes which could amplify the fitness costs. Additionally, I only tested the fitness costs of developmental delay and not the additional effect of the lack of *Minute* on reduced fertility. The inclusion of the full spectrum of *Minute* would almost certainly decrease fitness further and should also be quantified to give a comprehensive view of the effects of the *Minute* phenotype. Following this approach, additional quantifications of the fitness costs of *Minutes* were conducted by a project student under my supervision (A. Stephens). He tested the effects of developmental delay at a greater resolution, simulating developmental delay at 5h, 12h, 24h and 48h with multiple samples for each time period. These experiments showed that fitness costs arising from developmental delay were potentially sensitive to environment, resource availability and the density of individuals. Overall, significant fitness costs due to developmental delay were demonstrated in all experiments. The additional fitness costs arising from the effects of stacking multiple *Minute* genes as well as their

subfertility phenotypes means that the high fitness cost of *Minute* used as parameters for modelling the release of an underdominant killer-rescue population was realistic for the simulations performed.

The model itself demonstrated that the *Rp-Killer* and *Rescue* genes could successfully spread through a population for multiple generations while retaining its ability to be self-limiting. I simulated a range of *Rescue* fitness costs for this model in the absence of experimental data for the fitness costs associated with the *Rescue* gene. Here I found that a fitness cost of around 10% per *Rescue* allele would maximise the spread of the *Rescue* allele while preventing the *Rescue* allele from reaching fixation. Therefore, future work designing the underdominant killer-rescue construct should attempt to artificially engineer a fitness cost of around 10% per allele in order to maximise the ability of the system to spread while also retaining its self-limiting ability.

Models are built to trade-off between simplifying a complex system and adding enough complexity to be biologically relevant. The model I employed successfully demonstrated the spread of transgenic alleles from a release of the underdominance killer-rescue system into a wild population. However, improvements to the model could introduce additional factors such as migration and variation in population size, to build up a larger dataset for more accurate predictions of an underdominant killer-rescue release. This model was also not able to simulate the numbers of individuals in a wildtype (WT) population that have been subject to a release of an underdominant population. A new model that can simulate this would be welcome to test how effective the underdominance system would be not just for spreading the *Rp-Killer* and *Rescue* alleles in a population, but also for effectively suppressing pest populations.

Finally, while the modelling work demonstrated the effectiveness of the underdominant killer-rescue system, it is necessary to back this up with experimental proof of principle data.

Population cages containing just WT *D. melanogaster* and cages containing a mix of WT *D. melanogaster* and killer-rescue mutant *D. melanogaster* at different ratios would allow us to see how effectively the alleles spread over time and would produce some important baseline results for taking into semi-field trials.

7.4 **Where does this Work fit into the Broader Field of Control?**

Much research for insect pest control focusses on gene drive strategies (Gantz & Akbari, 2018) for maximum efficacy and low cost. The underdominant killer-rescue system could be a viable strategy, as it offers population suppression in addition to the spread of desired traits encoded by cargo genes. However, gene drives are a very new technology that can suffer from a negative public perception in many areas of the world and are subject to stringent regulations in many countries. Satyrization alleviates many of the gene drive concerns. For example, satyrization in its original form requires no genetic modification and therefore does not require such stringent regulation. While satyrization does not allow for such a targeted spread of desirable genes and phenotypes through a population like gene drive systems do, satyrization may become a viable additional contributor for use in integrated insect control strategies that employ multiple control methods to maximise control.

7.5 **Final Conclusions**

My work in this thesis has investigated and developed the use of satyrization and genetic underdominance as a method of insect control. Satyrization has rarely been studied up until the last few years and has only been identified in a few species. Here I have shown that satyrization is more widespread that was previously thought, even being present between multiple species pairs within the *D. melanogaster* species subgroup which has been a model organism for various aspects of biology for over 100 years. I have also discovered possible

metrics for easily identifying satyrization targets in nature. This will be useful for applications of satyrization in the wild, especially in conjunction with other insect suppression methods.

Additionally, my work here demonstrates that the underdominant killer-rescue strategy is a promising control strategy, with fitness parameters gained from experimental work showing that it is a self-limiting strategy that can be sustained in a population for multiple generations. However, the genetic construct in its current form is not suitable for use in *D. melanogaster* and requires further development.

Overall I have presented important groundwork for two insect control strategies, satyrization and genetic underdominance. This has provided new knowledge in both areas that can be taken forward to maximise the ability of these strategies to be used as effective methods of insect control.

References

Abeyasuriya, K. G. T. N., Nugapola, N. W. N. P., Perera, M. D. B. and Karunaratne. W. A. I. P. (2017) 'Effect of dengue mosquito control insecticide thermal fogging on non-target insects', *International Journal of Tropical Insect Science*, **37**(1), pp. 11-18. doi: 10.1017/s1742758416000254.

Ageep, T. B., Damiens, D., Alsharif, B., Ahmed, A., Salih, E. H. O., Ahmed, F. T. A., Diabaté, A., Lees, R. S., Gilles, J. R. L. and El Sayed, B. B. (2014) 'Participation of irradiated *Anopheles arabiensis* males in swarms following field release in Sudan', *Malaria Journal*, **13**(1), p. 484. doi: 10.1186/1475-2875-13-484.

Alexandrov, I. D., AlexandrovaI, M. V., Goryacheva, I., Rochina, N. V., ShaikevichI, N. V. and Zakharov, A. (2007) 'Removing endosymbiotic *Wolbachia* specifically decreases lifespan of females and competitiveness in a laboratory strain of *Drosophila melanogaster*', *Russian Journal of Genetics*, **43**(10), pp. 1147-1152. doi: 10.1134/s1022795407100080.

Alphey, L. (2002) 'Re-engineering the sterile insect technique', in *Insect Biochemistry and Molecular Biology*, **32**(10), pp. 1243-1247. doi: 10.1016/S0965-1748(02)00087-5.

Alphey, L., Beard, C. B., Billingsley, P., Coetzee, M., Crisanti, A., Curtis, C., Eggleston, P., Godfray, C., Hemingway, J., Jacobs-Lorena, M., James, A. A., Kafatos, F. C., Mukwaya, L. G., Paton, M., Powell, J. R., Schneider, W., Scott, T. W., Sina, B., Sinden, R., Sinkins, S., Spielman, A., Touré, Y. and Collins, F. H. (2002) 'Malaria control with genetically manipulated insect vectors', *Science*, **298**(5591), pp. 119-121. doi: 10.1126/science.1078278.

Alphey, L., Benedict, M., Bellini, R., Clark, G.G., Dame, D.A., Service, M.W. and Dobson, S.L. (2010) 'Sterile-insect methods for control', *Vector-Borne and Zoonotic Diseases*, **10**(3), pp. 295-311. doi: 10.1089/vbz.2009.0014.

Alphey, L., McKemey, A., Nimmo, D., Neira Oviedo, M., Lacroix, R., Matzen, K. and Beech, C. (2013) 'Genetic control of *Aedes* mosquitoes', *Pathogens and Global Health*, **107**(4), pp. 170-179. doi: 10.1179/2047773213y.0000000095.

Alphey, N., Bonsall, M. B., and Alphey, L. (2011) 'Modeling resistance to genetic control of insects', **270**(1), pp. 42-55. *Journal of Theoretical Biology*. doi: 10.1016/j.jtbi.2010.11.016.

Alto, B. W., Smartt, C.T., Shin, D., Bettinardi, D., Malicoate, J., Anderson, S.L. and Richards, S.L. (2014) 'Susceptibility of florida *Aedes aegypti* and *Aedes albopictus* to dengue viruses from Puerto Rico', **39**(2), pp. 406-13. *Journal of Vector Ecology*. doi: 10.1111/jvec.12116.

Ansai, S. and Kinoshita, M. (2014) 'Targeted mutagenesis using CRISPR/Cas system in medaka', *Biology Open*, **3**(5), pp. 362-71. doi: 10.1242/bio.20148177.

Arauz, M. J., Ridde, V., Hernández, L. M., Charris, Y., Carabali, M. and Villar, L. A. (2015) 'Developing a social autopsy tool for dengue mortality: a pilot study', *PLoS ONE*, **10**(2), p. e0117455. doi: 10.1371/journal.pone.0117455.

Arnqvist, G., Edvardsson, M., Friberg, U. and Nilsson, T. (2000) 'Sexual conflict promotes speciation in insects', *Proceedings of the National Academy of Sciences*, **97**(19), pp. 10460-10464. doi: 10.1073/pnas.97.19.10460.

Arthur, B. J., Sunayama-Morita, T., Coen, P., Murthy, M. and Stern, D. L. (2013) 'Multi-channel acoustic recording and automated analysis of *Drosophila* courtship songs', **11**(1), p. 11. *BMC biology*. doi: 10.1186/1741-7007-11-11.

Arthur, N. J. and Dyer, K. A. (2015) 'Asymmetrical sexual isolation but no postmating isolation between the closely related species *Drosophila suboccidentalis* and *Drosophila occidentalis*', *BMC Evolutionary Biology*, **15**(1), p. 18. doi: 10.1186/s12862-015-0328-y.

Austin, C. J. and Moehring, A. J. (2013) 'Optimal temperature range of a plastic species, *Drosophila simulans*', *Journal of Animal Ecology*, **82**(3), pp. 663-672. doi: 10.1111/1365-2656.12041.

Ayorinde, A., Oboh, B., Oduola, A. and Otubanjo, O. (2015) 'The insecticide susceptibility status of *Aedes aegypti* (Diptera: Culicidae) in farm and nonfarm sites of Lagos State, Nigeria', *Journal of Insect Science*, **15**(1), p. 75. doi: 10.1093/jisesa/iev045.

Barbash, D. A. (2010) 'Ninety years of *Drosophila melanogaster* hybrids', *Genetics*, 186(1), 1-8. doi: 10.1534/genetics.110.121459.

Bargielowski, I. E., Lounibos, L. P. and Carrasquilla, M. C. (2013) 'Evolution of resistance to satyrization through reproductive character displacement in populations of invasive dengue vectors', *Proceedings of the National Academy of Sciences*, **110**(8), pp. 2888–2892. doi: 10.1073/pnas.1219599110.

Bargielowski, I. and Lounibos, L. P. (2014) 'Rapid evolution of reduced receptivity to interspecific mating in the dengue vector *Aedes aegypti* in response to satyrization by invasive *Aedes albopictus*', *Evolutionary Ecology*, **28**(1) pp. 193-203. doi: 10.1007/s10682-013-9669-4.

Bargielowski, I., Honório, N. A., Blosser, E. M. and Lounibos, L. P. (2019) 'Rapid loss of resistance to satyrization in invasive mosquitoes and the effects of age on interspecific mating frequency', *Journal of Medical Entomology*, **56**(2), pp. 329-333. doi: 10.1093/jme/tjy153.

