Genetic tools for insect control

Thesis submitted for PhD

By

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Acknowledgements

It was during my undergraduate research project at Nottingham that Professor Fred Sablitzky suggested I should do a PhD. Before Fred planted that seed, the thought had never entered my head … thanks Fred ! I decided that I wanted to do applied research with potential impact and found a position studying the genetic control of pest insects. The advertised PhD project would give me the opportunity to study some of my key biological interests with scope for practical application of the research. So, I applied and to my genuine surprise was offered the position. Then a few months later I set off for UEA with the simple goal of eradicating malaria in four years.

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Abstract

Pest insects can impose strains on public health and food security and these are predicted to intensify as a consequence of population growth and climate change. In the 20th century, organosynthetic insecticides were considered the panacea for insect control. However, insecticides have become less effective, as pest species have evolved mechanisms of behavioural and genetic resistance. It imperative therefore, that novel modes of control are developed to eradicate or suppress wild, pest insect populations. In this thesis I tested various novel genetic tools and approaches for control of the Mediterranean Fruit Fly (*Ceratitis capitata;* medfly) and evaluated the Fall Armyworm (*Spodoptera frugiperda;* FAW) as a candidate species for Oxitec's established "self-limiting" control. I investigated environmental RNA interference (eRNAi) as a mode of control for the medfly and showed that neither medfly adults or eggs can easily be induced to become eRNAi competent via feeding or by exposure to electric current, respectively. I then sequenced and analysed the genome of a bacterium, aiming to modify it, for control of the medfly through paratransgenesis. The *Klebsiella* bacterium identified did not exhibit obvious signatures of transition to an obligate relationship with its medfly host. However, it was capable of degrading pectate and may provide essential nutrients to medfly larvae, which consume a large amount of fruit. Next, I manipulated medfly eggs to test whether vertical transmission of *Klebsiella* bacteria could occur by maternal 'egg smearing'. There was no clear evidence for this, though *Klebsiella* bacteria hold some potential for control of the medfly through paratransgenesis. Finally, I studied the mating and sperm precedence behaviours of the FAW and showed that this species has potential utility as an agent of self-limiting control. Overall, the thesis research gives novel insights in the context of genetic insect control and tactical suggestions for future investigations.

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Chapter 1 – General introduction

1.1) The requirement for insect control

Insects are the most abundant and species rich group of animals on Earth (Chapman, 2009; Mora et al., 2011). They are essential members of food webs (Boonpha et al., 2019; Damien et al., 2019; Menon et al., 2019) and provide critical ecosystem services, such as pollination (Eeraerts et al., 2019; Powney et al., 2019), decomposition of plant and animal matter (Beleza et al., 2019; Castro et al., 2019) and regulation of pest species (Ali et al., 2019; Zhang, Zhang, Liu, et al., 2019). However, some insect species are detrimental to the health of humans, animals and ecosystems, and are considered pests. Such pest insects vector pathogens to humans (Berté et al., 2019; Marklewitz et al., 2019; Matetovici et al., 2019; Mori et al., 2019; Mulatier et al., 2019) livestock (Kasozi et al., 2019; Muhanguzi et al., 2017; Rodríguez-Hidalgo et al., 2019) and crops (Hoh et al., 2010; Martinière et al., 2013; Willsey et al., 2019), while others decimate crops (Costa et al., 2019; D'Ambrosio et al., 2019) and forestry (Aukema et al., 2010) through herbivory.

The need for insect control is perhaps felt most keenly if one considers the cost to human health. The mosquitoes represent some of the world's most pernicious pest insects. Each year, *Anopheles* mosquitos infect ≈200 million people with malaria, which results in the death of one child every two minutes (WHO, 2018) and *Aedes* mosquitos vector dengue fever to ≈390 people resulting in ≈96 million clinical illnesses (Bhatt et al., 2013). The need for insect control can also be expressed in economic terms. For example, Bradshaw et al. (2016) estimate the global cost of invasive insects to be at least $77 billion a year, while Oliveira et al. (2014) estimate that in Brazil alone, the cost of pest insects is $17.7 billion per annum and Zalucki et al. (2012) estimate annual international losses of $4-5 billion due to damage caused by the diamondback moth (*Plutella xylostella*).

1.2) 20th Century developments in insect control

In the early 20th century, pest insects were controlled almost exclusively with broad spectrum organosynthetic insecticides (organochlorines, organophosphates, carbamates and pyrethroids) (Garcia et al., 2012; Horowitz et al., 2004). However, the use of such

organosynthetic insecticides is now becoming restricted for both practical and ethical reasons (Commission, 2019; NPIC, 2019).

In some cases, overuse of insecticides has selected for resistance in pest insects and this has diminished the effectiveness of those chemicals in the field (Aïzoun et al., 2013; Amelia-Yap et al., 2018; Busvine et al., 1969; Oduola et al., 2012; Osta et al., 2012; Reyes et al., 2015; Smith et al., 2016). Common mechanisms of insecticide resistance are: 1) Upregulation of enzymes which metabolise xenobiotic/insecticidal compounds, such as glutathione S-transferases (GST) (Chen et al., 2019; Safi et al., 2019; Zhang, Zhang, Shao, et al., 2019) and cytochrome P-450s (Uchibori-Asano et al., 2019; Wang et al., 2019; Zou et al., 2019), 2) Mutation of proteins that are targeted by insecticides (Fournier-Level et al., 2019; Grant et al., 2019; Ureña et al., 2019), 3) Behavioural modification of insects to avoid exposure (He et al., 2019; Kamau et al., 2018; Nansen et al., 2016). Some insects have been so overexposed to insecticides that they have evolved mechanisms of both behavioural and genetic resistance. For example, Nansen et al. (2016) found that a strain of the diamondback moth had evolved resistance to gamma-cyhalothrin and spinetoram insecticides, via two independent genetic processes. Furthermore, resistant DBM individuals had modified their egg laying behaviour and laid significantly fewer eggs on leaves that had been treated with insecticides than on untreated controls (Nansen et al., 2016).

Broad spectrum insecticides also inflict toxic, off-target effects on animal, bird and marine life. Off-target effects occur when organisms that are not targeted for control suffer the harmful effect of insecticides through direct contact (Baier et al., 2016; Carson, 1962; Řezáč et al., 2019; Sánchez-Bayo et al., 2019; Sarkar et al., 2017), or through secondary contact with insecticides that have persisted in the environment/food chain (Carson, 1962; Craddock et al., 2019; Furihata et al., 2019). For example, in the 1950s and 1960s the Global Malaria Eradication Programme (GMEP) exclusively controlled *Anopheles* mosquitos with dichloro-diphenyl-trichloroethane (DDT), which drastically reduced instances of malaria in certain territories (Cohn, 1973; Gray, 1974). However, DDT was linked with several off-target effects on other species (Carson, 1962), e.g. it can be detected in human breast milk (Hernández et al., 1993) and diminishes the quality of

peregrine falcon eggs (Ratcliffe, 1970). Hence, DDT has been globally banned for agricultural use since 2001 (POP, 2019).

In the 1960s, seeking to limit the environmental damage caused by insect control, Californian entomologists developed a novel system termed Integrated Pest Management (IPM) (Stern et al., 1959). IPM promotes the combined use of biological insect controls with organosynthetic insecticides. Biological controls include pheromone bait traps (Fan et al., 2019; Manoukis, 2016; Meagher et al., 2013; Park et al., 2019; Viklund et al., 2019) natural enemies (Ali et al., 2019; Zhang, Zhang, Liu, et al., 2019), habitat management (Ennis et al., 2019; Hassanali et al., 2008; Li et al., 2019) and the Sterile Insect Technique (SIT) (Knipling, 1955).

1.3) The Sterile Insect Technique

The Sterile Insect Technique (SIT) was conceived by E.F Knipling of the United States Department of Agriculture, while working with the New World Screwworm (*Cochliomyia hominivorax*; NWS) (Klassen et al., 2005). Knipling had observed that: 1) NWS matings are almost always initiated by males, 2) female NWS are monogamous. These observations led Knipling to form the fundamental hypothesis which underpins SIT; that mass release of sterile male insects into areas that contain wild-type females will cause the wild-type population to crash – provided the sterile males mate with wild-type females and those matings do not produce viable offspring (Knipling, 1955). Initially however, Knipling lacked the means to sterilise insects and could not test this hypothesis.

Subsequently, having read how sterility could be induced in *Drosophila* with irradiation (Muller, 1950), Knipling was inspired to investigate irradiation induced sterility in NWS (Klassen et al., 2005). Shortly after, R. C Bushland and D. E Hopkins found that 6 day old NWS pupae that had been treated with irradiation, gave rise to superficially normal, sexually competitive males (Bushland et al., 1953). Most importantly however, Bushland et al. (1953) were able to demonstrate that irradiated NWS males were sterile and did not produce viable offspring when mated with unirradiated females. These experiments provided the first empirical evidence in support of Knipling's SIT hypothesis.

The first successful SIT field trials were conducted in Curaçao, where the wild NWS population was eradicated in just 14 weeks with irradiated, sterile males (Baumhover et al., 1955). The Curaçao field trials led to field releases of irradiated NWS in the USA in May 1958 (Baumhover et al., 1959) and eventually the NWS became the first major success story for SIT control. Through international cooperation and the establishment of large mass-rearing facility in Mexico, the NWS was eradicated from the USA in 1982, then progressively south to Panama by 2001 (Klassen et al., 2005).

1.4) Shortcomings of the conventional Sterile Insect Technique

Irradiation-induced sterility has now been documented in a variety of insect species (Bond et al., 2019; Bushland et al., 1953; Lanouette et al., 2017; Limohpasmanee et al., 2017; Schwarz et al., 1985; Toledo et al., 2004). However, irradiation impairs locomotion, mate location and courtship behaviours and thus imposes significant performance costs on insects that can diminish the effectiveness of SIT control (Guerfali et al., 2011; Toledo et al., 2004). Irradiation dose and insect quality are inversely proportional but the relationship between irradiation dose and sterility is linear (Guerfali et al., 2011; Toledo et al., 2004). This paradox forces researchers to find an optimum dose of radiation that sterilises male insects but maintains their ability to locate, court and mate with females in the field (Bond et al., 2019; Guerfali et al., 2011; Lanouette et al., 2017; Limohpasmanee et al., 2017; Toledo et al., 2004).

In addition to the fitness costs that are associated with irradiation, conventional SIT can be hindered by any assortative mating which occurs when irradiated female insects mate with irradiated males in the field. Such assortative mating prevents irradiated males from mating with, and thus sterilising, wild, unirradiated females (Marec et al., 2005; Rendon et al., 2004). Assortative mating can however be mitigated if female insects are removed from SIT cohorts. Rendon et al. (2004) report that removal of irradiated females from a cohort of Mediterranean fruit flies (*Ceratitis capitata;* medfly) enhanced suppression of the wild population by nearly 600%. Furthermore, if females are removed in early development, then rearing costs are significantly reduced (Lutrat et al., 2019). However, sex-sorting of irradiated insects can be time consuming and expensive (Lutrat et al., 2019).

1.5) Genetic Sexing Strain (GSS) technology and The Moscamed Programme

In 1975 the USA, Mexico and Guatemala collaborated to establish the Moscamed programme, for control of the medfly (Enkerlin et al., 2017). The Moscamed programme applies IPM, relying heavily on the SIT (Knipling, 1955) but also employs surveillance (IAEA, 2003b) and chemical insecticides (Piñero et al., 2014). The Moscamed programme utilises Genetic Sexing Strain (GSS) Vienna 7 and Vienna 8 medflies (Caceres et al., 2004; Robinson, 2002), that are produced in a mass-rearing facility in Mexico (Schwarz et al., 1985).

Female Vienna7/8 medflies carry two closely linked mutations which facilitate their removal from SIT cohorts. During production, nearly 100% of female Vienna 7/8 eggs are killed with a 35^{o}C heat shock, due to a *temperature sex lethal* (*tsl*) mutation (Augustinos et al., 2017). The small number of females that survive the heat shock can be distinguished at the pupal stage, as a *wp* mutation confers them with characteristic white pupae (Augustinos et al., 2017). Remaining brown, male pupae are then sterilised with irradiation prior to release (Caceres et al., 2004).

The GSS (*tsl* and *wp)* loci occasionally recombine, which can hinder the removal of Vienna7/8 females from SIT cohorts. The Moscamed programme mitigates the recombination of *tsl/wp* with systematic colony filtration (Caceres, 2002). Moscamed Vienna7/8 SIT cohorts are generated from a small progenitor colony through two rounds of amplification (Caceres, 2002). During round one of amplification, heterozygous, recombinant females that survive heat shock and emerge from brown pupae are filtered from the population (Caceres, 2002). Homozygous recombinants are identified by a prolonged developmental period (Fisher et al., 2000) and are filtered from the population during round two (Caceres, 2002).

Moscamed GSS control is therefore impeded both by the negative effects of irradiation discussed above (Guerfali et al., 2011; Toledo et al., 2004) and the input of resources required for colony filtration (Caceres, 2002). However, the limitations of GSS technology can be circumvented to some extent by modern systems of genetic insect control.

1.6) Oxitec's self-limiting technology

Oxitec's "self-limiting" technology (previously known as RIDL) is a transgenic mode of insect control, that circumvents the fitness costs of irradiation and the financial costs of colony filtration that are associated with GSS. The self-limiting system induces female-specific mortality, by utilising tetracycline-repressible, female-specific genetic elements, to drive the expression of insecticidal proteins (Thomas et al., 2000). Self-limiting females die during early development if not provided with dietary tetracycline (Thomas et al., 2000). Hence, male-only cohorts of self-limiting insects are produced by subjecting larvae to a tetracycline deficient feeding regime (Thomas et al., 2000). However, the self-limiting model of control differs from conventional SIT, as self-limiting male/wild-type female matings produce fertile, male offspring.

In the absence of tetracycline, the dominant lethal effect of self-limiting technology kills female but not male heterozygotes. Hence, heterozygous male offspring that develop from self-limiting male/wild-type female matings in the field, survive in the absence of tetracycline and remain fertile, while heterozygous female offspring die if not supplied with dietary tetracycline (Thomas et al., 2000). However, the survival of male heterozygotes does not hinder control of pest populations, as self-limiting technology provides control at a rate equivalent to, or superior than, conventional SIT and GSS (Asadi et al., 2019; Gorman et al., 2016; Harvey-Samuel et al., 2015).

The self-limiting system was first tested in *Drosophila,* when Thomas et al. (2000) fused a tetracycline-repressible, toxic Ras-64B protein to the enhancer of the female-specific *yolk protein 3*. Thomas et al. (2000) reared self-limiting *Drosophila* on a tetracycline deficient diet and found that 0 females and >5000 males progressed to adulthood under those conditions (Thomas et al., 2000). As predicted, heterozygous female progeny of self-limiting male/wild-type female matings did not survive to adulthood unless provided with dietary tetracycline (Thomas et al., 2000).

Gong et al. (2005) refined the self-limiting system, utilising tetracycline-repressible transactivator protein (tTAV) as both toxic effector and transactivator. Under the Gong et al. (2005) system, prohibition of dietary tetracycline induces expression of tTAV, which

binds the tTAV regulatory element, inducing further tTAV expression. tTAV accumulates and reaches a toxic threshold due to the "squelching" of transcriptional apparatus, the acquisition of proteins required for ubiquitin-dependent proteolysis or a combination of those mechanisms (Gong et al., 2005). The Gong et al. (2005) construct induced up to 99% mortality in the medfly but the mechanism was not sex specific.

Fu et al. (2007) developed Gong et al.'s (2005) tTAV mechanism, placing it in an intron of the female-specific *transformer (tra)* gene. Prohibition of tetracycline completely eliminated females from a population of medflies carrying the Fu et al. (2007) tTAV-tra insert. Fu et al.'s (2007) self-limiting system has now been implemented in several insects and induces close to 100% female mortality for *Aedes aegypti* (Phuc et al., 2007), the olive fruit fly (Ant et al., 2012) and the diamondback moth (Harvey-Samuel et al., 2015).

Like conventional SIT, self-limiting control hinges on the ability of transgenic males to locate wild-type females and mate with them in the field. However, if self-limiting constructs insert at an unfavourable locus, this can negatively impact male fitness (Haghighat-Khah et al., 2015; Meza et al., 2014). To evaluate the costs of such "insertion effects", a Relative Sterility Index (RSI) (IAEA, 2003a) is calculated by forcing self-limiting males to compete with wild-type males for mating opportunities. An RSI is calculated based on the proportion of matings that occur between self-limiting males and wild-type females, in comparison to matings between wild-type males and wild-type females (IAEA, 2003a). An RSI score of 0.5 indicates equivalent mating competitivity between strains but SIT control can still be effectively applied when insects have RSI scores as low as 0.2 (IAEA, 2003a). All of Oxitec's self-limiting products score well above IAEA's (2003a) suggested RSI threshold of 0.2. For example, RSIs of 0.44, 0.47 and 0.42 have been recorded for self-limiting olive fruit fly, *Aedes aegypti* and medfly respectively (Ant et al., 2012; Asadi et al., 2019; Patil et al., 2015).

Field trials of self-limiting technology in Panama (Gorman et al., 2016) and Brazil (Oxitec, 2019) have documented efficient suppression of wild, *Aedes aegypti* populations. In addition, field-cage studies report efficient self-limiting control of the diamondback moth (Harvey-Samuel et al., 2015), medfly (Asadi et al., 2019; Leftwich et al., 2014) and olive fly (Ant et al., 2012). Unsurprisingly, several novel self-limiting strains such as the Fall

Armyworm (*Spodoptera frugiperda;* FAW), Spotted Wing *Drosophila* (*Drosophila suzukii*) and *Anopheles albimanus*/*Anopheles stephensi* mosquitos are currently under development (Oxitec, 2019). However, as self-limiting technology is classified as Genetic Modification (GM), its application is precluded in certain regions and countries.

1.7) RNA interference as a mode of insect control

RNA interference (RNAi) (Fire et al., 1998) is a gene silencing technique, that has the potential to be applied for insect control (Reviewed in:(Baum et al., 2014; Darrington et al., 2017; Huvenne et al., 2010). The chief interest in RNAi in the context of insect control stems from its technical classification, in that it is not currently classified as a GM technology and can therefore be applied in regions where GM is restricted.

Transmission of double-stranded RNA (dsRNA) into a eukaryotic cell simulates viral infection and initiates RNAi (Ding, 2010). Dicer RNAse III enzymes (Bernstein et al., 2001) bind and cleave cytoplasmic dsRNA, producing 21bp small interfering RNA (siRNA) duplexes (Elbashir, Harborth, et al., 2001). siRNAs are then captured by a RISC-loading complex (RLC) (Liang et al., 2015) and presented to an Argonaut protein (Ago) (Carmell et al., 2002). The Ago degrades a single "passenger" strand of the siRNA but retains the complementary 21bp "guide" strand (Matranga et al., 2005). The Ago and guide strand then coalesce, forming the functional component of RNAi, an RNA-Induced Silencing Complex (RISC) (Filipowicz, 2005). mRNAs that share homologous nucleotide sequences with the internalised guide strand are degraded by the RISC (Elbashir, Martinez, et al., 2001). Endogenously expressed genes can therefore be silenced with dsRNAs, if those dsRNAs exhibit cognate nucleotide sequence with target genes and can be efficiently transmitted into host cells.

dsRNAs have been demonstrated to induce mortality in pest insects (Shen et al., 2019; Tariq et al., 2019; Xu et al., 2019) and phenotypes beneficial to control, such as elevated sensitivity to insecticides (Liao et al., 2019; Ma et al., 2019) and environmental stressors (Gu et al., 2019). In addition, RNAi can induce sex-reversal (Liu et al., 2015; Pane et al., 2002; Peng et al., 2015; Salvemini et al., 2009; Shukla et al., 2012) making pest insects amenable to the SIT (Darrington et al., 2017). However, as control programs typically

require the simultaneous treatment of huge insect populations (Alphey et al., 2010) an RNAi based system of insect control would require a high-throughput, reliable method for distributing dsRNA into insect cells.

Exposure to environmental dsRNA is sufficient to induce RNAi (eRNAi) in some insects (Reviewed in: (Baum et al., 2014; Darrington et al., 2017)) and offers a simple and scalable method for delivery of effector molecules. Several insect species have been reported to express eRNAi when dsRNAs have been administered topically (Killiny et al., 2017; Niu et al., 2019; Whyard et al., 2015) or in food (Shen et al., 2019; Tariq et al., 2019; Xu et al., 2019). However, some insects are recalcitrant eRNAi, meaning that not all pest species can be controlled with this technique.

The genetic basis of eRNAi has been elucidated in the *Caenorhabditis elegans* nematode model. In *C. elegans,* eRNAi is facilitated by five *Systemic Interference Defective* (SID) genes (Jose et al., 2007), but is primarily regulated by SID2 (McEwan et al., 2012; Winston et al., 2007). McEwan et al. (2012) propose that SID2 codes for an intestinal transmembrane protein that endocytoses ingested dsRNA from the gut lumen. In support of the McEwan et al. (2012) hypothesis, Winston et al. (2007) demonstrated that SID2 deficient *C. elegans* mutants were recalcitrant to eRNAi of a GFP reporter. The presence or absence of SIDs has been used by some researchers to predict the capacity of different insect species to effect gene silencing via eRNAi (Tian et al., 2009; Tomoyasu et al., 2008). However, the presence of SIDs is not fully predictive of eRNAi potential – several studies have demonstrated that dipterans, which lack SIDs (Huvenne et al., 2010) are eRNAi competent (Gu et al., 2019; Whyard et al., 2015). In contrast, *Bombyx mori* possesses three SID orthologues, yet is not competent for eRNAi (Li, Zeng, et al., 2015).

In addition to the SID genes, clathrin mediated endocytosis appears to influence the ability of some insects to express eRNAi (Cappelle et al., 2016; Li, Dong, et al., 2015; Pinheiro et al., 2018; Xiao et al., 2015). Cappelle et al. (2016) report that inhibition of clathrin-mediated endocytosis with chlorpromazine hydrochloride severely diminished dsRNA uptake by the Colorado Potato Beetle (CPB). However, Cappelle et al. (2016) also report that eRNAi was mitigated in the CPB when SID genes were targeted for knockdown

with dsRNA. It therefore seems likely that eRNAi is regulated in the CPB by a suite of SID/endocytic genes. This is consistent with a study by Pinheiro et al. (2018) which suggests that both SIDs and endocytic genes mediate eRNAi in the Western Corn Rootworm.

Based on available data, eRNAi appears to be a viable mode of control for some pest insects. Several researchers have demonstrated that "naked" environmental molecules (which are unmodified when fed to the target insect) are sufficient to elicit eRNAi (Liao et al., 2019; Ma et al., 2019; Shen et al., 2019; Tariq et al., 2019; Xu et al., 2019). However, others have opted to vector dsRNAs within genetically modified bacteria, to elicit RNAi from their intended target insect (Bento et al., 2019; Caccia et al., 2019; Ganbaatar et al., 2017; Whitten et al., 2015).

1.8) Implementing RNAi mediated insect control with modified bacteria

When fed to certain pest insects, transgenic HT115 *Escherichia coli* (Kamath et al., 2001) elicit robust eRNAi (Bento et al., 2019; Caccia et al., 2019; Ganbaatar et al., 2017). For example, Bento et al. (2019) report that HT115 mediated eRNAi of *juvenile hormone inducible protein, chitin synthase A, carboxylesterase* and *arginine kinase,* all significantly increased rates of larval mortality in The South American Pinworm (*Tuta absoluta).*

The HT115 genome has been modified to be RNase deficient and to contain a T7 RNA polymerase (that is both promoter-specific and highly efficient), under the control of lactose regulatory elements (Kamath et al., 2001). The nucleotide sequence of a targeted host gene flanked by T7 promoters at each side, is transformed into HT115 bacteria on an L4440 expression vector. Induced by the allolactose mimic IPTG, T7 polymerases transcribe complementary strands of the target DNA sequence encoded by the L4440 vector. The complementary RNA strands base pair and form dsRNAs within the bacteria, which are then consumed by pest insects, inducing eRNAi (Bento et al., 2019; Caccia et al., 2019; Ganbaatar et al., 2017).

Synthesis of dsRNA by HT115 bacteria (Bento et al., 2019; Caccia et al., 2019; Ganbaatar et al., 2017) is inexpensive when compared to methods of *in vitro* synthesis (Liao et al., 2019; Ma et al., 2019; Shen et al., 2019; Tariq et al., 2019; Xu et al., 2019). HT115 bacteria

may therefore provide a cost-effective platform for control of large insect populations. However, the gene silencing effect of HT115 bacteria is likely to be transient as these bacteria are expected to fail to colonize the insect gut. The potential for engineering resident members of the gut microbiomes for dsRNA delivery (i.e. paratransgenesis) is also a potentially useful option.

Whitten et al. (2015) utilised modified, symbiotic *Rhodococcus rhodnii* bacteria to elicit eRNAi of *vitellogenin* in *Rhodnius prolixus*. *Rh. rhodnii* were reprogrammed to have similar properties to HT115 bacteria (in that they were RNAse-deficient), but RNA synthesis was constitutively active rather than being inducible. The fecundity of fifth instar nymphs that had been exposed to transgenic *Rh. rhodnii* was reduced 73% over a period of 200 days (Whitten et al., 2015). These results suggest that modification of symbiotic bacteria offers great potential for persistent control of pest species. However, it is important to note that modified bacteria, symbiotic or otherwise, will be subject to the same GM regulations as self-limiting technology.

1.9) Thesis overview

Throughout this thesis I utilise the medfly model system to investigate applied control techniques. I then interrogate the genome of a medfly associated microbe to discover if it has the potential to be modified for control purposes. Finally, I study the behaviours of the Fall Armyworm (*Spodoptera frugiperda;* FAW) and appraise its compatibility with self-limiting control. Although my studies focused on the medfly and FAW, it was my aim to develop technologies that could be applied for the control of all insects.

In chapter 2, I evaluated eRNAi as a means of control for the medfly. Self-limiting OX3864A strain medflies express a fluorescent dsRED2 marker gene (Asadi et al., 2019; Gong et al., 2005; Leftwich et al., 2014) which I targeted for knockdown with dsRNA. OX3864A adults and larvae were fed with HT115 bacteria (Kamath et al., 2001), naked (unmodified) dsRED2 dsRNA and dsRED2 dsRNA vectored in liposomes. I measured the effect of eRNAi both with biochemically with RT-qPCR and phenotypically, by quantifying the fluorescence of OX3864A flies that had been exposed to dsRNA.

In chapter 3, I investigated whether medfly eggs can be induced to become eRNAi competent by electroporation. As for chapter 2, I targeted the dsRED2 gene expressed by OX3864A medflies for knockdown and measured the effect of eRNAi with RT-qPCR. In a related set of experiments, I electroporated eggs with dsRNA targeting the *transformer 2* (Cctra2) gene, to induce the "pseudomale" phenotype from genetically female flies (Salvemini et al., 2009). eRNAi of Cctra2 was measured via the sex-ratio of fly populations that developed from eggs that had been treated with electric current. To determine the presence of cryptic pseudomales, phenotypically male flies were subjected to PCR targeting a male-specific gene.

In chapter 4 I assessed whether a *Klebsiella* bacterium resident in the medfly gut microbiome (hereafter "Medkleb") could be genetically modified for control of the medfly. Bacteria that have evolved physiological interdependence with pest insects can be manipulated to provide a stable platform for control (Whitten et al., 2015). I sequenced and annotated the Medkleb genome, then performed comparative bioinformatics and phenotypic assays to identify whether it exhibited characteristics that are frequently associated with bacterial symbionts of insects. Based on those data I evaluated whether Medkleb has evolved a co-dependent life history with the medfly that could be exploited for control purposes.

In Chapter 5, I conducted experiments to evaluate if *K. oxytoca* bacteria are maternally transmitted to medfly larvae via the egg chorion. I hypothesised that *K. oxytoca* are vertically transmitted by the medfly and that they offer a potential platform for control through paratransgenesis. I dechorionated medfly eggs and reared them in a sterile environment before analysing the microbiome of adult flies qualitatively with 16S metagenomics and quantitatively with qPCR.

In chapter 6, I evaluated the FAW as a candidate species for self-limiting control. I investigated traits that are predicted to affect the rate at which self-limiting FAW males will fertilise/sterilise the eggs of wild-type females in the field. First, I developed a system of spermatophore marking which enabled me to elucidate whether female FAW practice polyandry. Next, I employed a fluorescent marker gene and a system of sequential mating to clarify the sperm precedence phenotype in this species.

1.10) Attribution statement

Sections 1.8 and 1.9 of the general introduction and the introduction of Chapter 2 contain material published as Darrington et al. (2017) (Appendix). Darrington et al. (2017) is a review paper that I researched and wrote with three co-authors.