Barker, J. S. F. (1962) 'Sexual isolation between *Drosophila melanogaster* and *Drosophila simulans*', *The American Naturalist*, **96**(887), pp. 105-115. doi: 10.1086/282211

Basu, S., Aryan, A., Overcash, J. M., Samuel, G. H., Anderson, M. A. E., Dahlem, T. J., Myles, K. M. and Adelman, Z. N. (2015) 'Silencing of end-joining repair for efficient site-specific gene

insertion after TALEN/CRISPR mutagenesis in *Aedes aegypti*', *Proceedings of the National Academy of Sciences*, **112**(13), pp. 4038-4043 doi: 10.1073/pnas.1502370112.

Beckingham, K. M., Armstrong, J. D., Texada, M. J., Munjaal, R. and Baker, D.A. (2005) '*Drosophila melanogaster*--the model organism of choice for the complex biology of multi-cellular organisms', *Gravitational and Space Biology bulletin: Publication of the American Society for Gravitational and Space Biology*, **18**(2), pp. 17-29.

Beech, C. J., Nagaraju, J., Vasan, S. S., Rose, R. I., Othman, R. Y., Pillai, V. and Saraswathy, T. S. (2009) 'Risk analysis of a hypothetical open field release of a self-limiting transgenic *Aedes aegypti* mosquito strain to combat dengue', *Asia-Pacific Journal of Molecular Biology and Biotechnology*, **17**(3), pp. 99-111.

Belhaj, K., Chaparro-Garcia, A., Kamoun, S., Patron, N. J. and Nekrasov V. (2015) 'Editing plant genomes with CRISPR/Cas9', *Current Opinion in Biotechnology*, **32**(1), pp. 76-84. doi: 10.1016/j.copbio.2014.11.007

Bellini, R., Calvitti, M., Medici, A., Carrieri, M., Celli, G. and Maini, S. (2007) 'Use of the sterile insect technique against *Aedes albopictus* in Italy: First results of a pilot trial', *Area-Wide Control of Insect Pests: From Research to Field Implementation*, pp. 505-515. Springer, Dordrecht. doi: 10.1007/978-1-4020-6059-5_47.

Bellini, R., Medici, A., Puggioli, A., Balestrino, F and Carrieri, M. (2013) 'Pilot field trials with *Aedes albopictus* irradiated sterile males in Italian urban areas', *Journal of medical entomology*, **50**(2), pp. 317-325. doi: 10.1603/me12048

Berrigan, D. and Partridge, L. (1997) 'Influence of temperature and activity on the metabolic rate of adult *Drosophila melanogaster*', *Comparative Biochemistry and Physiology - A Physiology*, **118**(4), pp. 1301-1307. doi: 10.1016/S0300-9629(97)00030-3.

Bhatt, S., Gething, P. W., Brady, O. J., Messina, J. P., Farlow, A. W., Moyes, C. L., Drake, J. M., Brownstein, J. S., Hoen, A. G., Sankoh, O., Myers, M. F., George, D. B., Jaenisch, T., Wint, G.R., Simmons, C. P., Scott, T. W., Farrar, J. J. and Hay, S.I. (2013) 'The global distribution and burden of dengue', *Nature*, **496**(7446), pp. 504-507. doi: 10.1038/nature12060.

Billeter, J. C. and Wolfner, M. F. (2018) 'Chemical cues that guide female reproduction in *Drosophila melanogaster*', *Journal of Chemical Ecology*, **44**(9), pp. 750-769. doi: 10.1007/s10886-018-0947-z.

Black, W. C., Alphey, L. and James, A. A. (2011) 'Why RIDL is not SIT', *Trends in Parasitology*, **27**(8), pp. 362-370. doi: 10.1016/j.pt.2011.04.004.

Bouyer, J. and Marois, E. (2018) '14. Genetic control of vectors', in. *Pests and Vector-borne Diseases in the Livestock Industry*, pp. 435-451. Wageningen Academic Publishers. doi: 10.3920/978-90-8686-863-6_14.

Bracco, J. E., Capurro, M. L., Lourenço-de-Oliveira, R and Sallum, M. A. (2007) 'Genetic variability of *Aedes aegypti* in the Americas using a mitochondrial gene: Evidence of multiple introductions', *Memorias do Instituto Oswaldo Cruz*, **102**(5), pp. 573-580. doi: 10.1590/s0074-02762007005000062

Brelsfoard, C. L. and Dobson, S. L. (2011) '*Wolbachia* effects on host fitness and the influence of male aging on cytoplasmic incompatibility in *Aedes polynesiensis* (Diptera: Culicidae) ', *Journal of Medical Entomology*, **48**(5), pp. 1008-1015. doi: 10.1603/me10202.

Bretman, A., Lizé, A., Walling, C. A. and Price, T. A. R. (2014) 'The heritability of mating behaviour in a fly and its plasticity in response to the threat of sperm competition', *PLoS ONE*, **9**(2), p. e90236. doi: 10.1371/journal.pone.0090236.

Bretman, A., Gage, M. J. G. and Chapman, T. (2011) ‘Quick-change artists: male plastic behavioural responses to rivals’, *Trends in Ecology and Evolution,* **26**(9), pp. 467-473. doi: 10.1016/j.tree.2011.05.002.

Bull, J. J. and Turelli, M. (2013) ‘*Wolbachia* versus dengue: Evolutionary forecasts’, *Evolution, Medicine, and Public Health*, **2013**(1), pp. 197-207. doi: 10.1093/emph/eot018.

Burt, A. (2014) ‘Heritable strategies for controlling insect vectors of disease’, *Philosophical Transactions of the Royal Society B: Biological Sciences*, **369**(1645), p. 20130432 doi: 10.1098/rstb.2013.0432.

Carracedo, M. C., Suarez, C. and Casares, P. (2000) ‘Sexual isolation between *Drosophila melanogaster*, *D. simulans* and *D. mauritiana*: Sex and species specific discrimination’, *Genetica*, **108**(2), pp.155-162. doi: 10.1023/A:1004132414511.

Carvalho, D. O., McKemey, A. R., Garziera, L., Lacroix, R., Donnelly, C. A., Alphey, L., Malavasi, A. and Capurro M.L. (2015) ‘Suppression of a field population of *Aedes aegypti* in Brazil by sustained release of transgenic male mosquitoes’, *PLoS Neglected Tropical Diseases*, **9**(7), p. e0003864. doi: 10.1371/journal.pntd.0003864.

Castillo, J. A., Castrillón, J. C., Diosa-Toro, M., Betancur, J. G., St. Laurent, G 3rd., Smit. J. M. and Urcuqui-Inchima, S. (2016) ‘Complex interaction between dengue virus replication and expression of miRNA-133a’, *BMC Infectious Diseases*, **16**(1), p. 29. doi: 10.1186/s12879-016-1364-y.

Champer, J., Buchman, A. and Akbari, O. S. (2016) ‘Cheating evolution: Engineering gene drives to manipulate the fate of wild populations’, *Nature Reviews Genetics*, **17**(3), 146-159. doi: 10.1038/nrg.2015.34.

Chapman, T., Liddle, L. F., Kalb, J. M., Wolfner, M. F. and Partridge, L. (1995) 'Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products', *Nature*, **373**(6511), pp. 241-244. doi: 10.1038/373241a0.

Chapman, T., Herndon, L. A., Heifetz, Y., Partridge, L. and Wolfner, M.F. (2001) 'The Acp26Aa seminal fluid protein is a modulator of early egg hatchability in *Drosophila melanogaster*', *Proceedings of the Royal Society B: Biological Sciences*, **268**(1477), pp. 1647-1654. doi: 10.1098/rspb.2001.1684.

Chapman, T., Bangham, J., Vinti, G., Seifried, B., Lung, O., Wolfner, M. F., Smith, H. K. and Partridge, L. (2003) 'The sex peptide of *Drosophila melanogaster*: Female post-mating responses analyzed by using RNA interference', *Proceedings of the National Academy of Sciences*, **100**(17), pp. 9923-9928. doi: 10.1073/pnas.1631635100.

Chapman, T. and Davies, S. J. (2004) 'Functions and analysis of the seminal fluid proteins of male *Drosophila melanogaster* fruit flies', *Peptides*, **25**(9), pp. 1477-90. doi: 10.1016/j.peptides.2003.10.023.

Chapman, T. (2001) 'Seminal fluid-mediated fitness traits in *Drosophila*', *Heredity*, **87**(5), pp. 511-521. doi: 10.1046/j.1365-2540.2001.00961.x.

Chapman, T. (2008) 'The soup in my fly: Evolution, form and function of seminal fluid proteins', *PLoS Biology*, **6**(7), p. e179. doi: 10.1371/journal.pbio.0060179.

Chylinski, K., Le Rhun, A. and Charpentier, E. (2013) 'The tracrRNA and Cas9 families of type II CRISPR-Cas immunity systems', *RNA Biology*, **10**(5), pp. 726-37. doi: 10.4161/rna.24321.

Clark, M. E., Anderson, C. L., Cande, J. and Karr, T. L. (2005) 'Widespread prevalence of *Wolbachia* in laboratory stocks and the implications for *Drosophila* research', *Genetics*, **170**(4), pp. 1667–1675. doi: 10.1534/genetics.104.038901.

Cohet, Y. and David, J. (1978) ‘Control of the adult reproductive potential by preimaginal thermal conditions - A study in *Drosophila melanogaster*’, *Oecologia*, **36**(3), 295-306. doi: 10.1007/BF00348055.

Collins, J. P. (2018) ‘Gene drives in our future: Challenges of and opportunities for using a self-sustaining technology in pest and vector management’, *BMC Proceedings*, **12**(8), p. 9. doi: 10.1186/s12919-018-0110-4.

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W., Marraffini, L. A. and Zhang, F. (2013) ‘Multiplex genome engineering using CRISPR/Cas systems’, *Science*, **339**(6121), pp. 819-823. doi: 10.1126/science.1231143.

Coyne, J. A. and Orr, H. A. (1989) ‘Patterns of speciation in Drosophila’, *Evolution*, **43**(2), pp. 362-381. doi: 10.2307/2409213.

Crill, W. D., Huey, R. B. and Gilchrist, G. W. (1996) ‘Within- and between-generation effects of temperature on the morphology and physiology of *Drosophila Melanogaster*’, *Evolution*, **50**(3), 1205-1218. doi: 10.1111/j.1558-5646.1996.tb02361.x.

Curtis, C. F. (1968) ‘Possible use of translocations to fix desirable genes in insect pest populations’, *Nature*, **218(**5139), pp. 368-369. doi: 10.1038/218368a0.