The fluorescence quantification experiment in Chapter 2 (protocol 2.10) was carried out using an ImageJ macro written by Dr. Paul Thomas (https://people.uea.ac.uk/p_thomas).

In Chapter 4, I analyse the DNA of a *Klebsiella* bacterium. This bacterium was isolated from a medfly gut and its DNA was extracted (protocol 4.1) by Dr. Philip Leftwich (https://people.uea.ac.uk/p_leftwich). The DNA was sequenced by the Earlham Institute (Norwich Research Park, UK) (protocol 4.2) and I received the data in fasta format.

The Morocco flies used for microbiome analyses in Chapter 5 were captured from Argan fruit, by Elani Rachid on behalf of Oxitec Ltd in March 2014.

The experiment described in section 6.8.2 was carried out by Dr. Rodrigo Soares Ramos and myself. I wrote the experimental protocol with Dr. Neil Morrison and Dr. Catherine Reavey of Oxitec Ltd. Rodrigo and I carried out the assay and did the analysis together. Rodrigo had generated moths for the experiment prior to my arrival in Brazil.

1.11) References

Aïzoun, N., Aïkpon, R., Gnanguenon, V., Oussou, O., Agossa, F., Padonou, G., & Akogbéto, M. (2013). Status of organophosphate and carbamate resistance in *Anopheles gambiae* sensu lato from the south and north benin, West Africa. *Parasites & vectors, 6*, 274-274. doi:10.1186/1756-3305-6-274

Ali, M. P., Bari, M. N., Haque, S. S., Kabir, M. M. M., Afrin, S., Nowrin, F., . . . Landis, D. A. (2019). Establishing next-generation pest control services in rice fields: Eco-agriculture. *Scientific Reports, 9*(1). doi:10.1038/s41598-019-46688-6

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

Amelia-Yap, Z. H., Chen, C. D., Sofian-Azirun, M., & Low, V. L. (2018). Pyrethroid resistance in the dengue vector *Aedes aegypti* in Southeast Asia: Present situation and prospects for management. *Parasites & vectors, 11*(1), 332. doi:10.1186/s13071-018-2899-0

Ant, T., Koukidou, M., Rempoulakis, P., Gong, H.-F., Economopoulos, A., Vontas, J., & Alphey, L. (2012). Control of the olive fruit fly using genetics-enhanced sterile insect technique. *Bmc Biology, 10*(1), 51. doi:10.1186/1741-7007-10-51

Asadi, R., Elaini, R., Lacroix, R., Ant, T., Collado, A., Finnegan, L., . . . Koukidou, M. (2019). Preventative releases of self-limiting *Ceratitis capitata* provide pest suppression and protect fruit quality in outdoor netted cages. *International Journal of Pest Management*, 1-12. doi:10.1080/09670874.2019.1601293

Augustinos, A. A., Targovska, A., Cancio-Martinez, E., Schorn, E., Franz, G., Cáceres, C., . . . Bourtzis, K. (2017). *Ceratitis capitata* genetic sexing strains: Laboratory evaluation of strains from mass-rearing facilities worldwide. *Entomologia experimentalis et applicata, 164*(3), 305-317. doi:10.1111/eea.12612

Aukema, J. E., McCullough, D. G., Von Holle, B., Liebhold, A. M., Britton, K., & Frankel, S. J. (2010). Historical accumulation of nonindigenous forest pests in the continental United States. *BioScience, 60*(11), 886-897. doi:10.1525/bio.2010.60.11.5

Baier, F., Jedinger, M., Gruber, E., & Zaller, J. G. (2016). Temperature-dependence of glyphosate-based herbicide's effects on egg and tadpole growth of common toads. *Frontiers in Environmental Science, 4*(51). doi:10.3389/fenvs.2016.00051

Baum, J. A., & Roberts, J. K. (2014). Chapter five - progress towards RNAi-mediated insect pest management. In S. D. Tarlochan & S. G. Sarjeet (Eds.), *Advances in insect physiology* (Vol.47, pp. 249-295): Academic Press.

Baumhover, A. H., Graham, A. J., Bitter, B. A., Hopkins, D. E., New, W. D., Dudley, F. H., & Bushland, R. C. (1955). Screw-worm control through release of sterilized flies. *Journal of Economic Entomology, 48*(4), 462-466. doi:10.1093/jee/48.4.462

Baumhover, A. H., Husman, C. N., Skipper, C. C., & New, W. D. (1959). Field observations on the effects of releasing sterile screw-worms in Florida. *Journal of Economic Entomology, 52*(6), 1202-1206. doi:10.1093/jee/52.6.1202

Beleza, S., Campos, D., Nunes, B., & Pestana, J. L. T. (2019). Ecotoxicological responses of *Chironomus riparius* to eucalyptus and *alnus* leaf leachates: From biochemical to life-history effects. *Ecological Indicators, 106*. doi:10.1016/j.ecolind.2019.105473

Bento, F. M. M., Marques, R. N., Campana, F. B., Demétrio, C. G. B., Leandro, R. A., Parra, J. R. P., & Figueira, A. (2019). Gene silencing by RNAi via oral delivery of dsRNA by bacteria in the South American tomato pinworm, *Tuta absoluta*. *Pest Management Science*. doi:10.1002/ps.5513

Bernstein, E., Caudy, A. A., Hammond, S. M., & Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature, 409*(6818), 363-366. doi:10.1038/35053110

Berté, D., De Meeûs, T., Kaba, D., Séré, M., Djohan, V., Courtin, F., . . . Ravel, S. (2019). Population genetics of *Glossina palpalis palpalis* in sleeping sickness foci of Côte d'Ivoire before and after vector control. *Infection, Genetics and Evolution, 75*. doi:10.1016/j.meegid.2019.103963

Bhatt, S., Gething, P. W., Brady, O. J., Messina, J. P., Farlow, A. W., Moyes, C. L., . . . Hay, S. I. (2013). The global distribution and burden of dengue. *Nature, 496*(7446), 504-507. doi:10.1038/nature12060

Bond, J. G., Osorio, A. R., Avila, N., Gómez-Simuta, Y., Marina, C. F., Fernández-Salas, I., . . . Williams, T. (2019). Optimization of irradiation dose to *Aedes aegypti* and *Ae. albopictus* in a sterile insect technique program. *Plos One, 14*(2), e0212520. doi:10.1371/journal.pone.0212520

Boonpha, N., Duengkae, P., Wacharapluesadee, S., & Kaewket, C. (2019). Seasonal variation in sexual size dimorphism in the wrinkle-lipped free-tailed bat (*Chaerephon plicatus* buchannan, 1800) population in the Khao Chong Phran non-hunting area, Thailand. *Environment and Natural Resources Journal, 17*(3), 50-57. doi:10.32526/ennrj.17.3.2019.22

Bradshaw, C. J. A., Leroy, B., Bellard, C., Roiz, D., Albert, C., Fournier, A., . . . Courchamp, F. (2016). Massive yet grossly underestimated global costs of invasive insects. *Nature Communications, 7*, 12986. doi:10.1038/ncomms12986

Bushland, R. C., & Hopkins, D. E. (1953). Sterilization of screw-worm flies with x-rays and gamma-rays. *Journal of Economic Entomology, 46*(4), 648-656. doi:10.1093/jee/46.4.648

Busvine, J. R., & Pal, R. (1969). The impact of insecticide-resistance on control of vectors and vector-borne diseases. *Bull World Health Organ, 40*(5), 731-744.

Caccia, S., Astarita, F., Barra, E., Di Lelio, I., Varricchio, P., & Pennacchio, F. (2019). Enhancement of *Bacillus thuringiensis* toxicity by feeding *Spodoptera littoralis* larvae with bacteria expressing immune suppressive dsRNA. *Journal of Pest Science*. doi:10.1007/s10340-019-01140-6

Caceres, C. (2002). Mass rearing of temperature sensitive genetic sexing strains in the Mediterranean fruit fly (*Ceratitis capitata*). *Genetica, 116*(1), 107-116. doi:10.1023/A:1020967810703

Caceres, C., J, Cayol, J., P, Enkerlin, W., Franz, G., Hendrichs, J., & Robinson, A. S. (2004). Comparison of Mediterranean fruit fly *(Ceratitis capitata: Tephritidae)* bisexual and genetic sexing strains: Development, evaluation and economics. *Proceedings, of the 6th International Symposium on Fruit Flies of Economic Importance*, 367-381.

Cappelle, K., de Oliveira, C. F., Van Eynde, B., Christiaens, O., & Smagghe, G. (2016). The involvement of clathrin-mediated endocytosis and two sid-1-like transmembrane proteins in double-stranded RNA uptake in the Colorado potato beetle midgut. *Insect Mol Biol, 25*(3), 315-323. doi:10.1111/imb.12222

Carmell, M. A., Xuan, Z., Zhang, M. Q., & Hannon, G. J. (2002). The argonaute family: Tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. *Genes and Development, 16*(21), 2733-2742. doi:10.1101/gad.1026102

Carson, R. (1962). *Silent spring*: 40th anniversary edition. Boston : Houghton Mifflin, 2002. ©1962.

Castro, M., Centeno, N., & González-Vainer, P. (2019). An initial study of insect succession on pig carcasses in open pastures in the northwest of Uruguay. *Forensic Science International, 302*. doi:10.1016/j.forsciint.2019.05.053

Chapman, A., D. (2009). *Numbers of living species in Australia and the world* (2nd ed.): Australian Biodiversity Information Services.

Chen, X. D., Gill, T. A., Nguyen, C. D., Killiny, N., Pelz-Stelinski, K. S., & Stelinski, L. L. (2019). Insecticide toxicity associated with detoxification enzymes and genes related to transcription of cuticular melanization among color morphs of Asian citrus psyllid. *Insect Science, 26*(5), 843-852. doi:10.1111/1744-7917.12582

Cohn, E. J. (1973). Assessing the costs and benefits of anti-malaria programs: The Indian experience. *American journal of public health, 63*(12), 1086-1096. doi:10.2105/ajph.63.12.1086

Commission, E. (2019). Eu -pesticides database. Retrieved from https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public

Costa, T. L., Sarmento, R. A., Araújo, T. A. D., Pereira, P. S., Silva, R. S., Lopes, M. C., & Picanço, M. C. (2019). Economic injury levels and sequential sampling plans for *Bemisia tabaci (Hemiptera: Aleyrodidae)* biotype b on open-field melon crops. *Crop Protection, 125*. doi:10.1016/j.cropro.2019.104887

Craddock, H. A., Huang, D., Turner, P. C., Quirós-Alcalá, L., & Payne-Sturges, D. C. (2019). Trends in neonicotinoid pesticide residues in food and water in the United States, 1999-2015. *Environmental Health: A Global Access Science Source, 18*(1). doi:10.1186/s12940-018-0441-7

D'Ambrosio, D. A., Peele, W., Hubers, A., & Huseth, A. S. (2019). Seasonal dispersal of *Lygus lineolaris (Hemiptera: Miridae)* from weedy hosts into differently fragmented cotton landscapes in North Carolina. *Crop Protection, 125*. doi:10.1016/j.cropro.2019.104898

Damien, M., & Tougeron, K. (2019). Prey–predator phenological mismatch under climate change. *Current Opinion in Insect Science, 35*, 60-68. doi:10.1016/j.cois.2019.07.002

Darrington, M., Dalmay, T., Morrison, N. I., & Chapman, T. (2017). Implementing the sterile insect technique with RNA interference – a review. *Entomologia experimentalis et applicata, 164*(3), 155-175. doi:10.1111/eea.12575

Ding, S.-W. (2010). RNA-based antiviral immunity. *Nature Reviews Immunology, 10*(9), 632-644. doi:10.1038/nri2824

Eeraerts, M., Smagghe, G., & Meeus, I. (2019). Pollinator diversity, floral resources and semi-natural habitat, instead of honey bees and intensive agriculture, enhance pollination service to sweet cherry. *Agriculture, Ecosystems and Environment, 284*. doi:10.1016/j.agee.2019.106586

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., & Tuschl, T. (2001). Duplexes of 21-nucleotide rnas mediate RNA interference in cultured mammalian cells. *Nature, 411*(6836), 494-498. doi:10.1038/35078107

Elbashir, S. M., Martinez, J., Patkaniowska, A., Lendeckel, W., & Tuschl, T. (2001). Functional anatomy of sirnas for mediating efficient rnai in *Drosophila melanogaster* embryo lysate. *Embo Journal, 20*(23), 6877-6888. doi:10.1093/emboj/20.23.6877

Enkerlin, W. R., Gutiérrez Ruelas, J. M., Pantaleon, R., Soto Litera, C., Villaseñor Cortés, A., Zavala López, J. L., . . . Hendrichs, J. (2017). The Moscamed regional programme: Review of a success story of area-wide sterile insect technique application. *Entomologia experimentalis et applicata, 164*(3), 188-203. doi:10.1111/eea.12611

Ennis, K. K., & Philpott, S. M. (2019). Seasonal and microhabitat differences alter ant predation of a globally disruptive coffee pest. *Agriculture, Ecosystems and Environment, 284*. doi:10.1016/j.agee.2019.106597

Fan, J. T., Denux, O., Courtin, C., Bernard, A., Javal, M., Millar, J. G., . . . Roques, A. (2019). Multi-component blends for trapping native and exotic longhorn beetles at potential points-of-entry and in forests. *Journal of Pest Science, 92*(1), 281-297. doi:10.1007/s10340-018-0997-6

Filipowicz, W. (2005). RNAi: The nuts and bolts of the risc machine. *Cell, 122*(1), 17-20. doi:10.1016/j.cell.2005.06.023

Fire, A., Xu, S. Q., Montgomery, M. K., Kostas, S. A., Driver, S. E., & Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature, 391*(6669), 806-811. doi:10.1038/35888

Fisher, K., & CÃ¡ceres, C. (2000). A filter rearing system for mass reared genetic sexing strains of Mediterranean fruit fly (*Diptera: Tephritidae*). *Areawide Control of Fruit Flies and Other Insect Pests*, 543-550.