Dapper, A. L. and Wade, M. J. (2016) ‘The evolution of sperm competition genes: The effect of mating system on levels of genetic variation within and between species’, *Evolution*, **70**(2), pp. 502-511. doi: 10.1111/evo.12848.

David, J. R. and Capy, P. (1982) ‘Genetics and origin of a *Drosophila melanogaster* population recently introduced to the Seychelles’, *Genetical Research*, **40**(3), 295-303. doi: 10.1017/S0016672300019169.

David, M. R., Garcia, G. A., Valle, D. and Maciel-de-Freitas, R. (2018) 'Insecticide resistance and fitness: the case of four *Aedes aegypti* populations from different Brazilian regions', *BioMed Research International*. **2018**(1), p. 6257860. doi: 10.1155/2018/6257860.

Davis, S., Bax, N. and Grewe, P. (2001) 'Engineered underdominance allows efficient and economical introgression of traits into pest populations', *Journal of Theoretical Biology*, **212**(1), pp. 83-98. doi: 10.1006/jtbi.2001.2357.

De Bruin, L. M., Volpi, S. and Musunuru, K. (2015) 'Novel genome-editing tools to model and correct primary immunodeficiencies', *Frontiers in Immunology*, **2015**(6), p. 250. doi: 10.3389/fimmu.2015.00250.

De Bruyn, P. J. N., Tosh, C. A. and Bester, M. N. (2008) 'Sexual harassment of a king penguin by an Antarctic fur seal', *Journal of Ethology*, **26**(2):295-297 doi: 10.1007/s10164-007-0073-9.

Demok, S., Endersby-Harshman, N., Vinit, R., Timinao, L., Robinson, L. J., Susapu, M., Makita, L., Laman, M., Hofmann, A. and Karl, S. (2019) 'Insecticide resistance status of *Aedes aegypti* and *Aedes albopictus* mosquitoes in Papua New Guinea', *Parasites and Vectors*, **12**(1), p. 333. doi: 10.1186/s13071-019-3585-6

Denis, B., Claisse, G., Le Rouzic, A., Wicker-Thomas, C., Lepennetier, G. and Joly, D. (2017) 'Male accessory gland proteins affect differentially female sexual receptivity and remating in closely related *Drosophila* species', *Journal of Insect Physiology*, **2017**(99), pp. 67-77. doi: 10.1016/j.jinsphys.2017.03.008.

Deredec, A., Burt, A. and Godfray, H. C. J. (2008) 'The population genetics of using homing endonuclease genes in vector and pest management', *Genetics*, **179**(4), pp. 2013-26 doi: 10.1534/genetics.108.089037.

Dhole, S., Vella, M.R., Lloyd, A.L. and Gould, F. (2018) 'Invasion and migration of spatially self-limiting gene drives: A comparative analysis', *Evolutionary Applications*, **11**(5), pp. 794-808. doi: 10.1111/eva.12583.

DiCarlo, J. E., Chavez, A., Dietz, S. L., Esvelt, K. M. and Church, G. M. (2015) 'Safeguarding CRISPR-Cas9 gene drives in yeast', *Nature Biotechnology*, **33**(12), pp. 1250-1255 doi: 10.1038/nbt.3412.

Dida, G. O., Gelder, F. B., Anyona, D. N., Abuom, P. O., Onyuka, J. O., Matano, A. S., Adoka, S. O., Kanangire, C. K., Owuor, P. O., Ouma, C. and Ofulla, A. V. (2015) 'Presence and distribution of mosquito larvae predators and factors influencing their abundance along the Mara River, Kenya and Tanzania', *SpringerPlus*, **4**(1), p. 136 doi: 10.1186/s40064-015-0905-y.

Dighe S. N., Ekwudu, O., Dua, K., Chellappan, D. K., Katavic, P. L. and Collet T. A. (2019) 'Recent update on anti-dengue drug discovery', *European Journal of Medicinal Chemistry*, **176**(1), pp. 431–455. doi: 10.1016/j.ejmech.2019.05.010

Dillon, M. E., Cahn, L. R. Y. and Huey, R. B. (2007) 'Life history consequences of temperature transients in *Drosophila melanogaster*', *Journal of Experimental Biology*, **210**(16), pp. 2897-904. doi: 10.1242/jeb.007591.

Dodd, D. M. B. and Powell, J. R. (1985) 'Founder-flush speciation: an update of experimental results with *Drosophila*', *Evolution*, **39**(6), pp. 1388-1392. doi: 10.2307/2408795.

Dong, S., Lin, J., Held, N. L., Clem, R. J., Passarelli, A. L. and Franz, A.W. (2015) 'Heritable CRISPR/Cas9-mediated genome editing in the yellow fever mosquito, *Aedes aegypti*', *PLoS ONE*, **10**(3), p. e0122353. doi: 10.1371/journal.pone.0122353.

Dutra, H. L., Rocha, M. N., Dias, F. B., Mansur, S. B., Caragata, E. P. and Moreira, L. A. (2016) '*Wolbachia* blocks currently circulating zika virus isolates in Brazilian *Aedes aegypti* mosquitoes', *Cell Host and Microbe*, **19**(6), pp. 771-774. doi: 10.1016/j.chom.2016.04.021.

Edgington, M. P. and Alphey, L. S. (2018) ‘Population dynamics of engineered underdominance and killer-rescue gene drives in the control of disease vectors’, *PLoS Computational Biology*, **14**(3), p. e1006059. doi: 10.1371/journal.pcbi.1006059.

Ehrman, L. (1964) ‘Courtship and mating behavior as a reproductive isolating mechanism in *Drosophila*’, *Integrative and Comparative Biology*, **4**(1), pp. 147-153. doi: 10.1093/icb/4.2.147.

Eliminatedengue.com (2016) ‘Eliminate dengue - a natural method to reduce the spread of dengue’, *[online] Available at: http://www.eliminatedengue.com/our-research/wolbachia [Accessed 18 Apr. 2016]*.

Ellis, L. L. and Carney, G. E. (2009) ‘*Drosophila melanogaster* males respond differently at the behavioural and genome-wide levels to *Drosophila melanogaster* and *Drosophila simulans* females’, *Journal of Evolutionary Biology*, **22**(11), pp. 2183-91. doi: 10.1111/j.1420-9101.2009.01834.x.

Esu, E., Lenhart, A., Smith, L. and Horstick, O. (2010) ‘Effectiveness of peridomestic space spraying with insecticide on dengue transmission; Systematic review’, *Tropical Medicine and International Health*, **15**(5), pp. 619-631. doi: 10.1111/j.1365-3156.2010.02489.x.

Esvelt, K. M., Smidler, A. L., Catteruccia, F., and Church, G. M. (2014) ‘Concerning RNA-guided gene drives for the alteration of wild populations’, *eLife*, **2014**(3), pp. e03401. doi: 10.7554/elife.03401.

Fang, J. (2010) ‘Ecology: A world without mosquitoes’, *Nature*, **466**(7305), pp. 432-434 doi: 10.1038/466432a.

Fick, W. E. and MacQuarrie, C. J. K. (2018) ‘An artificial delay in emergence influences the number but not the fitness of adult emerald ash borer emerging from infested ash wood’, *Entomologia Experimentalis et Applicata*, **166**(3), pp. 171-182. doi: 10.1111/eea.12671.

Findlay, G. D., Sitnik, J. L., Wang, W., Aquadro, C. F., Clark, N. L., and Wolfner, M. F. (2014) 'Evolutionary rate covariation identifies new members of a protein network required for *Drosophila melanogaster* female post-mating responses', *PLoS Genetics*, **10**(1), p. e1004108 doi: 10.1371/journal.pgen.1004108.

Findlay, G. D. Yi, X., Maccoss, M.J. and Swanson, W.J. (2008) 'Proteomics reveals novel *Drosophila* seminal fluid proteins transferred at mating', *PLoS Biology*, **6**(7), p. e178. doi: 10.1371/journal.pbio.0060178.

Fry, A. J., Palmer, M. R. and Rand, D. M. (2004) 'Variable fitness effects of *Wolbachia* infection in *Drosophila melanogaster*', *Heredity*, **93**(4), pp. 379-89. doi: 10.1038/sj.hdy.6800514.

Fu, Y., Foden, J. A., Khayter, C., Maeder, M. L., Reyon, D., Joung, J. K. and Sander, J. D. (2013) 'High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells', *Nature Biotechnology*, **31**(9), pp. 822-826. doi: 10.1038/nbt.2623.

Gabrieli, P., Smidler, A. and Catteruccia, F. (2014) 'Engineering the control of mosquito-borne infectious diseases', *Genome biology*, **15**(11), p. 535. doi: 10.1186/s13059-014-0535-7.

Gantz, V. M., Jasinskiene, N., Tatarenkova, O., Fazekas, A., Macias, V. M., Bier, E., and James, A. A. (2015) 'Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*', *Proceedings of the National Academy of Sciences*, **112**(49), p. E6736-43. doi: 10.1073/pnas.1521077112.

Gantz, V. M. and Akbari, O. S. (2018) 'Gene editing technologies and applications for insects', *Current Opinion in Insect Science*, **2018**(28), pp. 66–72.

Gavrilets, S. (2000) 'Rapid evolution of reproductive barriers driven by sexual conflict', *Nature*, 403(**6772**), pp. 886-889. doi: 10.1038/35002564.

Gay, L., Eady, P. E., Vasudev, R., Hosken, D. J. and Tregenza, T. (2009) 'Does reproductive isolation evolve faster in larger populations via sexually antagonistic coevolution?', *Biology Letters*, **5**(5):693-696. doi: 10.1098/rsbl.2009.0072.

Gibson, G., Souza-Santos, R., Brasil, P., Pacheco, A.G., Cruz, O.G., Honório, N.A., Kubelka, C. and Carvalho, M.S. (2013) 'From primary care to hospitalization: clinical warning signs of severe dengue fever in children and adolescents during an outbreak in Rio de Janeiro, Brazil', *Cadernos de Saúde Pública*, **29**(1), pp. 82-90. doi: 10.1590/S0102-311X2013000100010.

Gilchrist, A. S. and Partridge, L. (2000) 'Why it is difficult to model sperm displacement in *Drosophila melanogaster*: The relation between sperm transfer and copulation duration', *Evolution*, **54**(2), pp. 534-542. doi: 10.1111/j.0014-3820.2000.tb00056.x.

Godwin, J. L., Spurgin, L. G., Michalczyk, Ł., Martin, O. Y., Lumley, A. J., Chapman, T. and Gage, M. J. G (2018) 'Lineages evolved under stronger sexual selection show superior ability to invade conspecific competitor populations', *Evolution Letters*, **2**(5), pp. 522-523. doi: 10.1002/evl3.80.

Goldim, J. R. (2015) 'Genetics and ethics: a possible and necessary dialogue', *Journal of Community Genetics*, **6**(3): pp. 193–196. doi: 10.1007/s12687-015-0232-6.

Gould, F., Huang, Y., Legros, M. and Lloyd, A.L. (2008) 'A killer-rescue system for self-limiting gene drive of anti-pathogen constructs', *Proceedings of the Royal Society B: Biological Sciences*, **275**(1653), pp. 2823-2839. doi: 10.1098/rspb.2008.0846.

Gratz, S. J., Cummings, A.M., Nguyen, J.N., Hamm, D.C., Donohue, L.K., Harrison, M.M., Wildonger, J. and O'Connor-Giles, K.M. (2013) 'Genome engineering of *Drosophila* with the CRISPR RNA-guided Cas9 nuclease', *Genetics*, **194**(4), pp. 1029-1035. doi: 10.1534/genetics.113.152710.

Grissa, I., Vergnaud, G. and Pourcel, C. (2007) ‘The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats’, *BMC Bioinformatics*, **2007**(8), p. 172. doi: 10.1186/1471-2105-8-172.

Gromko, M. H. and Markow, T. A. (1993) ‘Courtship and remating in field populations of *Drosophila*’, *Animal Behaviour*, **45**(2), pp. 253-262. doi: 10.1006/anbe.1993.1031.

Gröning, J. and Hochkirch, A. (2008) ‘Reproductive interference between animal species.’, *The Quarterly review of biology*, **83**(3), pp. 257-282. doi: 10.1086/590510

Hackett, S. C. and Bonsall, M. B. (2019) ‘Insect pest control, approximate dynamic programming, and the management of the evolution of resistance’, *Ecological Applications*, **29**(2), p. e01851. doi: 10.1002/eap.1851.

Haerty, W., Jagadeeshan, S., Kulathinal, R. J., Wong, A., Ram, K. R., Sirot, L. K., Levesque, L., Artieri, C. G., Wolfner, M. F., Civetta A. and Singh, R. S. (2007) ‘Evolution in the fast lane: Rapidly evolving sex-related genes in *Drosophila*’, *Genetics*, **177**(3), pp. 1321-1335. doi: 10.1534/genetics.107.078865.

Hammond, A. M. and Galizi, R. (2017) ‘Gene drives to fight malaria: current state and future directions’, *Pathogens and Global Health*, **111**(8), pp. 412–423 doi: 10.1080/20477724.2018.1438880.

Hammond, A., Galizi, R., Kyrou, K., Simoni, A., Siniscalchi, C., Katsanos, D., Gribble, M., Baker, D., Marois, E., Russell, S., Burt, A., Windbichler, N., Crisanti, A. and Nolan, T. (2016) ‘A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*’, *Nature Biotechnology*, **34**(1), pp. 78-83. doi: 10.1038/nbt.3439.

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’, *Nature Biotechnology*, **29**(11), pp. 1034–1037. doi: 10.1038/nbt.2019.

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., Collado, A., Stevenson, J., Scaife, S., Dafa'alla, T., Fu, G., Phillips, C., Miles, A., Raduan, N., Kelly, N., Beech, C., Donnelly, C. A., Petrie, W. D. and Alphey, L. (2012) 'Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes', *Nature Biotechnology*, **30**(9), pp. 828-830. doi: 10.1038/nbt.2350.

Harvey-Samuel, T., Ant, T. and Alphey, L. (2017) 'Towards the genetic control of invasive species', *Biological Invasions*, **19**(6), pp. 1683–1703. doi: 10.1007/s10530-017-1384-6.

Heifetz, Y., Lung, O., Frongillo, E. A. and Wolfner, M. F. (2000) 'The *Drosophila* seminal fluid protein Acp26Aa stimulates release of oocytes by the ovary', *Current Biology*, **10**(2), pp. 99-102. doi: 10.1016/S0960-9822(00)00288-8.

Helinski, M. E. H., Parker, A. G. and Knols, B. G. J. (2009) 'Radiation biology of mosquitoes', *Malaria Journal*, **8**(2), p. S6. doi: 10.1186/1475-2875-8-S2-S6.

Hemingway, J., Vulcan, J., Rodriguez, S. D., Moore, E., Chung, H., Mitra, S., Cordova, J. J., Martinez, K. J. L., Moon, A. S., Kulkarni, A., Ettestad, P., Melman, S., Xu, J., Buenemann, M., Hanley, K. A. and Hansen, I. A. (1989) 'Mechanisms of insecticide resistance in *Aedes aegypti* (L.) (Diptera: Culicidae) from Puerto Rico', *Bulletin of Entomological Research*, **14**(2), p. e0212693. doi: 10.1017/S0007485300018630.

Hibino, Y. and Iwahashi, O. (1991) 'Appearance of wild females unreceptive to sterilized males on Okinawa Is. in the eradication program of the melon fly, *Dacus cucurbitae* COQUILLETT (Diptera:Tephritidae)', *Applied Entomology and Zoology*, **26**(2), pp. 265-270 doi: 10.1303/aez.26.265.

Hoffmann, A. A. Montgomery, B.L., Popovici, J., Iturbe-Ormaetxe, I., Johnson, P. H., Muzzi, F., Greenfield, M., Durkan, M., Leong, Y. S., Dong, Y., Cook, H., Axford, J., Callahan, A. G.,

Kenny, N., Omodei, C., McGraw, E. A., Ryan, P. A., Ritchie, S. A., Turelli, M. and O'Neill, S. L. (2011) ‘Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission’, *Nature*, **476**(7361), pp. 454-457. doi: 10.1038/nature10356.

Hoffmann, A. A. (2014) ‘Facilitating *Wolbachia* invasions’, *Austral Entomology*, **52**(2), pp. 125-134. doi: 10.1111/aen.12068.

Hoffmann, A. A., Ross, P. A. and Rašić, G. (2015) ‘*Wolbachia* strains for disease control: Ecological and evolutionary considerations’, *Evolutionary Applications*, **8**(8), pp. 751-768. doi: 10.1111/eva.12286.

Hollis, B., Koppik, M., Wensing, K. U., Ruhmann, H., Genzoni, E., Erkosar, B., Kawecki, T. J., Fricke, C. and Keller, L. (2019) ‘Sexual conflict drives male manipulation of female postmating responses in *Drosophila melanogaster*’, *Proceedings of the National Academy of Sciences*, **116**(17), pp. 8437-8444. doi: 10.1073/pnas.1821386116.

Honma, A., Kumano, N. and Noriyuki, S. (2019) ‘Killing two bugs with one stone: a perspective for targeting multiple pest species by incorporating reproductive interference into sterile insect technique’, *Pest Management Science*, **75**(3), pp. 571-577, doi: 10.1002/ps.5202.

Horvath, P. and Barrangou, R. (2010) ‘CRISPR/Cas, the immune system of bacteria and archaea’, *Science*. **327**(5962), pp. 167-170. doi: 10.1126/science.1179555.

Hsu, P. D., Lander, E. S. and Zhang, F. (2014) ‘Development and applications of CRISPR-Cas9 for genome engineering’, *Cell*, **57**(6), pp. 1262-1278. doi: 10.1016/j.cell.2014.05.010.

Huang, Y. *et al.* (2007) ‘Introducing transgenes into insect populations using combined gene-drive strategies: Modeling and analysis’, *Insect Biochemistry and Molecular Biology*. doi: 10.1016/j.ibmb.2007.06.002.

Hugo, L. E., Magori, K., Lloyd, A. L. and Gould F. (2014) ‘Adult survivorship of the dengue mosquito *Aedes aegypti* varies seasonally in central Vietnam’, *PLoS Neglected Tropical Diseases*, **37**(10), pp. 1054-1063. doi: 10.1371/journal.pntd.0002669.

Hugo, R.L.E., Stassen, L., La, J., Gosden, E., Ekwudu, O.M., Winterford, C., Viennet, E., Faddy, H.M., Devine, G.J. and Frentiu, F.D. (2019) ‘Vector competence of Australian *Aedes aegypti* and *Aedes albopictus* for an epidemic strain of Zika virus’ *PLoS neglected tropical diseases*, **13**(4), p. e0007281. doi: 10.1371/journal.pntd.0007281.

Ikeya, T., Broughton S., Alic, N., Grandison, R. and Partridge L. (2009) ‘The endosymbiont *Wolbachia* increases insulin/IGF-like signalling in *Drosophila*’, *Proceedings of the Royal Society B: Biological Sciences*, **276**(1674), pp. 3799-3807. doi: 10.1098/rspb.2009.0778.

Ishii, T. (2015) ‘Germline genome-editing research and its socioethical implications’, *Trends in Molecular Medicine*, **21**(8), pp. 473-481. doi: 10.1016/j.molmed.2015.05.006.

Iturbe-Ormaetxe, I., Walker, T. and O’Neill, S. L. (2011) ‘*Wolbachia* and the biological control of mosquito-borne disease’, *EMBO Reports*, **12**(6), pp. 508-518. doi: 10.1038/embor.2011.84.

Jamart, J. A., Casares, P., Carracedo, M. C and Piñeiro, R. (1995) ‘Consequences of homo- and heterospecific rapid remating on the fitness of *Drosophila melanogaster* females’, *Journal of Insect Physiology*, **41**(12), pp. 1019-1026. doi: 10.1016/0022-1910(95)00090-H.

James, A. A. (2005) ‘Gene drive systems in mosquitoes: Rules of the road’, *Trends in Parasitology*, **21**(2), pp. 64-67. doi: 10.1016/j.pt.2004.11.004.

Jinek, M. *et al.* (2012) ‘A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity’, *Science*, **337**(6096), pp. 816-821. doi: 10.1126/science.1225829.

Johnson, B.W., Chambers, T.V., Crabtree, M.B., Filippis, A.M., Vilarinhos, P.T., Resende, M.C., Maria de Lourdes, G.M. and Miller, B.R. (2002) ‘Vector competence of Brazilian *Aedes aegypti*

and *Ae. albopictus* for a Brazilian yellow fever virus isolate', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **96**(6), pp. 611-613. doi: 10.1016/S0035-9203(02)90326-3

Jones, C. D. (2005) 'The genetics of adaptation in *Drosophila sechellia*', in *Genetica*, **123**(1-2), pp. 137-145. doi: 10.1007/s10709-004-2728-6.

Joshi, D., McFadden, M. J., Bevins, D., Zhang, F. and Xi, Z. (2014) '*Wolbachia* strain *wAlbB* confers both fitness costs and benefit on *Anopheles stephensi*', *Parasites and Vectors*, **7**(1), p. 336. doi: 10.1186/1756-3305-7-336.