Fournier-Level, A., Good, R. T., Wilcox, S. A., Rane, R. V., Schiffer, M., Chen, W., . . . Robin, C. (2019). The spread of resistance to imidacloprid is restricted by thermotolerance in natural populations of *Drosophila melanogaster*. *Nature Ecology and Evolution, 3*(4), 647-656. doi:10.1038/s41559-019-0837-y

Fu, G., Stainton, K., J Epton, M., Gong, P., Jin, L., Condon, G., . . . Alphey, L. (2007). Female-specific insect lethality engineered using alternative splicing. *25*, 353-357. doi:10.1038/nbt1283

Furihata, S., Kasai, A., Hidaka, K., Ikegami, M., Ohnishi, H., & Goka, K. (2019). Ecological risks of insecticide contamination in water and sediment around off-farm irrigated rice paddy fields. *Environmental Pollution*, 628-638. doi:10.1016/j.envpol.2019.05.009

Ganbaatar, O., Cao, B., Zhang, Y., Bao, D., Bao, W., & Wuriyanghan, H. (2017). Knockdown of *Mythimna separata* chitinase genes via bacterial expression and oral delivery of RNAi effectors. *BMC Biotechnol, 17*(1), 9. doi:10.1186/s12896-017-0328-7

Garcia, F. P., Ascencio, S. Y. C., Gaytán-Oyarzún, J., CerueloHernandez, A., & Alavarado, P. V. (2012). Pesticides: Classification, uses and toxicity. Measures of exposure and genotoxic risks. *Journal of Research in Environmental Science and Toxicology, 1*, 279-293.

Gong, P., Epton, M. J., Fu, G. L., Scaife, S., Hiscox, A., Condon, K. C., . . . Alphey, L. (2005). A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly. *Nature Biotechnology, 23*(4), 453-456. doi:10.1038/nbt1071

Gorman, K., Young, J., Pineda, L., Marquez, R., Sosa, N., Bernal, D., . . . Caceres, L. (2016). Short-term suppression of *Aedes aegypti* using genetic control does not facilitate *Aedes albopictus*. *Pest Manag Sci, 72*(3), 618-628. doi:10.1002/ps.4151

Grant, C., Jacobson, R., Ilias, A., Berger, M., Vasakis, E., Bielza, P., . . . Bass, C. (2019). The evolution of multiple-insecticide resistance in UK populations of tomato leafminer, *Tuta absoluta*. *Pest Management Science, 75*(8), 2079-2085. doi:10.1002/ps.5381

Gray, R. H. (1974). The decline of mortality in Ceylon and the demographic effects of malaria control. *Popul Stud (Camb), 28*(2), 205-229.

Gu, X., Li, Z., Su, Y., Zhao, Y., & Liu, L. (2019). Imaginal disc growth factor 4 regulates development and temperature adaptation in *Bactrocera dorsalis*. *Scientific Reports, 9*(1), 931. doi:10.1038/s41598-018-37414-9

Guerfali, M. M., Parker, A., Fadhl, S., Hemdane, H., Raies, A., & Chevrier, C. (2011). Fitness and reproductive potential of irradiated mass-reared Mediterranean fruit fly males *Ceratitis capitata* (*Diptera: Tephritidae*): Lowering radiation doses. *Florida Entomologist, 94*(4), 1042-1050.

Haghighat-Khah, R. E., Scaife, S., Martins, S., St John, O., Matzen, K. J., Morrison, N., & Alphey, L. (2015). Site-specific cassette exchange systems in the *Aedes aegypti* mosquito and the *Plutella xylostella* moth. *Plos One, 10*(4), e0121097. doi:10.1371/journal.pone.0121097

Harvey-Samuel, T., Morrison, N. I., Walker, A. S., Marubbi, T., Yao, J., Collins, H. L., . . . Alphey, L. (2015). Pest control and resistance management through release of insects carrying a male-selecting transgene. *Bmc Biology, 13*(1), 49. doi:10.1186/s12915-015-0161-1

Hassanali, A., Herren, H., Khan, Z. R., Pickett, J. A., & Woodcock, C. M. (2008). Integrated pest management: The push-pull approach for controlling insect pests and weeds of cereals, and its potential for other agricultural systems including animal husbandry. *Philos Trans R Soc Lond B Biol Sci, 363*(1491), 611-621. doi:10.1098/rstb.2007.2173

He, Z., Zhang, J., Shi, Z., Liu, J., Zhang, J., Yan, Z., & Chen, B. (2019). Modification of contact avoidance behaviour associated with pyrethroid resistance in *Anopheles sinensis (Diptera: Culicidae)*. *Malaria Journal, 18*(1). doi:10.1186/s12936-019-2765-3

Hernández, L. M., Fernández, M. A., Hoyas, E., González, M. J., & García, J. F. (1993). Organochlorine insecticide and polychlorinated biphenyl residues in human breast milk in Madrid (Spain). *Bulletin of Environmental Contamination and Toxicology, 50*(2), 308-315. doi:10.1007/BF00191738

Hoh, F., Uzest, M., Drucker, M., Plisson-Chastang, C., Bron, P., Blanc, S., & Dumas, C. (2010). Structural insights into the molecular mechanisms of cauliflower mosaic virus transmission by its insect vector. *Journal of Virology, 84*(9), 4706. doi:10.1128/JVI.02662-09

Horowitz, A. R., & Ishaaya, I. (2004). *Insect pest management : Field and protected crops*: Springer, Berlin ; London

Huvenne, H., & Smagghe, G. (2010). Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: A review. *Journal of Insect Physiology, 56*(3), 227-235. doi:10.1016/j.jinsphys.2009.10.004

IAEA. (2003a). *Manual for product quality control and shipping procedures for sterile mass-reared Tephritid fruit flies* (5 ed.): FAO/IAEA/USDA.

IAEA, I. A. E. A. (2003b). *Trapping guidelines for area-wide fruit fly programmes*. Vienna: International Atomic Energy Agency.

Jose, A. M., & Hunter, C. P. (2007). Transport of sequence-specific RNA interference information between cells. *Annual Review of Genetics, 41*, 305-330. doi:10.1146/annurev.genet.41.110306.130216

Kamath, R. S., Martinez-Campos, M., Zipperlen, P., Fraser, A. G., & Ahringer, J. (2001). Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. *Genome Biology, 2*(1).

Kamau, A., Mwangangi, J. M., Rono, M. K., Mogeni, P., Omedo, I., Midega, J., . . . Bejon, P. (2018). Variation in the effectiveness of insecticide treated nets against malaria and outdoor biting by vectors in Kilifi, Kenya. *Wellcome Open Research, 2*. doi:10.12688/wellcomeopenres.11073.4

Kasozi, K. I., Namayanja, M., Gaithuma, A. K., Mahero, M., Matovu, E., Yamagishi, J., . . . MacLeod, E. (2019). Prevalence of hemoprotozoan parasites in small ruminants along a human-livestock-wildlife interface in Western Uganda. *Veterinary Parasitology: Regional Studies and Reports, 17*. doi:10.1016/j.vprsr.2019.100309

Killiny, N., & Kishk, A. (2017). Delivery of dsRNA through topical feeding for RNA interference in the citrus sap piercing-sucking hemipteran, *Diaphorina citri*. *Archives of Insect Biochemistry and Physiology, 95*(2). doi:10.1002/arch.21394

Klassen, W., & Curtis, C. F. (2005). History of the sterile insect technique. In V. A. Dyck, J. Hendrichs, & A. S. Robinson (Eds.), *Sterile insect technique: Principles and practice in area-wide integrated pest management* (pp. 3-36). Dordrecht: Springer Netherlands.

Knipling, E. F. (1955). Possibilities of insect control or eradication through the use of sexually sterile males. *Journal of Economic Entomology, 48*(4), 459.

Lanouette, G., Brodeur, J., Fournier, F., Martel, V., Vreysen, M., Cáceres, C., & Firlej, A. (2017). The sterile insect technique for the management of the spotted wing *Drosophila, Drosophila suzukii*: Establishing the optimum irradiation dose. *Plos One, 12*(9), e0180821. doi:10.1371/journal.pone.0180821

Leftwich, P. T., Koukidou, M., Rempoulakis, P., Gong, H.-F., Zacharopoulou, A., Fu, G., . . . Alphey, L. (2014). Genetic elimination of field-cage populations of Mediterranean fruit flies. *Proceedings of the Royal Society B-Biological Sciences, 281*(1792). doi:10.1098/rspb.2014.1372

Li, J., Zhou, Y., Zhou, B., Tang, H., Chen, Y., Qiao, X., & Tang, J. (2019). Habitat management as a safe and effective approach for improving yield and quality of tea (*Camellia sinensis)* leaves. *Scientific Reports, 9*(1). doi:10.1038/s41598-018-36591-x

Li, X., Dong, X., Zou, C., & Zhang, H. (2015). Endocytic pathway mediates refractoriness of insect *Bactrocera dorsalis* to RNA interference. *Scientific Reports, 5*. doi:10.1038/srep08700

Li, Z., Zeng, B., Ling, L., Xu, J., You, L., Aslam, A. F. M., . . . Huang, Y. (2015). Enhancement of larval RNAi efficiency by over-expressing argonaute2 in *Bombyx mori*. *International Journal of Biological Sciences, 11*(2), 176-185. doi:10.7150/ijbs.10235

Liang, C., Wang, Y., Murota, Y., Liu, X., Smith, D., Siomi, M. C., & Liu, Q. (2015). Taf11 assembles the RISC loading complex to enhance RNAi efficiency. *Molecular Cell, 59*(5), 807-818. doi:10.1016/j.molcel.2015.07.006

Liao, X., Jin, R., Zhang, X., Ali, E., Mao, K., Xu, P., . . . Wan, H. (2019). Characterization of sulfoxaflor resistance in the brown planthopper, *Nilaparvata lugens* (stål). *Pest Management Science, 75*(6), 1646-1654. doi:10.1002/ps.5282

Limohpasmanee, W., Kongratarpon, T., & Tannarin, T. (2017). The effect of gamma radiation on sterility and mating ability of brown planthopper, *Nilaparvata lugens* (stål) in field cage. *Journal of Physics: Conference Series, 860*, 012004. doi:10.1088/1742-6596/860/1/012004

Liu, G., Wu, Q., Li, J., Zhang, G., & Wan, F. (2015). RNAi-mediated knock-down of transformer and transformer 2 to generate male-only progeny in the oriental fruit fly, *Bactrocera dorsalis* (hendel). *Plos One, 10*(6). doi:10.1371/journal.pone.0128892

Lutrat, C., Giesbrecht, D., Marois, E., Whyard, S., Baldet, T., & Bouyer, J. (2019). Sex sorting for pest control: It's raining men! *Trends in Parasitology, 35*(8), 649-662. doi:10.1016/j.pt.2019.06.001

Ma, K., Tang, Q., Zhang, B., Liang, P., Wang, B., & Gao, X. (2019). Overexpression of multiple cytochrome p450 genes associated with sulfoxaflor resistance in *Aphis gossypii* (glover). *Pesticide Biochemistry and Physiology, 157*, 204-210. doi:10.1016/j.pestbp.2019.03.021

Manoukis, N. C. (2016). To catch a fly: Landing and capture of *Ceratitis capitata* in a jackson trap with and without an insecticide. *Plos One, 11*(2), e0149869. doi:10.1371/journal.pone.0149869

Marec, F., Neven, L. G., Robinson, A. S., Vreysen, M., Goldsmith, M. R., Nagaraju, J., & Franz, G. (2005). Development of genetic sexing strains in *Lepidoptera*: From traditional to transgenic approaches. *J Econ Entomol, 98*(2), 248-259. doi:10.1603/0022-0493-98.2.248

Marklewitz, M., Dutari, L. C., Paraskevopoulou, S., Page, R. A., Loaiza, J. R., & Junglen, S. (2019). Diverse novel phleboviruses in sandflies from the Panama canal area, central Panama. *Journal of General Virology, 100*(6), 938-949. doi:10.1099/jgv.0.001260

Martinière, A., Bak, A., Macia, J.-L., Lautredou, N., Gargani, D., Doumayrou, J., . . . Drucker, M. (2013). A virus responds instantly to the presence of the vector on the host and forms transmission morphs. *eLife, 2*, e00183. doi:10.7554/eLife.00183

Matetovici, I., De Vooght, L., & Van Den Abbeele, J. (2019). Innate immunity in the tsetse fly (*Glossina*), vector of African trypanosomes. *Dev Comp Immunol, 98*, 181-188. doi:10.1016/j.dci.2019.05.003

Matranga, C., Tomari, Y., Shin, C., Bartel, D. P., & Zamore, P. D. (2005). Passenger-strand cleavage facilitates assembly of siRNA into ago2-containing RNAi enzyme complexes. *Cell, 123*(4), 607-620. doi:10.1016/j.cell.2005.08.044

McEwan, D., Weisman, A., & Hunter, C. P. (2012). Uptake of extracellular double-stranded RNA by sid-2. *Molecular Cell, 47*(5), 746-754. doi:10.1016/j.molcel.2012.07.014

Meagher, R. L., Nagoshi, R. N., Armstrong, J. S., Niogret, J., Epsky, N. D., & Flanders, K. L. (2013). Captures and host strains of Fall Armyworm (*Lepidoptera: Noctuidae*) males in traps baited with different commercial pheromone blends. *Florida Entomologist, 96*(3), 729-740. doi:10.1653/024.096.0305

Menon, T., Sridhar, H., & Shahabuddin, G. (2019). Effects of extractive use on forest birds in Western Himalayas: Role of local and landscape factors. *Forest Ecology and Management, 448*, 457-465. doi:10.1016/j.foreco.2019.06.033

Meza, J. S., Díaz-Fleischer, F., Sánchez-Velásquez, L. R., Zepeda-Cisneros, C. S., Handler, A. M., & Schetelig, M. F. (2014). Fitness cost implications of phic31-mediated site-specific integrations in target-site strains of the Mexican fruit fly, *Anastrepha ludens (Diptera: Tephritidae). Plos One, 9*(10), e109690. doi:10.1371/journal.pone.0109690

Mora, C., Tittensor, D. P., Adl, S., Simpson, A. G. B., & Worm, B. (2011). How many species are there on earth and in the ocean? *PLoS Biol, 9*(8), e1001127. doi:10.1371/journal.pbio.1001127

Mori, T., Hirai, M., & Mita, T. (2019). See-through observation of malaria parasite behaviors in the mosquito vector. *Scientific Reports, 9*(1). doi:10.1038/s41598-019-38529-3

Muhanguzi, D., Mugenyi, A., Bigirwa, G., Kamusiime, M., Kitibwa, A., Akurut, G. G., . . . Tweyongyere, R. (2017). African animal trypanosomiasis as a constraint to livestock health and production in Karamoja region: A detailed qualitative and quantitative assessment. *BMC veterinary research, 13*(1), 355-355. doi:10.1186/s12917-017-1285-z

Mulatier, M., Camara, S., Koffi, A., Carrasco, D., Porciani, A., Chandre, F., . . . Cohuet, A. (2019). Efficacy of vector control tools against malaria-infected mosquitoes. *Scientific Reports, 9*(1). doi:10.1038/s41598-019-43195-6

Muller, H. J. (1950). Radiation damage to the genetic material. *Am Sci, 38*(1), 33-59.

Nansen, C., Baissac, O., Nansen, M., Powis, K., & Baker, G. (2016). Behavioral avoidance - will physiological insecticide resistance level of insect strains affect their oviposition and movement responses? *Plos One, 11*(3), e0149994-e0149994. doi:10.1371/journal.pone.0149994

Niu, J., Yang, W. J., Tian, Y., Fan, J. Y., Ye, C., Shang, F., . . . Wang, J. J. (2019). Topical dsRNA delivery induces gene silencing and mortality in the pea aphid. *Pest Management Science*. doi:10.1002/ps.5457

NPIC, N. P. I. C. (2019). State pesticide regulation. Retrieved from http://npic.orst.edu/reg/regstate.html

Oduola, A. O., Idowu, E. T., Oyebola, M. K., Adeogun, A. O., Olojede, J. B., Otubanjo, O. A., & Awolola, T. S. (2012). Evidence of carbamate resistance in urban populations of *Anopheles gambiae* mosquitoes resistant to ddt and deltamethrin insecticides in Lagos, South-western Nigeria. *Parasites & vectors, 5*(1), 116. doi:10.1186/1756-3305-5-116

Oliveira, C., Auad, A. M., Mendes, S., & Frizzas, M. (2014). Crop losses and the economic impact of insect pests on Brazilian agriculture. *Crop Protection, 56*, 50–54. doi:10.1016/j.cropro.2013.10.022

Osta, M. A., Rizk, Z. J., Labbé, P., Weill, M., & Knio, K. (2012). Insecticide resistance to organophosphates in *Culex pipiens* complex from Lebanon. *Parasites & vectors, 5*(1), 132. doi:10.1186/1756-3305-5-132

Oxitec. (2019). Oxitec ltd. Retrieved from https://www.oxitec.com/

Pane, A., Salvemini, M., Bovi, P. D., Polito, C., & Saccone, G. (2002). The transformer gene in *Ceratitis capitata* provides a genetic basis for selecting and remembering the sexual fate. *Development, 129*(15), 3715-3725.

Park, I. K., Lee, H. R., Jung, C. S., & Koh, S. H. (2019). Synergic effect of sex pheromone (7r,8s)-cis-7,8-epoxy-2-methyloctadec-17-ene on (+)-disparlure of the Asian gypsy moth, *Lymantria dispar*, in Korea. *Journal of Forestry Research, 30*(3), 1119-1122. doi:10.1007/s11676-018-0668-8

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

Peng, W., Zheng, W., Handler, A. M., & Zhang, H. (2015). The role of the transformer gene in sex determination and reproduction in the tephritid fruit fly, *Bactrocera dorsalis* (hendel). *Genetica, 143*(6), 717-727. doi:10.1007/s10709-015-9869-7

Phuc, H. K., Andreasen, M. H., Burton, R. S., Vass, C., Epton, M. J., Pape, G., . . . Alphey, L. (2007). Late-acting dominant lethal genetic systems and mosquito control. *Bmc Biology, 5*. doi:10.1186/1741-7007-5-11

Piñero, J. C., Enkerlin, W., & Epsky, N. D. (2014). Recent developments and applications of bait stations for integrated pest management of Tephritid fruit flies. *Trapping and the detection, control, and regulation of Tephritid fruit flies: Lures, area-wide programs, and trade implications* (pp. 457-492). Dordrecht: Springer Netherlands.

Pinheiro, D. H., Vélez, A. M., Fishilevich, E., Wang, H., Carneiro, N. P., Valencia-Jiménez, A., . . . Siegfried, B. D. (2018). Clathrin-dependent endocytosis is associated with RNAi response in the western corn rootworm, *Diabrotica virgifera virgifera* (leconte). *Plos One, 13*(8), e0201849. doi:10.1371/journal.pone.0201849

POP, S. C. o. P. O. P. (2019). Stockholm convention on persistent organic pollutants. Retrieved from http://www.pops.int/Implementation/PesticidePOPs/DDT

Powney, G. D., Carvell, C., Edwards, M., Morris, R. K. A., Roy, H. E., Woodcock, B. A., & Isaac, N. J. B. (2019). Widespread losses of pollinating insects in Britain. *Nature Communications, 10*(1). doi:10.1038/s41467-019-08974-9

Ratcliffe, D. A. (1970). Changes attributable to pesticides in egg breakage frequency and eggshell thickness in some British birds. *Journal of Applied Ecology, 7*(1), 67-115. doi:10.2307/2401613

Rendon, P., McInnis, D., Lance, D., & Stewart, J. (2004). Medfly (*Diptera: Tephritidae*) genetic sexing: Large-scale field comparison of males-only and bisexual sterile fly releases in Guatemala. *J Econ Entomol, 97*(5), 1547-1553. doi:10.1603/0022-0493-97.5.1547

Reyes, M., Barros-Parada, W., Ramírez, C. C., & Fuentes-Contreras, E. (2015). Organophosphate resistance and its main mechanism in populations of codling moth (*Lepidoptera: Tortricidae*) from central Chile. *Journal of Economic Entomology, 108*(1), 277-285, 279.

Řezáč, M., Řezáčová, V., & Heneberg, P. (2019). Contact application of neonicotinoids suppresses the predation rate in different densities of prey and induces paralysis of common farmland spiders. *Scientific Reports, 9*(1). doi:10.1038/s41598-019-42258-y

Robinson, A. S. (2002). Genetic sexing strains in medfly, *Ceratitis capitata*, sterile insect technique programmes. *Genetica, 116*(1), 5-13. doi:10.1023/A:1020951407069

Rodríguez-Hidalgo, R., Tapia-Chiriboga, A., Arciniegas, S., Vanwambeke, S. O., & Benítez-Ortiz, W. (2019). Epidemiological analysis of the new world screwworm (*Cochliomyia hominivorax*) in Ecuador. *Transboundary and Emerging Diseases, 66*(2), 968-977. doi:10.1111/tbed.13113

Safi, N. H. Z., Ahmadi, A. A., Nahzat, S., Warusavithana, S., Safi, N., Valadan, R., . . . Hemingway, J. (2019). Status of insecticide resistance and its biochemical and molecular mechanisms in *Anopheles stephensi (Diptera: Culicidae)* from Afghanistan. *Malaria Journal, 18*(1). doi:10.1186/s12936-019-2884-x

Salvemini, M., Robertson, M., Aronson, B., Atkinson, P., Polito, L. C., & Saccone, G. (2009). *Ceratitis capitata* transformer-2 gene is required to establish and maintain the autoregulation of cctra, the master gene for female sex determination. *International Journal of Developmental Biology, 53*(1), 109-120. doi:10.1387/ijdb.082681ms

Sánchez-Bayo, F., & Wyckhuys, K. A. G. (2019). Worldwide decline of the entomofauna: A review of its drivers. *Biological Conservation, 232*, 8-27. doi:10.1016/j.biocon.2019.01.020

Sarkar, S., & Roy, S. (2017). Monitoring the effects of a *Lepidopteran* insecticide, flubendiamide, on the biology of a non-target dipteran insect, *Drosophila melanogaster*. *Environmental Monitoring and Assessment, 189*(11). doi:10.1007/s10661-017-6287-6

Schwarz, A. J., Zambada, A., Orozco, D. H. S., Zavala, J. L., & Calkins, C. O. (1985). Mass production of the Mediterranean fruit fly at Metapa, Mexico. *The Florida Entomologist, 68*(3), 467-477. doi:10.2307/3495135

Shen, G.-M., Chen, W., Li, C.-Z., Ou, S.-Y., & He, L. (2019). RNAi targeting ecdysone receptor blocks the larva to adult development of *Tetranychus cinnabarinus*. *Pesticide Biochemistry and Physiology*. doi:10.1016/j.pestbp.2019.05.020

Shukla, J. N., & Palli, S. R. (2012). Sex determination in beetles: Production of all male progeny by parental RNAi knockdown of transformer. *Scientific Reports, 2*. doi:10.1038/srep00602

Smith, L. B., Kasai, S., & Scott, J. G. (2016). Pyrethroid resistance in *Aedes aegypti* and *Aedes albopictus*: Important mosquito vectors of human diseases. *Pesticide Biochemistry and Physiology, 133*, 1-12. doi:10.1016/j.pestbp.2016.03.005

Stern, V., Smith, R., van den Bosch, R., & Hagen, K. (1959). The integration of chemical and biological control of the spotted alfalfa aphid: The integrated control concept. *Hilgardia, 29*, 81-101.

Tariq, K., Ali, A., Davies, T. G. E., Naz, E., Naz, L., Sohail, S., . . . Ullah, F. (2019). RNA interference-mediated knockdown of voltage-gated sodium channel (mpnav) gene causes mortality in peach-potato aphid, *Myzus persicae*. *Scientific Reports, 9*(1), 5291. doi:10.1038/s41598-019-41832-8

Thomas, D. D., Donnelly, C. A., Wood, R. J., & Alphey, L. S. (2000). Insect population control using a dominant, repressible, lethal genetic system. *Science, 287*(5462), 2474.

Tian, H., Peng, H., Yao, Q., Chen, H., Xie, Q., Tang, B., & Zhang, W. (2009). Developmental control of a Lepidopteran pest *Spodoptera exigua* by ingestion of bacteria expressing dsRNA of a non-midgut gene. *Plos One, 4*(7). doi:10.1371/journal.pone.0006225

Toledo, J., Rull, J., Oropeza, A., Hernandez, E., & Liedo, P. (2004). Irradiation of *Anastrepha obliqua* (*Diptera: Tephritidae*) revisited: Optimizing sterility induction. *J Econ Entomol, 97*(2), 383-389.

Tomoyasu, Y., Miller, S. C., Tomita, S., Schoppmeier, M., Grossmann, D., & Bucher, G. (2008). Exploring systemic RNA interference in insects: A genome-wide survey for RNAi genes in *Tribolium*. *Genome Biology, 9*(1). doi:10.1186/gb-2008-9-1-r10

Uchibori-Asano, M., Jouraku, A., Uchiyama, T., Yokoi, K., Akiduki, G., Suetsugu, Y., . . . Shinoda, T. (2019). Genome-wide identification of tebufenozide resistant genes in the smaller tea tortrix, *Adoxophyes honmai (Lepidoptera: Tortricidae)*. *Scientific Reports, 9*(1). doi:10.1038/s41598-019-40863-5

Ureña, E., Guillem-Amat, A., Couso-Ferrer, F., Beroiz, B., Perera, N., López-Errasquín, E., . . . Hernández-Crespo, P. (2019). Multiple mutations in the nicotinic acetylcholine receptor ccα6 gene associated with resistance to spinosad in medfly. *Scientific Reports, 9*(1). doi:10.1038/s41598-019-38681-w

Viklund, L., Rahmani, R., Bång, J., Schroeder, M., & Hedenström, E. (2019). Optimizing the attractiveness of pheromone baits used for trapping the four-eyed spruce bark beetle *Polygraphus poligraphus*. *Journal of Applied Entomology, 143*(7), 721-730. doi:10.1111/jen.12641

Wang, J., Tanaka, Y., Ohno, H., Jia, J., Mori, T., Xiao, T., . . . Hirai, H. (2019). Biotransformation and detoxification of the neonicotinoid insecticides nitenpyram and dinotefuran by *Phanerochaete sordida* yk-624. *Environmental Pollution*, 856-862. doi:10.1016/j.envpol.2019.06.022

Whitten, M. M. A., Facey, P. D., del Sol, R., Fernández-Martínez, L. T., Evans, M. C., Mitchell, J. J., . . . Dyson, P. J. (2015). Symbiont-mediated RNA interference in insects. *Proceedings of the Royal Society B: Biological Sciences, 283*(1825). doi:10.1098/rspb.2016.0042

WHO. (2018). *World malaria report*.

Whyard, S., Erdelyan, C. N., Partridge, A. L., Singh, A. D., Beebe, N. W., & Capina, R. (2015). Silencing the buzz: A new approach to population suppression of mosquitoes by feeding larvae double-stranded RNAs. *Parasites & vectors, 8*(1), 716-716. doi:10.1186/s13071-015-0716-6

Willsey, T., Chatterton, S., & Cárcamo, H. (2019). Interactions between the root rot pathogen *Fusarium avenaceum* and the pea leaf weevil (*Sitona lineatus*) in field pea. *Crop Protection, 116*, 108-114. doi:10.1016/j.cropro.2018.08.027

Winston, W. M., Sutherlin, M., Wright, A. J., Feinberg, E. H., & Hunter, C. P. (2007). *Caenorhabditis elegans* sid-2 is required for environmental RNA interference. *Proceedings of the National Academy of Sciences of the United States of America, 104*(25), 10565-10570. doi:10.1073/pnas.0611282104

Xiao, D., Gao, X., Xu, J., Liang, X., Li, Q., Yao, J., & Zhu, K. Y. (2015). Clathrin-dependent endocytosis plays a predominant role in cellular uptake of double-stranded RNA in the red flour beetle. *Insect Biochemistry and Molecular Biology, 60*, 68-77. doi:10.1016/j.ibmb.2015.03.009

Xu, Q.-Y., Deng, P., Mu, L.-L., Fu, K.-Y., Guo, W.-C., & Li, G.-Q. (2019). Silencing taiman impairs larval development in *Leptinotarsa decemlineata*. *Pesticide Biochemistry and Physiology*. doi:10.1016/j.pestbp.2019.06.013

Zalucki, M. P., Shabbir, A., Silva, R., Adamson, D., Shu-Sheng, L., & Furlong, M. J. (2012). Estimating the economic cost of one of the world's major insect pests, *Plutella xylostella (Lepidoptera: Plutellidae):* Just how long is a piece of string? *J Econ Entomol, 105*(4), 1115-1129. doi:10.1603/ec12107

Zhang, X. R., Zhang, J. Q., Shao, Y. Y., Xing, X. R., Wang, J., Liu, Z. X., . . . Wu, F. A. (2019). Identification of glutathione-s-transferase genes by transcriptome analysis in *Meteorus pulchricornis (Hymenoptera: Braconidae)* and their expression patterns under stress of phoxim and cypermethrin. *Comparative Biochemistry and Physiology - Part D: Genomics and Proteomics, 31*. doi:10.1016/j.cbd.2019.100607

Zhang, Y. B., Zhang, G. F., Liu, W. X., & Wan, F. H. (2019). Variable temperatures across different stages have novel effects on behavioral response and population viability in a host-feeding parasitoid. *Scientific Reports, 9*(1). doi:10.1038/s41598-018-38087-0

Zou, F., Guo, Q., Shen, B., & Zhu, C. (2019). A cluster of cyp6 gene family associated with the major quantitative trait locus is responsible for the pyrethroid resistance in *Culex pipiens* (pallen). *Insect Molecular Biology, 28*(4), 528-536. doi:10.1111/imb.12571

Chapter 2 – Investigating methods for implementing environmental RNA interference (eRNAi) in the Mediterranean Fruit Fly (Ceratitis capitata)

2.1) Abstract

The Mediterranean Fruit Fly is a highly invasive, destructive pest of over 300 plant species, many of which are of global economic importance. The Sterile Insect Technique (SIT) has proved to be an effective mode of medfly control but conventional methods for producing SIT insects diminish their fitness and vigour, which limits their performance in the field. Oxitec Ltd developed "self-limiting" technology to overcome the performance issues associated with conventionally produced SIT insects. However, self-limiting technology requires genetic modification (GM) of pest species and is therefore restricted from use in certain countries and regions. Here I investigated whether non-GM, environmental RNA interference (eRNAi) methods, which are starting to be tested in various insect models, could provide control of the medfly in regions where GM is restricted. I subjected self-limiting OX3864A strain medflies to various eRNAi treatments, to knock down expression of a fluorescent reporter gene. My results showed that the medfly may be sensitive to liposomally-vectored environmental dsRNA, but not to unmodified dsRNA or dsRNA vectored in modified bacteria. Further investigations are required to definitively validate this potentially promising technique.