Kalb, J. M., DiBenedetto, A. J. and Wolfner, M. F. (1993) 'Probing the function of Drosophila melanogaster accessory glands by directed cell ablation.', *Proceedings of the National Academy of Sciences*, **90**(17), pp. 8093-8097. doi: 10.1073/pnas.90.17.8093.

Kambris, Z. Cook, P. E., Phuc, H. K. and Sinkins, S. P. (2009) 'Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes', *Science*, **326**(5949), pp. 134-136. doi: 10.1126/science.1177531.

Kishi, S. and Nakazawa, T., 2013. Analysis of species coexistence co-mediated by resource competition and reproductive interference. Population ecology, **55**(2), pp. 305-313. 10.1007/s10144-013-0369-2.

Kistler, K. E., Vosshall, L. B. and Matthews, B. J. (2015) 'Genome engineering with CRISPR-Cas9 in the mosquito *Aedes aegypti*', *Cell Reports*, **11**(1), pp. 51-60 doi: 10.1016/j.celrep.2015.03.009.

Kittayapong, P., Kaeothaisong, N. O., Ninphanomchai, S. and Limohpasmanee, W. (2018) 'Combined sterile insect technique and incompatible insect technique: Sex separation and quality of sterile *Aedes aegypti* male mosquitoes released in a pilot population suppression trial in Thailand', *Parasites and Vectors*, **11**(Suppl 2), p. 657. doi: 10.1186/s13071-018-3214-9.

Kliot, A. and Ghanim, M. (2012) ‘Fitness costs associated with insecticide resistance’, *Pest Management Science*, **68**(11), pp. 1431-1437. doi: 10.1002/ps.3395.

Knipling, E. F. (2015) ‘Possibilities of insect control or eradication through the use of sexually sterile males’, *Journal of Economic Entomology*, **48**(4), pp. 459-462. doi: 10.1093/jee/48.4.459.

Kodric-Brown, A. and West, R. J. D. (2014) ‘Asymmetries in premating isolating mechanisms in a sympatric species flock of pupfish (Cyprinodon)’, *Behavioral Ecology*, **25**(1), pp. 69-75. doi: 10.1093/beheco/art087.

Koou, S. Y., Chong, C. S., Vythilingam, I., Lee, C. Y. and Ng, L. C. (2014) ‘Insecticide resistance and its underlying mechanisms in field populations of *Aedes aegypti* adults (Diptera: Culicidae) in Singapore’, *Parasites and Vectors*, **7**(1), p. 471 doi: 10.1186/s13071-014-0471-0.

Kraemer, M. U. G., Reiner, R. C., Brady, O. J., Messina, J. P., Gilbert, M., Pigott, D. M., Yi, D., Johnson, K., Earl, L., Marczak, L. B., Shirude, S., Davis Weaver, N., Bisanzio, D. Perkins, T. A., Lai, S., Lu, X., Jones, P., Coelho, G. E., Carvalho, R. G., Van Bortel, W., Marsboom, C., Hendrickx, G., Schaffner, F., Moore, C. G., Nax, H. H., Bengtsson, L., Wetter, E., Tatem, A. J. Brownstein, J. S., Smith, D. L., Lambrechts, L., Cauchemez, S., Linard, C., Faria, N. R., Pybus, O. G., Scott, T. W., Liu, Q., Yu, H., Wint, G. R. W., Hay, S. I. and Golding, N. (2019) ‘Past and future spread of the arbovirus vectors *Aedes aegypti* and *Aedes albopictus*’, *Nature Microbiology*, **4**(5), pp. 854-863. doi: 10.1038/s41564-019-0376-y.

Kraemer, M. U., Sinka, M. E., Duda, K. A., Mylne, A. Q., Shearer, F. M., Barker, C. M., Moore, C. G., Carvalho, R. G., Coelho, G. E., Van Bortel, W., Hendrickx, G., Schaffner, F., Elyazar, I. R., Teng, H. J., Brady, O. J., Messina, J. P., Pigott, D. M., Scott, T. W., Smith, D. L., Wint, G. R.,

Golding, N. and Hay, S. I. (2015) 'The global distribution of the arbovirus vectors *Aedes aegypti* and *Ae. albopictus*', *eLife*, **4**, p. e08347 doi: 10.7554/elife.08347.

Kumar, S., Thakur, K., Sharma, B., Bhardwaj, T. R., Prasad, D. N. and Singh, R. K. (2019) 'Recent advances in vaccine development for the treatment of emerging infectious diseases', *Indian Journal of Pharmaceutical Education and Research*, **53**(3), pp. 343-354. doi: 10.5530/ijper.53.3.68.

Kunin, V., Sorek, R. and Hugenholtz, P. (2007) 'Evolutionary conservation of sequence and secondary structures in CRISPR repeats', *Genome Biology*, **8**(4), p. R61. doi: 10.1186/gb-2007-8-4-r61.

Kyrou, K., Hammond, A. M., Galizi, R., Kranjc, N., Burt, A., Beaghton, A. K., Nolan, T. and Crisanti, A. (2018) 'A CRISPR-Cas9 gene drive targeting doublesex causes complete population suppression in caged *Anopheles gambiae* mosquitoes', *Nature biotechnology*, **36**(11), pp. 1062–1066. doi: 10.1038/nbt.4245.

Labbé, G. M. C., Scaife, S., Morgan, S. A., Curtis, Z. H. and Alphey, L. (2012) 'Female-specific flightless (fsRIDL) phenotype for control of *Aedes albopictus*', *PLoS Neglected Tropical Diseases*, **6**(7), p. e1724. doi: 10.1371/journal.pntd.0001724.

Lachaise, D., Cariou, M., David, J. R., Lemeunier, F., Tsacas, L. and Ashburner M. (1988) 'Historical Biogeography of the *Drosophila melanogaster* Species Subgroup', in *Evolutionary Biology*, **22**, pp. 159-225. Springer, Boston, MA. doi: 10.1007/978-1-4613-0931-4_4.

Lacroix, R., McKemey, A. R., Raduan, N., Kwee Wee, L., Hong Ming, W., Guat Ney, T., Rahidah, A. A. S., Salman, S., Subramaniam, S., Nordin, O., Hanum, A. T. N., Angamuthu, C., Marlina Mansor, S., Lees, R. S., Naish, N., Scaife, S., Gray, P., Labbé, G., Beech, C., Nimmo, D., Alphey, L., Vasan, S. S., Han Lim, L., Wasi, A. N. and Murad, S. (2012) 'Open field release of genetically

engineered sterile male *Aedes aegypti* in Malaysia', *PLoS ONE*, **7**(8), p. e42771 doi: 10.1371/journal.pone.0042771.

Lahondère, C., Vinauger, L., Okubo, R. P., Wolff, G., Akbari O. S. and Riffell, J. A. (2019) 'The olfactory basis of orchid pollination by mosquitoes', *bioRxiv* [PREPRINT], p.643510. doi: 10.1101/643510. Preprint at: https://www.biorxiv.org/content/10.1101/643510v1.full.

Lambrechts, L. Ferguson, N. M., Harris, E., Holmes, E. C., McGraw, E. A., O'Neill, S. L., Ooi, E. E., Ritchie, S.A., Ryan, P. A., Scott, T. W., Simmons, C. P. and Weaver, S. C. (2015) 'Assessing the epidemiological effect of *Wolbachia* for dengue control', *The Lancet Infectious Diseases*, **15**(7), pp. 862-866. doi: 10.1016/S1473-3099(15)00091-2.

Landolt, P. J. and Heath, R. R. (1987) 'Role of female-produced sex pheromone in behavioral reproductive isolation between *Trichoplusia ni* (Hübner) and *Pseudoplusia includens* (Walker) (Lepidoptera: Noctuidae, plusiinae)', *Journal of Chemical Ecology*, **13**(5), pp. 1005-1018. doi: 10.1007/BF01020534.

Lee, R. C. P. (1983) 'An asymmetric species-isolating mechanism between two sympatric species of *Zaprionus* (Diptera, Drosophilidae)', *Animal Behaviour*, **31**(1), pp. 11-25. doi: 10.1016/S0003-3472(83)80169-9.

Lees, R. S., Gilles, J. R. L., Hendrichs, J., Vreysen, M. J. B. and Bourtzis, K. (2015) 'Back to the future: the sterile insect technique against mosquito disease vectors', *Current Opinion in Insect Science*, **10**, pp. 156-162. doi: 10.1016/j.cois.2015.05.011.

Leftwich, P. T., Bolton, M. and Chapman, T. (2016) 'Evolutionary biology and genetic techniques for insect control', *Evolutionary Applications*, **9**(1), pp. 212-230 doi: 10.1111/eva.12280.

Legrand, D., Tenaillon, M. I., Matyot, P., Gerlach, J., Lachaise D. and Cariou, M. (2009) 'Species-wide genetic variation and demographic history of *Drosophila sechellia*, a species

lacking population structure', *Genetics*, **182**(4), pp. 1197-1206. doi: 10.1534/genetics.108.092080.

Li, T. and Shen, X. (2019). 'Pleiotropy complicates human gene editing: CCR5Δ32 and beyond', *Frontiers in Genetics*, **10**, p. 669.

Liu, Z., Zhou, T., Lai, Z., Zhang, Z., Jia, Z., Zhou, G., Williams, T., Xu, J., Gu, J., Zhou, X., Lin, L., Yan, G. and Chen, X. (2017) 'Competence of *Aedes aegypti*, *Ae. albopictus*, and *Culex quinquefasciatus* mosquitoes as Zika virus vectors, China', *Emerging Infectious Diseases*, **23**(7), p. 1085. doi: 10.3201/eid2307.161528.

Lizé, A., Price, T. A. R., Marcello, M., Smaller, E. A., Lewis, Z. and Hurst, G. D. D. (2012) 'Males do not prolong copulation in response to competitor males in the polyandrous fly *Drosophila bifasciata*', *Physiological Entomology*. doi: 10.1111/j.1365-3032.2012.00836.x.

Llopart, A., Lachaise, D. and Coyne, J. A. (2005) 'Multilocus analysis of introgression between two sympatric sister species of *Drosophila*: *Drosophila yakuba* and *D. santomea*', *Genetics*, **171**(1), pp. 197-210. doi: 10.1534/genetics.104.033597.

Louca, V., Lucas, M. C., Green, C., Majambere, S., Fillinger, U. and Lindsay, S. W. (2009) 'Role of fish as predators of mosquito larvae on the floodplain of the Gambia River', *Journal of Medical Entomology*, **46**(3), pp. 546–556, doi: 10.1603/033.046.0320.

Lowe, R. E., Fowler, J. E. F., Bailey, D. L., Dame, D. A. and Savage, K. E. (1981) 'Separation of sexes of adult *Anopheles albimanus* by feeding of insecticide-laden blood', *Mosquito News*, **41**, pp. 634–638.

Ma, Y., Zhang, L. and Huang, X. (2014) 'Genome modification by CRISPR/Cas9', *FEBS Journal*, **281**(23), pp. 5186-93. doi: 10.1111/febs.13110.

Madakacherry, O., Lees, R. S. and Gilles, J. R. L. (2014) '*Aedes albopictus* (Skuse) males in laboratory and semi-field cages: Release ratios and mating competitiveness', *Acta Tropica*, **132**(Supplement), pp. S124-S129. doi: 10.1016/j.actatropica.2013.11.020.

Magori, K. and Gould, F. (2006) 'Genetically engineered underdominance for manipulation of pest populations: A deterministic model', *Genetics*, **172**(4), pp. 2613-2620. doi: 10.1534/genetics.105.051789.

Mair, M. M., Seifert, N. and Ruther, J. (2018) 'Previous interspecific courtship impairs female receptivity to conspecifics in the parasitoid wasp *Nasonia longicornis* but not in *N. Vitripennis*', *Insects*, **9**(3): 112. doi: 10.3390/insects9030112.

Malcolm, C. A. and Wood, R. J. (1982) 'Location of a gene conferring resistance to knockdown by permethrin and bioresmethrin in adults of the BKPM3 strain of *Aedes aegypti*', *Genetica*, **59**(3), pp. 233–237. doi: 10.1007/BF00056546.

Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., DiCarlo, J. E., Norville, J. E. and Church, G. M. (2013) 'RNA-guided human genome engineering via Cas9', *Science*, **339**(6121), pp. 823-826. doi: 10.1126/science.1232033.

Markow, T. A. (1981) 'Courtship behavior and control of reproductive isolation between *Drosophila mojavensis* and *Drosophila arizonensis*', *Evolution*, **35**(5), 1022-1026, doi: 10.2307/2407873.

Marois, E., Scali, C., Soichot, J., Kappler, C., Levashina, E. A. and Catteruccia, F. (2012) 'High-throughput sorting of mosquito larvae for laboratory studies and for future vector control interventions', *Malaria Journal*, **11**(1), p.302. doi: 10.1186/1475-2875-11-302.

Marshall, J. M. (2009) 'The effect of gene drive on containment of transgenic mosquitoes', *Journal of Theoretical Biology*, **258**(2), pp. 250-265. doi: 10.1016/j.jtbi.2009.01.031.

Martin, O. Y. and Hosken, D. J. (2004) 'Reproductive consequences of population divergence through sexual conflict', *Current Biology,* **14**(10), 906-910. doi: 10.1016/j.cub.2004.04.043.

Martin, O. Y. and Hosken, D. J. (2003) 'The evolution of reproductive isolation through sexual conflict', *Nature*, **423**(6943), pp. 979-982. doi: 10.1038/nature01752.

Martinez, J., Ok, S., Smith, S., Snoeck, K., Day, J. P. and Jiggins, F. M. (2015) 'Should symbionts be nice or selfish? Antiviral effects of *Wolbachia* are costly but reproductive parasitism is not', *PLoS Pathogens*, **11**(7), p. e1005021. doi: 10.1371/journal.ppat.1005021.

Marygold, S. J., Roote, J., Reuter, G., Lambertsson, A., Ashburner, M., Millburn, G. H., Harrison, P. M., Yu, Z., Kenmochi, N., Kaufman, T. C., Leevers, S.J. and Cook, K.R. (2007) 'The ribosomal protein genes and *Minute* loci of *Drosophila melanogaster*', *Genome Biology*, **8**(10), p. R216. doi: 10.1186/gb-2007-8-10-r216.

Massonnet-Bruneel, B., Corre-Catelin, N., Lacroix, R., Lees, R. S., Hoang, K. P., Nimmo, D., Alphey, L. and Reiter, P. (2013) 'Fitness of transgenic mosquito *Aedes aegypti* males carrying a dominant lethal genetic system', *PLoS ONE*, **8**(5), p. e62711. doi: 10.1371/journal.pone.0062711.

Mastrangelo, T., Kovaleski, A., Botteon, V., Scopel, W. and Costa, M. L. Z. (2018) 'Optimization of the sterilizing doses and overflooding ratios for the South American fruit fly', *PLoS ONE*, **3**(7), p. e0201026. doi: 10.1371/journal.pone.0201026.

Mattingly, P. F. (1967) 'Taxonomy of *Aedes aegypti* and related species', *Bulletin of the World Health Organization*, **36**(4), pp. 552–554.

Matute, D. R., Gavin-Smyth, J. and Liu, G. (2014) 'Variable post-zygotic isolation in *Drosophila melanogaster*/*D. simulans* hybrids', *Journal of Evolutionary Biology*, **27**(8), pp. 1691-1705. doi: 10.1111/jeb.12422.

Mazzi, D., Kesäniemi J., Hoikkala, A. and Klappert, K. (2009) ‘Sexual conflict over the duration of copulation in *Drosophila montana*: Why is longer better?’, *BMC Evolutionary Biology*, **9**(1), p. 132 doi: 10.1186/1471-2148-9-132.

McInnis, D. O., Lance, D. R. and Jackson, C. G. (1996) ‘Behavioral resistance to the sterile insect technique by Mediterranean fruit fly (Diptera: Tephritidae) in Hawaii’, *Annals of the Entomological Society of America*, **89**(5), pp. 739-744. doi: 10.1093/aesa/89.5.739.

McInnis, D. O., Tam, S., Grace, C. and Miyashita, D. (1994) ‘Population supression and sterility rates induced by variable sex ratio, sterile insect releases of *Ceratitis capitata* (Diptera: Tephritidae) in Hawaii’, *Annals of the Entomological Society of America*, **87**(2), pp. 231-240. doi: 10.1093/aesa/87.2.231.

Medina, R. F. (2018) ‘Gene drives and the management of agricultural pests’, *Journal of Responsible Innovation*, **5**(Supplementary), pp. S255-S262. doi: 10.1080/23299460.2017.1407913.

Mendelson, T. C. (2003) ‘Evidence of intermediate and asymmetrical behavioral isolation between orangethroat and orangebelly darters (Teleostei: Percidae)’, *The American Midland Naturalist*, **150**(2), pp. 343-347. doi: 10.1674/0003-0031(2003)150[0343:eoiaab]2.0.co;2.

Minekawa, K., Miyatake, T., Ishikawa, Y. and Matsuo, T. (2018) ‘The adaptive role of a species-specific courtship behaviour in coping with remating suppression of mated females’, *Animal Behaviour*, **144**, pp. 29-37. doi: 10.1016/j.anbehav.2018.04.002.

Montchamp-Moreau, C. (1983) ‘Interspecific competition between *Drosophila melanogaster* and *Drosophila simulans*: temperature effect on competitive ability and fitness components’, *Genetics Selection Evolution*, **15**(3), pp. 367–378. doi: 10.1186/1297-9686-15-3-367.

Morin, J., Moreteau, B., Pétavy, G., Imasheva A. G. and David, J. R. (1996) ‘Body size and developmental temperature in *Drosophila simulans*: Comparison of reaction norms with

sympatric *Drosophila melanogaster*', *Genetics Selection Evolution*, **28**(5), p. 415. doi: 10.1186/1297-9686-28-5-415.

Morrill, S. and Amon, A. (2019) 'Why haploinsufficiency persists', *Proceedings of the National Academy of Sciences*, **116**(24), pp. 11866–11871. doi: 10.1073/pnas.1900437116

Mossman, J.A., Mabeza, R.M.S., Blake, E., Mehta, N. and Rand, D.M., (2019) 'Age of both parents influences reproduction and egg dumping behavior in *Drosophila melanogaster*', Journal of Heredity, **110**(3), pp.300-309.

Moulin, B., Aubin, T. and Jallon, J. M. (2004) 'Why there is a one-way crossability between *D. melanogaster* and *D. simulans*? An ontogenic explanation', *Genetica*, **120**(1-3), pp. 285-292. doi: 10.1023/B:GENE.0000017650.45464.f4.

Mueller, J. L., Ravi Ram, K., McGraw, L. A., Bloch Qazi, M. C., Siggia, E. D., Clark, A. G. Aquadro, C. F. and Wolfner, M. F. (2005) 'Cross-species comparison of *Drosophila* male accessory gland protein genes', *Genetics*, **171**(1), pp. 131–143. doi: 10.1534/genetics.105.043844.

Mumford, J. D. (2012) 'Science, regulation, and precedent for genetically modified insects', *PLoS Neglected Tropical Diseases*, **6**(1), p. e1504. doi: 10.1371/journal.pntd.0001504.

Munhenga, G., Brooke, B. D., Gilles, J. R. L., Slabbert, K., Kemp, A., Dandalo, L. C., Wood, O. R., Lobb, L. N., Govender, D., Renke, M. and Koekemoer L. L. (2016) 'Mating competitiveness of sterile genetic sexing strain males (GAMA) under laboratory and semi-field conditions: Steps towards the use of the sterile insect technique to control the major malaria vector *Anopheles arabiensis* in South Africa', *Parasites and Vectors*, **9**(1), p. 122. doi: 10.1186/s13071-016-1385-9.

Namciu, S. J., Blochlinger, K. B. and Fournier, R. E. K. (1998) 'Human matrix attachment regions insulate transgene expression from chromosomal position effects in *Drosophila*

melanogaster', *Molecular and Cellular Biology*, **18**(4), pp. 2382-2391. doi: 10.1128/mcb.18.4.2382.

National Academies of Sciences Engineering and Medicine. (2016) *Gene drives on the horizon: advancing science, navigating uncertainty, and aligning research with public values.* National Academies Press. doi: 10.17226/23405.

Noble, C., Min, J., Olejarz, J., Buchthal, J., Chavez, A., Smidler, A. L., DeBenedictis, E. A., Church, G. M., Nowak, M. A. and Esvelt, K. M. (2019) 'Daisy-chain gene drives for the alteration of local populations', *Proceedings of the National Academy of Sciences*, **116**(17), pp. 8275-8282. doi: 10.1073/pnas.1716358116.

Nguyen, T. H., Nguyen, H. L., Nguyen, T. Y., Vu, S. N., Tran, N. D., Le, T. N., Vien, Q. M., Bui, T. C., Le, H. T., Kutcher, S., Hurst, T. P., Duong, T. T., Jeffery, J. A., Darbro, J. M., Kay, B. H., Iturbe-Ormaetxe, I., Popovici, J., Montgomery, B. L., Turley, A. P., Zigterman, F., Cook, H., Cook, P. E., Johnson, P. H., Ryan, P. A., Paton, C. J., Ritchie, S. A., Simmons, C. P., O'Neill, S. L. and Hoffmann, A. A. (2015) 'Field evaluation of the establishment potential of *w*MelPop *Wolbachia* in Australia and Vietnam for dengue control', *Parasites and Vectors*, **8**(1), p.563. doi: 10.1186/s13071-015-1174-x.