2.2) Introduction

The Mediterranean Fruit Fly (*Ceratitis capitata*; medfly) is a member of the *Tephritidae* family of "true fruit flies" (White et al., 1992). The medfly is native to West Africa (Gasperi et al., 1991) but is particularly invasive (Malacrida et al., 2006) and has spread to over 100 countries on 7 continents (CABI, 2019). Countries invaded by the medfly have suffered significant agricultural and economic impacts (Enkerlin et al., 1997; Lysandrou, 2009; Siebert et al., 1995).

Female medflies oviposit through the tough outer layer of ripening fruit, placing eggs within the fleshy mesocarp. As larvae develop, they damage plants actively by consuming the mesocarp (Liquido et al., 1991), and passively via the transmission of microbial phytopathogens (Behar et al., 2008; Ordax et al., 2015). Medfly larvae can successfully utilise over 300 plant species (Liquido et al., 1991) but are particularly renowned pests of economically important citrus and stone fruits (Ahmed, 2015; Mazih et al., 2016). Current programs for medfly control rely on the use of broad-spectrum insecticides (Couso-Ferrer et al., 2011; Mangan et al., 2014), pheromone bait traps (Manoukis, 2016), natural enemies (Argov et al., 2008) and the Sterile Insect Technique (SIT) (Barry et al., 2004; Enkerlin et al., 2017; Gonzalez et al., 2007).

The SIT approach (Knipling, 1955) has been effective for controlling the medfly in both North and South America (Enkerlin et al., 2017). The SIT is applied via the strategic mass release of sterile, male insects (Marec et al., 2005; Rendon et al., 2004) into the field. The released males subsequently engage in sterile matings with wild females, causing the wild population to crash (Knipling, 1955). Conventionally, insects for SIT release have been sterilised with gamma radiation but this has well documented negative impacts on insect performance (Guerfali et al., 2011; Hooper, 1972; Toledo et al., 2004).

OX3864A medflies are a next-generation SIT technology that circumvent the fitness costs associated with sterilising irradiation (Fu et al., 2007; Gong et al., 2005). The OX3864A medflies belong to Oxitec's "self-limiting" product line (Thomas et al., 2000) and have been engineered to exhibit female-specific, tetracycline repressible lethality and ubiquitous expression of a fluorescent dsRED2 marker (Fu et al., 2007; Gong et al., 2005).

When provided with tetracycline, OX3864A medflies can be reared effectively in the lab, but removal of dietary tetracycline results in almost 100% mortality of females and the production of male-only OX3864A cohorts (Fu et al., 2007). Release of OX3864A males into the field is predicted to be an effective mode of population suppression. In field cage trials, OX3864A male/wild-type female matings produce non-viable female offspring, leading to rapid population extinction (Asadi et al., 2019; Leftwich et al., 2014). Furthermore, field trials of self-limiting technology implemented in the *Aedes aegypti* system demonstrate up to 96% suppression of target populations (Oxitec, 2019).

A drawback of the self-limiting technology is that it is classified as GM and hence not permitted for use in certain countries or regions of the world. In regions where GM technology is not permitted, an alternative, innovative approach such as RNA interference (RNAi) (Fire et al., 1998) could be used to produce medflies for use with SIT programmes (Darrington et al., 2017).

RNAi is systematic manipulation of the canonical small interfering RNA (siRNA) pathway. The siRNA pathway is a conserved anti-viral gene silencing mechanism (Ding, 2010) initiated via the introduction, by various means, of double-stranded RNA (dsRNA) into target cells. Eukaryotic cells recognise intracellular molecules of double-stranded RNA (dsRNA) as a signal of viral infection, and process them into 21bp small interfering RNAs (siRNA) with Dicer enzymes (Elbashir et al., 2001). siRNAs are subsequently bound to an Argonaut protein (Carmell et al., 2002), forming an RNA-induced silencing complex (RISC). Finally, RISCs degrade cellular mRNAs that have nucleotide sequence homologous to the siRNA component (Maniataki et al., 2005; Rand et al., 2005), which prevents the translation of mRNAs encoded by parasitic viral dsRNA. However, if dsRNAs with specific homology to host genes are transmitted into insect cells, then the siRNA pathway is hijacked and RNAi is initiated.

The interest of effecting RNAi in pest species is to silence target genes that could be useful for achieving insect control. RNAi is a potentially valuable tool for insect control (Reviewed in: (Baum et al., 2014; Darrington et al., 2017; Huvenne et al., 2010)) as genes that induce mortality (Shen et al., 2019; Tariq et al., 2019; Xu et al., 2019) or induce

beneficial phenotypes (Gu, Zhao, et al., 2019; Zhang et al., 2019) can be specifically targeted for knockdown.

The overarching objective of this study was to utilise RNAi to produce medflies that could be used in SIT programmes (Darrington et al., 2017). The principles by which RNAi might offer an alternative route for the induction of sterility, as well as other potentially useful manipulations for insect control, were explored in a study using *Aedes aegypti* (Whyard et al., 2015). The scenario envisaged by Whyard et al. (2015) requires knockdown of at least two genes in the target insects. First, females would be targeted through silencing of a gene in the sexual differentiation cascade to turn them into pseudomales, i.e., genetic females which are phenotypically male (Pane et al., 2002; Salvemini et al., 2009). Next, genes that could induce male (and pseudomale) sterility would be targeted in order to produce a 100% sterile male release cohort (Whyard et al., 2015). However, two equally important conditions must be met before this technique can be applied in the field. The primary condition of RNAi-based SIT is that the sex reversal target must reliably produce a male-only cohort (Marec et al., 2005; Rendon et al., 2004). The second condition is to ensure that silencing of neither the sex reversal nor the sterility target unduly reduces insect performance. Evidence is so far scant, but does suggests that these conditions can be met, in principle.

Salvemini et al. (2009) were able to produce a medfly cohort which was 95.6% phenotypically male, by inducing eggs to express RNAi of *transformer-2* (*Cctra2*). Karyotypic analysis of phenotypically male flies (n = 20) demonstrated that they were 55% genetically female. Most importantly, XX pseudomales expressed male-specific courtship rituals, which should allow them to attract and copulate with females (Briceño et al., 2003). In addition, Gabrieli et al. (2016) report that RNAi of *innexin-5* in medflies produced spermless, sterile males. Spermless males remained sexually competitive with wild-type rivals and were able to induce similar post-mating responses. It is plausible therefore that simultaneous RNAi of *Cctra2* and *innexin-5* could produce a male-only, sterile cohort of medflies that could be used for SIT. It is important to note however that Salvemini et al. (2009) and Gabrieli et al. (2016) microinjected insect eggs with dsRNA, a technique that is incompatible with the large-scale required by SIT.

If RNAi is to produce insects in sufficient numbers for SIT, effector dsRNA molecules need to be administered environmentally (eRNAi). However, for reasons that are as yet not entirely clear, the capacity of insects to express eRNAi varies both within and between species (Baum et al., 2014; Darrington et al., 2017; Li et al., 2015). Therefore, a primary objective of this study was to discover methods of environmental dsRNA delivery that would induce efficient eRNAi in the medfly system.

Initially, I investigated if eRNAi could be induced in OX3864A flies via feeding them with HT115 strain *E. coli* bacteria, that had been engineered to express dsRNA homologous to the dsRED2 gene. HT115 bacteria are RNAse deficient and their genome has been transformed to include the highly specific and prolific T7 RNA polymerase (Kamath et al., 2001). Although originally developed for eRNAi of *C. elegans* (Kamath et al., 2001), the HT115 strain has since been utilised to implement eRNAi in several insects (Bento et al., 2019; Caccia et al., 2019; Ganbaatar et al., 2017). I predicted that successful eRNAi of dsRED2 in OX3864A medflies would be simple to quantify via: 1) the reduction or loss of the dsRED2 fluorescent phenotype measured as fluorophore intensity with ImageJ (Schindelin et al., 2012; Waters, 2009), and 2) a reduction in level of dsRED2 gene expression, measured with RT-qPCR (Gu, Zhao, et al., 2019; Kyre et al., 2019; Shen et al., 2019; Tariq et al., 2019; Zhang et al., 2019).

Next, I supplied discrete populations of larvae and adults with diets that had been supplemented with dsRED2 dsRNA either in an unmodified (naked) form or vectored within liposomes. Naked dsRNA is reported to elicit eRNAi in many eRNAi competent insects (Gu, Zhao, et al., 2019; Kyre et al., 2019; Shen et al., 2019; Tariq et al., 2019; Zhang et al., 2019). Liposomally vectored dsRNA can elicit a gene silencing response from insects that are recalcitrant to eRNAi implemented with naked molecules (Taning et al., 2016; Whyard et al., 2009). As for the HT115 assay I predicted that I would be able to detect knockdown of dsRED2 via RT-qPCR.

2.3) Methods

2.1) Medfly strains used for eRNAi investigations

eRNAi experiments were conducted using OX3864A medflies supplied by Oxitec Ltd (Fu et al., 2007; Gong et al., 2005). Control experiments to assess if dsRNA effector molecules had introduced noise to RT-qPCR analyses (see supplementary materials 2.2) were performed on the Toliman wild-type medfly strain. Toliman was originally collected in Guatemala in 1990 and was obtained from a lab reared population, maintained by Oxitec (Oxitec, 2019) since 2004.

2.2) OX3864A medfly husbandry

OX3864A medflies were reared in a controlled environment: 25^{o}C temperature, 51% relative humidity and 12L: 12D photoperiod. Day 1: approximately 500 eggs (<24 hrs old) were collected by pipette from adult cages and transferred to 100ml of larval diet (Agar 12.5g/L; Sucrose 73.5g/L; Maize 67g/L; Brewer's yeast 47.5g/L; 10% Nipagin 25ml/L; Propionic acid 2ml/L; Tetracycline 0.1g/L) in a plastic bottle. Day 7: the bottle was laid down in 2cm of childrens' play-sand in a plastic pupation box (170mm x 130mm x 50mm) that had a mesh breathing hole (50mm Ø) cut in the lid. From Day 8 to Day 15: L3 stage larvae naturally dispersed from the bottles to pupate in the sand. Day 16: 100 pupae were collected from the sand and transferred to a plastic cage (120mm x 120mm x 135mm) which had a 90mm (Ø) opening cut into the side enclosed with fine nylon mesh. In the cage, flies had *ad libitum* access to ddH_2O that contained 0.1g/l of tetracycline (Sigma) and standard artificial diet (3:1 sucrose: hydrolysed yeast). Day 24: caged females laid eggs through the nylon mesh which dropped into a reservoir of ddH_2O to be collected for infestation onto larval diet for the next generation.

2.3) Strains of HT115 *E. coli* that were fed to OX3864A medflies to elicit eRNAi

Two strains (treatment and +ve-control) of HT115 (DE3) *E. coli* (Timmons et al., 2001) were fed to OX3864A medflies to elicit eRNAi. Both the treatment and +ve-control HT115 strains were supplied by Oxitec Ltd (Oxitec, 2019). Treatment bacteria were modified to synthesise a 612bp dsRNA construct with 100% homology to bases 1-612 of the deRED2

gene sequence (Gong et al., 2005). +ve-control bacteria were modified to express a 710bp dsRNA construct homologous to bases 5406-6116 of the GFP protein (Shimomura, 2005) (see supplementary materials 2.5/2.6).

2.4) Propagation of HT115 bacteria with selective media

Tetracycline and ampicillin antibiotics were employed as selective agents in HT115 media, as the HT115 chromosome and L4440 expression vector confer resistance to these compounds (Timmons et al., 2001). Bacteria were cultured in LB broth (5g/L NaCl; 5g/L yeast extract; 1.5g/L glucose; 10g/L tryptone) or on LB agar plates (5g/L NaCl; 5g/L yeast extract; 1.5g/L glucose; 10g/L tryptone; 15g/L agar) that had been supplemented with 12.5µg/µl tetracycline and 10µg/µl ampicillin.

2.5) Labelling of HT115 bacteria with Brilliant Blue FCF to determine if medflies would consume bacterial cells contained within the diet

Two feeding regimes (treatment and control) were established in discrete, caged fly populations (n=100). The treatment and control regimes had immediate *ad libitum* access to ddH_2O (0.1g/l tetracycline) (Sigma) but diets were not introduced to cages until 48hrs after the first fly had eclosed. To prepare diet for the treatment feeding regime, 2ml of HT115 treatment broth culture (OD600 ≈1) was pelleted by centrifuge (5000RPM for 10 mins) and supernatant was removed. The bacterial pellet was re-suspended by vortex in 500µl of 0.1% w/v Brilliant Blue FCF (BFCF) in glacial acetic acid (ThermoFisher), then centrifuged at 5000RPM for a further 10 mins. The blue pellet was re-suspended in 400µl of molecular water (ThermoFisher) and 300µl of the suspension was pipetted onto 3g of artificial diet (3:1 sucrose: hydrolysed yeast). Diet for the control regime was prepared by centrifugation of 2ml of sterile LB broth (5000RPM for 10 mins). Supernatant was removed and 500µl of 0.1% w/v BFCF in 100% acetic acid was added to the tube which was then vortexed and centrifuged (5000RPM for 10 mins). The supernatant was removed and 400µl of molecular water (ThermoFisher) was added. 300µl of the suspension was then pipetted onto 3g of artificial diet (3:1 sucrose: hydrolysed yeast). Treatment and control diets were air dried for 1.5hrs before being transferred to regime cages. Flies were left to feed undisturbed for 24 hrs before being euthanised by cold

exposure (-20^{o}C). Guts were then dissected from these flies and visualised for blue staining (see below).

2.6) Preparation and administration of diet infected with HT115 bacteria to elicit eRNAi in OX3864A medflies

Caged fly populations (n=100) were subjected to one of three dietary regimes: 1) -ve control – standard artificial diet, 2) treatment - artificial diet infected with treatment bacteria, 3) +ve control - artificial diet infected with +ve-control bacteria. Feeding regimes had immediate *ad libitum* access to ddH_2O (0.1g/l tetracycline) (Sigma). Diets were not introduced to cages until 48hrs after the first fly had eclosed, then fresh food was provided every 24hrs. Cultures of treatment and +ve-control broth (feeding broth inoculums; FEEB) were prepared at the beginning of the experiment to provide a continuous pool of bacteria to those regimes. To prepare a FEEB, 20ml of broth was inoculated with a loop of HT115 bacteria then placed in an orbital incubator set at 37^{o}C and 200 RPM. When the FEEB had reached a late-log phase (OD600 between 1.0 – 1.2) it was stored at 4^{o}C for the duration of the feeding assay. Each day during the experiment, daily feeding broths (DAILY) to be administered to the treatment and +ve-control regimes were prepared by inoculating 10ml of broth with a volume of the stored FEEBs. The volume of FEEB added to DAILY varied (according to equation 1), to provide each regime with a standardised dose of bacteria over the course of the experiment (see supplementary materials 2.4).

Equation 1

(OD600 of treatment FEEB on day 1/OD600 of FEEB at time of DAILY preparation) X 100

When inoculated with a FEEB, DAILYs were incubated at 37^{o}C and 200 RPM for 3 hrs in an orbital incubator (New Brunswick Scientific Innova 44) to reach an OD600 ≈ 0.8. 0.4mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma) was then added to induce the synthesis of dsRNA effector molecules and DAILYs were further incubated at 37^{o}C for a 2.5hrs at 200 RPM. Bacteria were pelleted from 2ml aliquots of DAILY (5000RPM for 10 mins), the supernatant was removed, and pellets were re-suspended in 400µl of molecular water (ThermoFisher) by vortex. 300µl of the bacterial suspension

was pipetted onto 3g of artificial diet (3:1 sucrose: hydrolysed yeast) and the mixture was air dried for 1.5hrs. Once diets were completely dry, they were supplied to the appropriate feeding regime. Every 24hrs, 9 individuals were extracted from each regime and transferred to 2ml Eppendorf tubes in groups of three. Eppendorfs were immediately dropped into liquid nitrogen and flies were stored at -80^{o}C.

2.7) Feeding OX3864A larvae with dsRED2 dsRNA to induce eRNAi of the dsRED2 gene

dsRED2 dsRNAs were *in vitro* synthesised by Genolution, Korea (Genolution, 2019) and supplied by Oxitec Ltd (Oxitec, 2019) (see supplementary materials 2.5). OX3864A larvae were subjected to three feeding treatment regimes to elicit eRNAi of the dsRED2 gene: 1) naked – larval diet supplemented with unmodified dsRED2 dsRNA, 2) Lipo 10 – larval diet supplemented with dsRED2 dsRNA that had been conjugated to Lipofectamine™ 2000 liposomes (ThermoFisher). Liposomes had been diluted 10:1 with molecular water (ThermoFisher) and 3) Lipo 20 – larval diet supplemented with dsRED2 dsRNA that had been conjugated to Lipofectamine™ 2000 liposomes (ThermoFisher). Liposomes had been diluted 20:1 with molecular water (ThermoFisher). For the Lipo 10 and Lipo 20 feeding regimes, dsRNA was conjugated to liposomes according to the manufacturer's instructions. Diets for each regime were prepared by mixing 556.75µg of appropriately conditioned dsRED2 dsRNA with 5ml of larval diet in a 35mm petri dish. To initiate feeding, L2 larvae (<72hrs old) were transferred to the diets with a stainless-steel dissection needle that had been sterilised with 70% ethanol then RNAseZap (ThermoFisher). Every 24hrs, nine larvae were extracted from feeding regimes with a sterile, stainless-steel dissection needle and transferred in groups of three to sterile 2ml Eppendorf tubes. Eppendorfs were immediately dropped into liquid nitrogen and stored at -80^{o}C.

2.8) Feeding of adult OX3864A medflies with dsRED2 dsRNA to induce eRNAi of the dsRED2 gene

dsRED2 dsRNAs were *in vitro* synthesised by Genolution, Korea (Genolution, 2019) and supplied by Oxitec Ltd (Oxitec, 2019). Individual OX3864A adults were subjected to three distinct feeding regimes to elicit eRNAi of the dsRED2 gene: 1) naked – larval diet

supplemented with naked dsRED2 dsRNA, 2) Lipo 10 – larval diet supplemented with dsRED2 dsRNA that had been conjugated to Lipofectamine™ 2000 liposomes (ThermoFisher). Liposomes had been diluted 10:1 with molecular water (ThermoFisher) and 3) Lipo 20 – larval diet supplemented with dsRED2 dsRNA that had been conjugated to Lipofectamine™ 2000 liposomes (ThermoFisher). Liposomes had been diluted 20:1 with molecular water (ThermoFisher). For the Lipo 10 and Lipo 20 feeding regimes, dsRNA was conjugated to liposomes according to the manufacturer's instructions. For each regime, diets were prepared by overlaying 556.75µg of appropriately conditioned dsRED2 dsRNA on 0.5g of artificial diet with a pipette. Diets were air dried for 1.5hrs then administered to flies (48 hrs post eclosion) that had been isolated in plastic pots (50mm x 50mm x 50mm). Every 24 hrs diets were replenished, and three flies were extracted from each regime for subsequent RT-qPCR analysis. Extracted flies were placed individually in 2ml Eppendorf tubes and immediately dropped into liquid nitrogen, then stored at -80^{o}C.

2.9) Quantifying dsRED2 expression in OX3864A adults and larvae with RT-qPCR

2.9.1) Extraction and purification of RNA from fly matter

Adult flies and larvae that had been extracted from eRNAi experiments were stored at -80^{o}C in 2ml Eppendorf tubes. To render fly material brittle, Eppendorfs were placed in liquid nitrogen. The brittle fly matter was then pulverised inside Eppendorfs using a micropestle that had been sterilised with 10 mins exposure to UV radiation (Labcaire PCR workstation) and wiped with RNAseZap (ThermoFisher). RNA extraction was carried out on pulverised fly material using a mirVana kit (ThermoFisher) according to the manufacturer's instructions. To digest all residual genomic DNA contained in the mirVana solution, the suspension was treated with Turbo DNAase (ThermoFisher) according to the manufacturer's instructions.

2.9.2) Synthesis of first strand cDNA from RNA templates

CDNA was synthesised using a Revertaid first strand synthesis kit (ThermoFisher). 0.5µg of mRNA was incubated with 1µl of OligodT (10µM) in molecular water (ThermoFisher) (11.5µl total volume) at 65˚C for 5 mins. The remaining Revertaid components (4µl 5X

reaction buffer; 0.5µl Ribolock; 2µl 10mM dNTP mix; 1µl Revertaid) were then added to the incubated suspension and incubated for 60 mins at 42˚C, then 5 mins at 70˚C.

2.9.3) Quantitative reverse transcriptase PCR (RT-qPCR)

RT-qPCR was carried out on a StepOnePlus thermocycler (Applied Biosystems) using Taqman gene expression mastermix (Applied Biosystems). Primers and probes for the dsRED2 target and two reference genes (Rpl13a and L27) (Nash et al., 2018) were purchased from Eurofins Genomics (see supplementary materials 2.1). Each RT-qPCR well contained 1µl of CDNA (25ng total CDNA assuming 100% recovery during reverse transcription), 10µl of primers (1.6µl of each primer at 15.63µM), 12.5µl of 2X reaction mix and 1.25µl of fluorescent probes (0.416µl of each probe at 15.63µM).

2.9.4) Analysis of RT-qPCR data

Relative dsRED2 expression was calculated using the 2–ΔΔCt method (Pfaffl, 2001) for all assays other than the HT115 feeding assay. For the HT115 feeding assay, relative dsRED2 expression was calculated with qBASE+ software (Hellemans et al., 2007). All relative expression data were analysed with the tidyverse packages (Wickham, 2017) in R Studio (Team, 2016).

2.10) Quantifying fluorescence emitted by OX3864A medflies as a proxy for dsRED2 expression

OX3864A medflies were subjected to the treatment and -ve-control HT115 feeding regimes described in section 2.6 for five days. Every 24hrs, approximately 30 flies were individually transferred from each feeding regime to 2ml Eppendorf tubes and flash frozen in liquid nitrogen. Before being analysed for fluorescence, flies were thawed for ten minutes at room temperature and legs were removed. Remaining fly sections were assessed for fluorescence under a Leica MZ75 stereo microscope with a 100W mercury lamp adapter and imaged with a GXCAM HiChrome-S camera (Vision, 2019) using GX capture software (version 8.5). The GC capture settings were as follows: exposure = 1sec 420ms; gain = 0; gamma = 11; contrast = 8; saturation = 8; red = 0.44; green = 0; blue = 0; denoise = 5; sharpen = 5. Images were analysed with ImageJ version 1.48S (Schindelin et

al., 2012). For each image, a region of interest (ROI) was drawn around the fly and three (100 pixels X 100 pixels) regions of non-fluorescent background. To enhance the fluorescent signal-to-noise ratio, the mean background fluorescence of each image was subtracted from the mean ROI fluorescence (Waters, 2009) according to equation 2.

Equation 2

Corrected pixel intensity = mean pixel intensity of ROI – mean background pixel intensity

2.4) Results

2.1) Verifying consumption of HT115 bacteria by OX3864A medflies

OX3864A medflies were supplied with diet supplemented with HT115 bacteria that had been labelled with Brilliant Blue FCF (BFCF) for 24hrs. Consumption of HT115 cells by flies was then verified by the presence of blue labelling in the gut (Li et al., 2011). Internal blue colouration of the anterior, ventral abdomen was observed for flies that had been exposed to labelled bacteria (n=30) (Figure 2.1). Blue dye was also visible throughout the dissected guts of flies that had been exposed to labelled diet. Blue colouration verified that OX3864A medflies were consuming HT115 bacteria, as flies that had been exposed to a control feeding condition (n=30) did not exhibit any blue staining of the abdomen or gut.

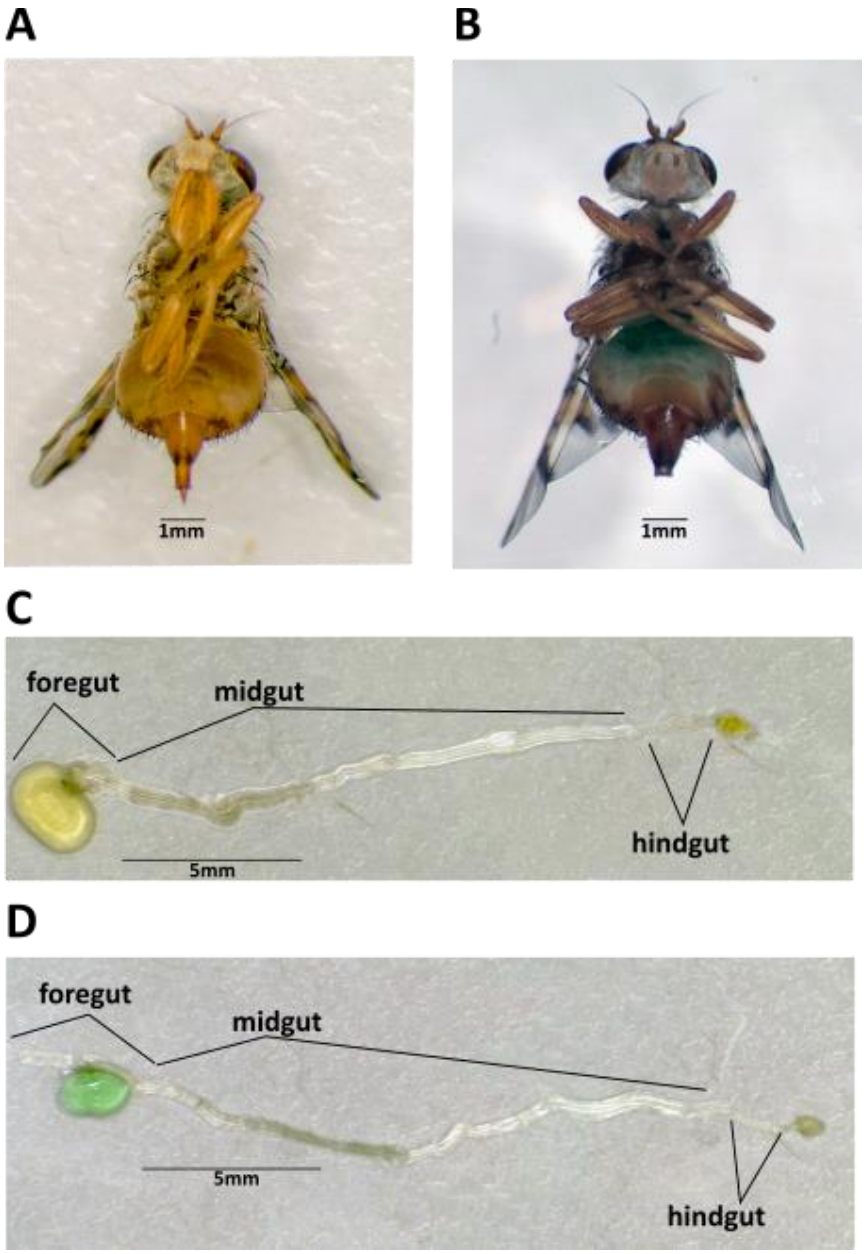

Figure 2.1 Images of adult medflies and guts captured following exposure to brilliant blue-labelled bacteria vs a standard diet control. A) A medfly that was reared on standard artificial diet for 24hrs. The anterior abdomen exhibits the yellow/brown phenotype that is typical of this species. B) A medfly that was reared on diet infested with blue labelled bacteria for 24hrs. The anterior abdomen exhibits obvious blue colouring. C) A gut extracted from a medfly that had been reared on standard diet. The gut exhibits yellow staining in the crop, anterior midgut (peritrophic matrix) and rectum which is likely to be caused by residual yeast. D) A medfly gut extracted from an individual that had been reared on diet infected with brilliant blue labelled bacteria. The gut exhibits blue staining in the crop, anterior midgut (peritrophic matrix) and rectum. Blue staining in the gut lumen provided evidence that dietary bacteria had been consumed.