Obbard, D. J., Maclennan, J., Kim, K. W., Rambaut, A., O'Grady, P. M. and Jiggins, F. M. (2012) 'Estimating divergence dates and substitution rates in the *Drosophila* phylogeny', *Molecular Biology and Evolution*, **29**(11), pp. 3459-3473. doi: 10.1093/molbev/mss150.

Olcott, M. H., Henkels, M. D., Rosen, K. L., Walker, F. L., Sneh, B., Loper, J. E. and Taylor, B. J. (2010) 'Lethality and developmental delay in *Drosophila melanogaster* larvae after ingestion of selected *Pseudomonas fluorescens* strains', *PLoS ONE*, **5**(9), p. e12504. doi: 10.1371/journal.pone.0012504.

Oliva, C. F., Jacquet, M., Gilles, J., Lemperiere, G., Maquart, P. O., Quilici, S., Schooneman, F., Vreysen, M. J. and Boyer, S. (2012) 'The sterile insect technique for controlling populations of *Aedes albopictus* (Diptera: Culicidae) on reunion island: mating vigour of sterilized males', *PLoS ONE*, **7**(11), p. e49414. doi: 10.1371/journal.pone.0049414.

Oliva, C. F., Maier, M.J., Gilles, J., Jacquet, M., Lemperiere, G., Quilici, S., Vreysen, M. J., Schooneman, F., Chadee, D. D. and Boyer, S. (2013) 'Effects of irradiation, presence of females, and sugar supply on the longevity of sterile males *Aedes albopictus* (Skuse) under semi-field conditions on Reunion Island', *Acta Tropica*, **125**(3), pp. 287-293. doi: 10.1016/j.actatropica.2012.11.010.

Orr, H. A. (1996) 'Dobzhansky, Bateson, and the genetics of speciation', *Genetics*, **144**(4), pp. 1331-1335.

O'Shea, K. L. and Singh, N. D. (2015) 'Tetracycline-exposed *Drosophila melanogaster* males produce fewer offspring but a relative excess of sons', *Ecology and Evolution*, **5**(15), pp. 3130–3139. doi: 10.1002/ece3.1535.

Oye, K. A., Esvelt, K., Appleton, E., Catteruccia, F., Church, G., Kuiken, T., Bar-Yam Lightfoot, S., McNamara, J., Smidler, A. and Collins, J. P. (2014) 'Regulating gene drives', *Science*, **345**(6197), pp. 626-628. doi: 10.1126/science.1254287.

Papathanos, P. A., Bossin, H. C., Benedict, M. Q., Catteruccia, F., Malcolm, C. A., Alphey, L. and Crisanti, A. (2009) 'Sex separation strategies: Past experience and new approaches', *Malaria Journal*, **8**(2), pp. S5. doi: 10.1186/1475-2875-8-S2-S5.

Pardy, J.A., Rundle, H.D., Bernards, M.A. and Moehring, A.J., (2019) 'The genetic basis of female pheromone differences between *Drosophila melanogaster* and *D. simulans*', *Heredity*, **122**(1), p. 93. doi: 10.1038/s41437-018-0080-3.

Patil, P. B., Reddy, B. P., Gorman, K., Reddy, K. V., Barwale, S. R., Zehr, U. B., Nimmo, D., Naish, N. and Alphey, L. (2015) ‘Mating competitiveness and life-table comparisons between transgenic and Indian wild-type *Aedes aegypti* L.’, *Pest Management Science*, **71**(7), pp. 957-965, doi: 10.1002/ps.3873.

Phuc, H., Andreasen, M. H., Burton, R. S., Vass, C., Epton, M. J., Pape, G., Fu, G., Condon, K. C., Scaife, S., Donnelly, C. A., Coleman, P. G., White-Cooper, H. and Alphey L. (2007) ‘Late-acting dominant lethal genetic systems and mosquito control’, *BMC Biology*, **5**(1), p. 11. doi: 10.1186/1741-7007-5-11.

Pimentel, D. (2009) ‘Pest control in world agriculture’, *Agricultural Science*, **2**, pp. 272–293.

Pimentel, D., Dewey, J. E. and Schwardt, H. H. (1951) ‘An Increase in the Duration of the Life Cycle of DDT-Resistant Strains of the House Fly’, *Journal of Economic Entomology*, **44**(4), pp. 477-481. doi: 10.1093/jee/44.4.477.

Pitnick, S., Brown, W. D. and Miller, G. T. (2001) ‘Evolution of female remating behaviour following experimental removal of sexual selection’, *Proceedings of the Royal Society B: Biological Sciences*, **268**(1467), pp. 557–563. doi: 10.1098/rspb.2000.1400.

Polačik, M. and Reichard, M. (2011) ‘Asymmetric reproductive isolation between two sympatric annual killifish with extremely short lifespans’, *PLoS ONE*, **6**(8), p.e22684. doi: 10.1371/journal.pone.0022684.

Pollack, R. (2015) ‘Eugenics lurk in the shadow of CRISPR’, *Science*. **348**(6237), p. 871 doi: 10.1126/science.348.6237.871-a.

Pryce, J., Choi, L., Richardson, M. and Malone, D. (2018) ‘Insecticide space spraying for preventing malaria transmission’, *Cochrane Database of Systematic Reviews*. **11**. doi: 10.1002/14651858.CD012689.pub2.

Quétier, F. (2016) ‘The CRISPR-Cas9 technology: Closer to the ultimate toolkit for targeted genome editing’, *Plant Science*, **242**, 65-76. doi: 10.1016/j.plantsci.2015.09.003.

R Core Team (2012) ‘R: A language and environment for statistical computing’, *R Foundation for Statistical Computing*. doi: ISBN 3-900051-07-0.

Reed, F. A., Traulsen, A. and Altrock, P. M. (2013) ‘Underdominance’ *Brenner’s Encyclopedia of Genetics*, **2**(7), pp. 247-249. Elsevier Science. doi: 10.1016/B978-0-12-374984-0.01601-6.

Reed, F. A., Aquino-Michaels, T. G., Costantini, M. S., Láruson, Á. J. and Sutton, J. T. (2018) ‘RPM-Drive: A robust, safe, and reversible gene drive system that remains functional after 200+ generations’, *arXiv* [PREPRINT]. doi: arXiv:1806.05304. Preprint at: https://arxiv.org/abs/1806.05304.

Reeves, R. G. Bryk, J., Altrock, P. M., Denton, J. A., Reed, F. A. (2014) ‘First steps towards underdominant genetic transformation of insect populations’, *PLoS ONE*, **9**(5), p. e97557 doi: 10.1371/journal.pone.0097557.

Rendón, P., McInnis, D., Lance, D. and Stewart, J. (2009) ‘Medfly (Diptera:Tephritidae) genetic sexing: large-scale field comparison of males-only and bisexual sterile fly releases in Guatemala’, *Journal of Economic Entomology*, **97**(5): pp. 1547-1553 doi: 10.1603/0022-0493-97.5.1547.

REX Consortium. (2013) ‘Heterogeneity of selection and the evolution of resistance’, *Trends in Ecology and Evolution*, **28**(2), pp. 110-118. doi: 10.1016/j.tree.2012.09.001.

Ribeiro, J. M. C. and Spielman, A. (1986) ‘The satyr effect: a model predicting parapatry and species extinction’, *The American Naturalist*, **128**(4), pp. 513-528. doi: 10.1086/284584.

Ritchie, S. A., Montgomery, B. L. and Hoffmann, A. A. (2013) ‘Novel estimates of *Aedes aegypti* (Diptera: Culicidae) population size and adult survival based on *Wolbachia* releases’, *Journal of Medical Entomology*, **50**(3), pp. 624-631 doi: 10.1603/me12201.

Ritchie, S. A., Townsend, M., Paton, C. J., Callahan, A. G. and Hoffmann, A. A. (2015) ‘Application of *w*MelPop *Wolbachia* strain to crash local populations of *Aedes aegypti*’, *PLoS Neglected Tropical Diseases*, **9**(7), p. e0003930. doi: 10.1371/journal.pntd.0003930.

Rode, N. O., Estoup, A., Bourguet, D., Courtier-Orgogozo, V. and Débarre, F. (2019) ‘Population management using gene drive: molecular design, models of spread dynamics and assessment of ecological risks’, *Conservation Genetics*, **20**(4), pp. 671-690. doi: 10.1007/s10592-019-01165-5.

Rodriguez, S. D., Brar, R. K., Drake, L. L., Drumm, H. E., Price, D. P., Hammond, J. I., Urquidi, J. and Hansen, I. A. (2013) ‘The effect of the radio-protective agents ethanol, trimethylglycine, and beer on survival of X-ray-sterilized male *Aedes aegypti*’, *Parasites and Vectors*, **6**(1), p.211. doi: 10.1186/1756-3305-6-211.

Rottschaefer, S. M. and Lazzaro, B. P. (2012) ‘No effect of *Wolbachia* on resistance to intracellular infection by pathogenic bacteria in *Drosophila melanogaster*’, *PLoS ONE*, **7**(7), p.e40500. doi: 10.1371/journal.pone.0040500.

Rozera, R., Verma S., Kumar, R., Haque, A. and Attri, A. (2019) ‘Herbal remedies, vaccines and drugs for dengue fever: Emerging prevention and treatment strategies’, *Asian Pacific Journal of Tropical Medicine*, **12**(4), pp. 147-152. doi: 10.4103/1995-7645.257113.

Rubinstein, C. D. and Wolfner, M. F. (2013) ‘*Drosophila* seminal protein ovulin mediates ovulation through female octopamine neuronal signaling’, *Proceedings of the National Academy of Sciences*, **110**(43), pp. 17420-17425 doi: 10.1073/pnas.1220018110.

Seehausen, O., Van Alphen, J. J. M. and Witte, F. (1997) ‘Cichlid fish diversity threatened by eutrophication that curbs sexual selection’, *Science*, **277**(5333), pp. 1808-1811. doi: 10.1126/science.277.5333.1808.

Serbus, L. R., Casper-Lindley, C., Landmann, F. and Sullivan, W. (2008) 'The Genetics and Cell Biology of *Wolbachia* -Host Interactions', *Annual Review of Genetics*, **42**, pp. 683-707. doi: 10.1146/annurev.genet.41.110306.130354.

Sharma, M. D., Mitchell. C., Hunt, J., Tregenza, T. and Hosken, D.J. (2012) 'The genetics of cuticular hydrocarbon profiles in the fruit fly *Drosophila simulans*', *Journal of Heredity*, **103**(2), pp. 230-239. doi: 10.1093/jhered/esr132.

Shuker, D. M. and Burdfield-steel, E. R. (2017) 'Reproductive interference in insects', *Ecological Entomology*, **42**(S1), pp. 65-75. doi: 10.1111/een.12450.

Sinkins, S. P. (2004) 'Wolbachia and cytoplasmic incompatibility in mosquitoes', in *Insect Biochemistry and Molecular Biology*, **34**(7), pp. 723-729. doi: 10.1016/j.ibmb.2004.03.025.

Sirot, L. K., LaFlamme, B. A., Sitnik, J. L., Rubinstein, C. D., Avila, F. W., Chow, C. Y. and Wolfner, M. F. (2009a) 'Molecular social interactions: *Drosophila melanogaster* seminal fluid proteins as a case study.', in *Advances in genetics*, **68**, pp. 23-56. doi: 10.1016/S0065-2660(09)68002-0.

Sirot, L. K., Buehner, N. A., Fiumera, A. C. and Wolfner, M. F. (2009b) 'Seminal fluid protein depletion and replenishment in the fruit fly, *Drosophila melanogaster*: An ELISA-based method for tracking individual ejaculates', *Behavioral Ecology and Sociobiology*, **63**(10), pp. 1505-1513. doi: 10.1007/s00265-009-0806-6.

Sirot, L. K., Findlay, G. D., Sitnik, J. L., Frasheri, D., Avila, F. W. and Wolfner, M. F. (2014) 'Molecular characterization and evolution of a gene family encoding both female-and male-specific reproductive proteins in *Drosophila*', *Molecular Biology and Evolution*. **31**(6), pp. 1554-67. doi: 10.1093/molbev/msu114.

Sirot, L. K., Wong, A., Chapman, T. and Wolfner, M. F. (2015) ‘Sexual conflict and seminal fluid proteins: A dynamic landscape of sexual interactions’, *Cold Spring Harbor Perspectives in Biology*, **7**(2), p. a017533. doi: 101101/cshperspect.a017533.

Slosek, J. (1986) ‘*Aedes aegypti* mosquitoes in the Americas: A review of their interactions with the human population’, *Social Science and Medicine*, **23**(3), pp. 249-257. doi: 10.1016/0277-9536(86)90345-X.

Sowilem, M. M., Kamal, H. A. and Khater, E. I. (2013) ‘Life table characteristics of *Aedes aegypti* (Diptera: Culicidae) from Saudi Arabia’, *Tropical Biomedicine*, **30**(2), pp. 301-314.

Sperlich, D. (1962) ‘Hybrids between *Drosophila melanogaster* and *D. simulans* in nature’, *Drosophila Information Service*, **36**, p. 118.

Sternberg, S. H., Redding, S., Jinek, M., Greene, E. C. and Doudna, J. A. (2014) ‘DNA interrogation by the CRISPR RNA-guided endonuclease Cas9’, *Nature*, **507**(7490), pp. 62-67 doi: 10.1038/nature13011.

Stockley, P. (1997) ‘Sexual conflict resulting from adaptations to sperm competition’, *Trends in Ecology and Evolution*, **12**(4), 154-159 doi: 10.1016/S0169-5347(97)01000-8.

Stouthamer, R., Breeuwer, J. A. J. and Hurst, G. D. D. (1999) ‘*Wolbachia pipientis*: microbial manipulator of arthropod reproduction’, *Annual Review of Microbiology*, **53**, pp. 71-102. doi: 10.1146/annurev.micro.53.1.71.

Sturtevant, A. (1920) ‘Genetic studies on *Drosophila simulans*. I. Introduction. Hybrids with *Drosophila melanogaster*.’, *Genetics*, **5**(5), pp. 488–500. doi: 10.1002/jez.b.21128.

Suman, D. S. (2019) ‘Evaluation of enhanced oviposition attractant formulations against *Aedes* and *Culex* vector mosquitoes in urban and semi-urban areas’, *Parasitology Research*, **118**(3), pp. 743-750. doi: 10.1007/s00436-019-06228-7.

Swanson, W. J., Yang, Z., Wolfner, M. F. and Aquadro, C.F. (2001) ‘Positive Darwinian selection drives the evolution of several female reproductive proteins in mammals’, *Proceedings of the National Academy of Sciences*, **98**(5), pp. 2509-2514 doi: 10.1073/pnas.051605998.

Swanson, W. J. and Vacquier, V. D. (2002) ‘The rapid evolution of reproductive proteins’, *Nature Reviews Genetics*, **3**(2), pp. 137-44. doi: 10.1038/nrg733.

Takahashi, A., Fujiwara-Tsujii, N., Yamaoka, R., Itoh, M., Ozaki, M. and Takano-Shimizu, T. (2012) ‘Cuticular hydrocarbon content that affects male mate preference of *Drosophila melanogaster* from West Africa’, *International Journal of Evolutionary Biology*. **2012**. doi: 10.1155/2012/278903.

Teixeira, L., Ferreira, Á. and Ashburner, M. (2008) ‘The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*’, *PLoS Biology*, **6**(12), p. e2. doi: 10.1371/journal.pbio.1000002.

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**(5462), pp. 2474-2476. doi: 10.1126/science.287.5462.2474.

Thurmond, J., Goodman, J. L., Strelets, V. B., Attrill, H. 2., Gramates, L. S., Marygold, S. J., Matthews, B. B., Millburn, G., Antonazzo, G., Trovisco, V., Kaufman, T. C. and Calvi, B. R.; FlyBase Consortium. (2019) ‘FlyBase 2.0: the next generation’, *Nucleic Acids Research*, **47**(D1), pp. D759-D765. doi: 10.1093/nar/gky1003.

Traver, B. E., Anderson, M. A. E. and Adelman, Z. N. (2009) ‘Homing endonucleases catalyze double-stranded DNA breaks and somatic transgene excision in *Aedes aegypti*’, *Insect Molecular Biology*, **18**(5), pp. 623-633. doi: 10.1111/j.1365-2583.2009.00905.x.

Tripet, F., Lounibos, L. P., Robbins, D., Moran, J., Nishimura, N. and Blosser, E. M. (2011) ‘Competitive reduction by satyrization? Evidence for interspecific mating in nature and asymmetric reproductive competition between invasive mosquito vectors’, *American Journal of Tropical Medicine and Hygiene*, **85**(2), pp. 265-270. doi: 10.4269/ajtmh.2011.10-0677.

Tsuda, M. and Aigaki, T. (2016) ‘Evolution of sex-peptide in Drosophila’, *Fly*, **10**(4), pp. 172-177. doi: 10.1080/19336934.2016.1193655.

Turissini, D. A., McGirr, J. A., Patel, S. S., David, J. R. and Matute, D. R. (2018) ‘The rate of evolution of postmating-prezygotic reproductive isolation in *Drosophila*’, *Molecular Biology and Evolution*, **35**(2), pp. 312-334. doi: 10.1093/molbev/msx271.

Van Doorn, G. S., Edelaar, P. and Weissing, F. J. (2009) ‘On the origin of species by natural and sexual selection’, *Science*, **326**(5960), pp. 1704-1707. doi: 10.1126/science.1181661.

Vreysen, M. J. B. Saleh, K. M., Ali, M. Y., Abdulla, A. M., Zhu, Z. R., Juma, K. G., Dyck, V. A., Msangi, A. R., Mkonyi, P. A. and Feldmann, H. U. (2009) ‘*Glossina austeni* (Diptera: Glossinidae) eradicated on the island of Unguja, Zanzibar, using the sterile insect technique’, *Journal of Economic Entomology*, **93**(1), pp. 123-35. doi: 10.1603/0022-0493-93.1.123.

Walker, T., Johnson, P. H., Moreira, L. A., Iturbe-Ormaetxe, I., Frentiu, F. D., McMeniman, C. J., Leong, Y. S., Dong, Y., Axford, J., Kriesner, P., Lloyd, A. L., Ritchie, S. A., O'Neill, S. L. and Hoffmann, A. A. (2011) ‘The *w*Mel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations’, *Nature*, **476**(7361), pp. 450-453. doi: 10.1038/nature10355.

Watanabe, M., Miura, K., Hunter, M. S., and Wajnberg, E. (2011) ‘Superinfection of cytoplasmic incompatibility-inducing Wolbachia is not additive in *Orius strigicollis* (Hemiptera: Anthocoridae)’, *Heredity*, **106**(4), pp. 642–648. doi: 10.1038/hdy.2010.100.

Wei, Y., Terns, R. M. and Terns, M. P. (2015) 'Cas9 function and host genome sampling in type II-A CRISPR–cas adaptation', *Genes and Development*, **29**(4), pp. 356–361. doi: 10.1101/gad.257550.114.

Wolfner, M. F. (1997) 'Tokens of love: Functions and regulation of *Drosophila* male accessory gland products', *Insect Biochemistry and Molecular Biology*, **27**(3), pp. 179-192. doi: 10.1016/S0965-1748(96)00084-7.

World Health Organization (2014) 'A Global brief on vector-borne diseases', *World Health Organization*, Report at: https://apps.who.int/iris/handle/10665/111008.

Wyss, J. H. (2000) 'Screwworm eradication in the Americas', *Annals of the New York Academy of Sciences*, **916**, pp. 186-193. doi: 10.1111/j.1749-6632.2000.tb05289.x.

Yassin, A. and David, J. R. (2016) 'Within-species reproductive costs affect the asymmetry of satyrization in *Drosophila'*, *Journal of Evolutionary Biology*, **29**(2), pp.455-460. doi: 10.1111/jeb.12784.

Yi, H., Devkota, B.R., Yu, J.S., Oh, K.C., Kim, J. and Kim, H.J (2014) 'Effects of global warming on mosquitoes & mosquito-borne diseases and the new strategies for mosquito control', *Entomological Research*, **44**(6), pp. 215-235 doi: 10.1111/1748-5967.12084.

Zhang, B., Salieb-Beugelaar, G.B., Nigo, M.M., Weidmann, M. and Hunziker, P (2015) 'Diagnosing dengue virus infection: Rapid tests and the role of micro/nanotechnologies', *Nanomedicine: Nanotechnology, Biology, and Medicine*, **11**(7), pp. 1745-1761. doi: 10.1016/j.nano.2015.05.009.

Zhang, F., Wen, Y. and Guo, X. (2014) 'CRISPR/Cas9 for genome editing: Progress, implications and challenges', *Human Molecular Genetics*, **23**(R1), pp. R40-R46. doi: 10.1093/hmg/ddu125.