2.2) Quantifying fluorescence emmitted by OX3864A medflies as an estimate of dsRED2 expression

OX3864A medflies were supplied with diet that had been infected with treatment strain HT115 bacteria or a -ve-control diet for five days. It was hypothesised that flies that had been exposed to treatment bacteria would initiate eRNAi of the dsRED2 gene (Ganbaatar et al., 2017; Lopez et al., 2019; Taracena et al., 2015; Whyard et al., 2015) and exhibit reduced fluorescence in comparison to -ve-controls (Waters, 2009). Flies were extracted from feeding regimes every 24hrs and 8bit fluorescent images (n≈30) were captured with a HiChrome-S camera (Vision, 2019) (Figure 2.2). The mean corrected pixel intensity of fly images served as a proxy for fluorophore concentration (Waters, 2009) and thus dsRED2 expression. From a possible 8-bit range of 255 intensity values, the pixel intensity values captured from all images varied from 30 to 147 (Figure 2.3). The distribution of pixel intensities was not normal (Shap.Wilk, $W=0.97$, $p<0.0001$) and could not be transformed to normality. Therefore, the fluorescence data were analysed with a non-parametric ANCOVA model using the fANCOVA package (Wang et al., 2010) in R Studio (Team, 2016). Controlling for time as a covariate, the fluorescence exhibited by flies that consumed HT115 treatment bacteria was not significantly different from flies that consumed -ve-control diet (fANCOVA, $T=297.6$, $p>0.5$).

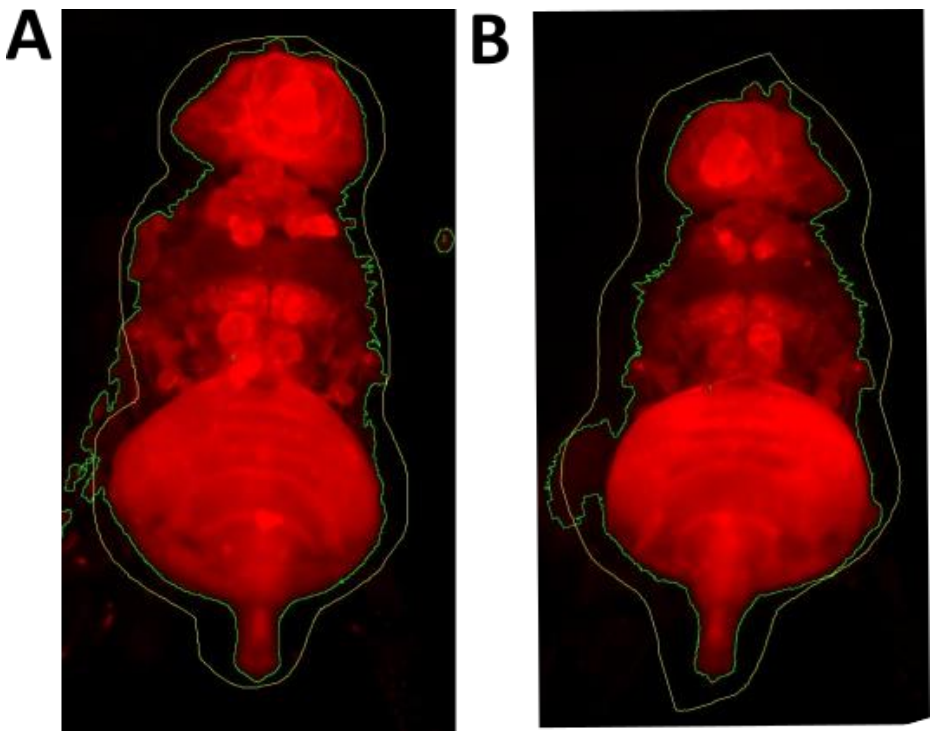

Figure 2.2 Fluorescent images of two OX3864A medflies demonstrating the upper lower range of mean pixel intensity captured with the HiChrome-S camera. Yellow/green outlines represent a region of interest (ROI) that was designated for analysis using ImageJ (Schindelin et al., 2012). The ROI surrounding fly A exhibited a mean pixel intensity of 49.6 and the ROI surrounding fly B exhibited a mean pixel intensity of 125.5.

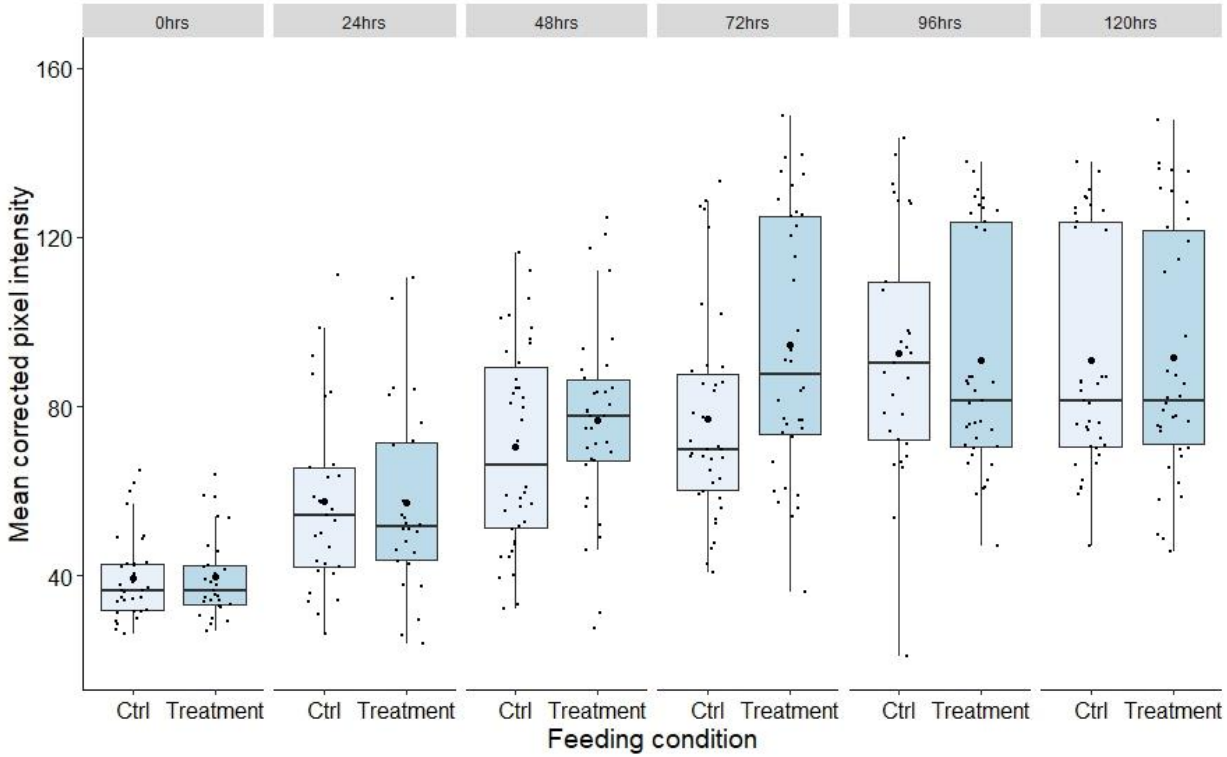

Figure 2.3 The pixel intensities of OX3864A flies that were exposed to either HT115 treatment or -ve-control feeding conditions over a period of five days. Control (light blue boxes) and treatment (dark blue boxes) feeding conditions are represented on the x-axis. The x-axis is faceted according to the period that flies were exposed to a given feeding condition. Mean corrected pixel intensities exhibited by flies are represented on the y-axis. The upper and lower hinges of boxplots represent the first and third quartiles of pixel intensity exhibited by groups of flies (n≈30) extracted from a feeding regime at a particular time point. Median pixel intensity is represented by a horizontal line and mean signal is represented by a circle. Unique data points included in boxplots are represented by small black dots. Controlling for the effect of time as a covariate, there was no significant difference in the pixel intensities exhibited by flies subjected to the treatment or -ve-control feeding regimes (fANCOVA, $T = 297.6$, $p>0.5$).

2.3) Quantication of dsRED2 expression by OX3864A medflies subsequent to feeding with HT115 bacteria

OX3864A medflies were exposed to HT115 treatment, HT115 +ve-control and -ve-control feeding conditions for four days. It had been predicted that exposure to treatment bacteria would suppress expression of dsRED2 by OX3864A medflies relative to the controls (Ganbaatar et al., 2017; Lopez et al., 2019; Taracena et al., 2015; Whyard et al., 2015). Every 24hrs, three groups of flies (n=3) were extracted from each feeding environment and subjected to RT-qPCR of dsRED2 and two reference genes (L27 and Rpl13a) (Nash et al., 2018). This procedure generated a total of 45 specimens for RT-qPCR analysis, that were processed over three RT-qPCR runs. Relative expression of dsRED2 between all 45 specimens collected over the three RT-qPCR runs was calculated with qBASE+ software (Hellemans et al., 2007). qBASE+ consolidates data accumulated from multiple RT-qPCR runs via "inter-run calibration" and is MIQE (Bustin et al., 2009) compliant. The relative expression of dsRED2 calculated by qBASE+ was log10 transformed to create a normal distribution (Shap.Wilk, $W=0.96$, $p>0.05$) with homogenous variance (Bartlett's $K^2= 2.4$, $df = 3$, p-value>0.05) for parametric analysis (Figure 2.4). Data were analysed with an ANCOVA model that included an interaction between feeding condition and time as this was found to be superior to a model that controlled for additive effects of feeding condition and time (Akaike Information Criterion = 161 vs 171). There were significant differences in the expression of dsRED2 between groups of flies that were reared on distinct diets (ANCOVA, $F(2,39) =5.92$, $p<0.05$), but dsRED2 expression was not significantly different due to time (ANCOVA, $F(1,39) =3.15$, $p>0.05$). However, a post-hoc Tukey test revealed that dsRED2 expression was not significantly between the treatment and control groups as had been predicted (Figure 2.5).

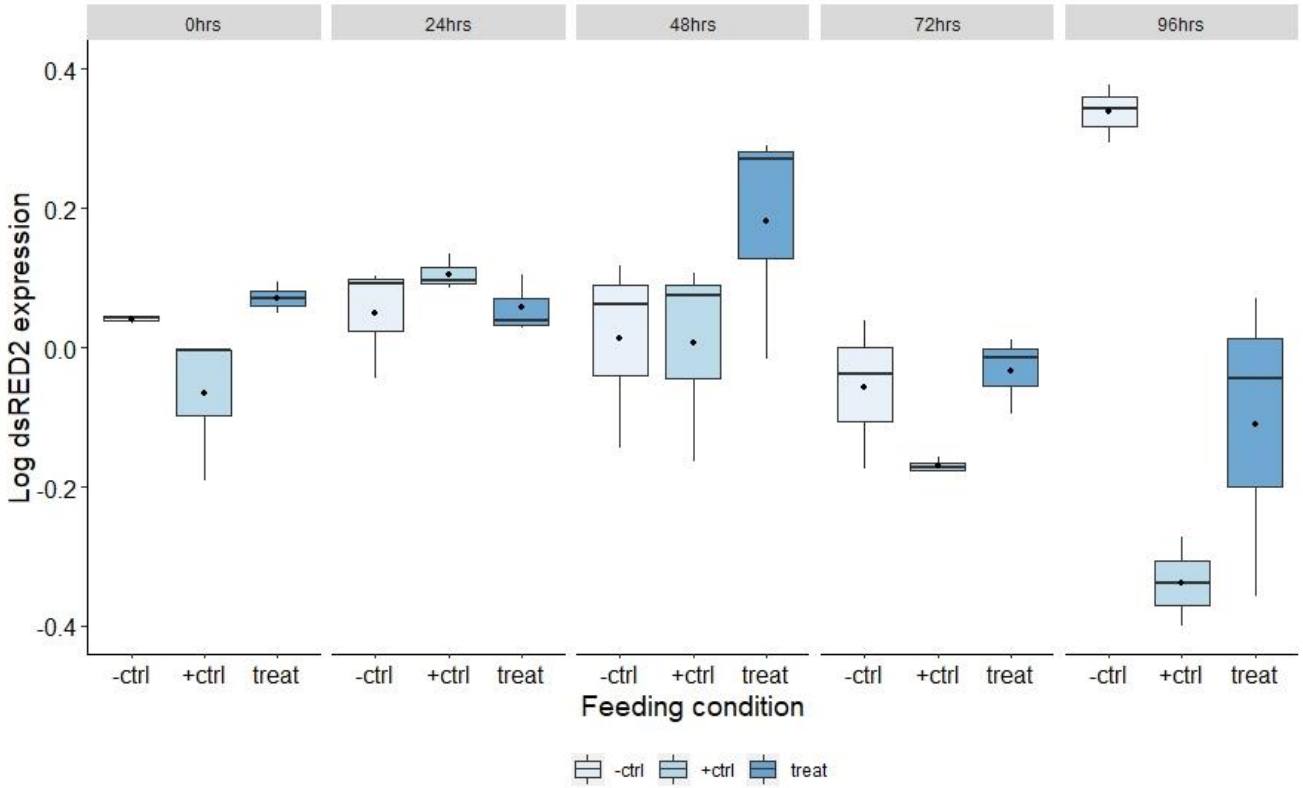

Figure 2.4 Relative expression of dsRED2 by OX3864A flies that had been exposed to either HT115 treatment, HT115 +ve-control or -ve-control feeding conditions over a period of four days. Discrete feeding conditions are represented on the x-axis. The x-axis is faceted according to the period that flies were exposed to a given feeding condition. Log10 relative expression of the dsRED2 gene is represented on the y-axis. The upper and lower hinges of boxplots represent the first and third quartiles of gene expression exhibited by three groups of flies (n=3) extracted from a feeding regime at a particular time point. Median gene expression is represented by a horizontal line and mean signal is represented by a dot. Controlling for the interacting covariate effects of time, flies subjected to different feeding regimes exhibited significant difference in dsRED2 expression (ANCOVA, $F(2,39) = 5.92$, $p<0.05$).

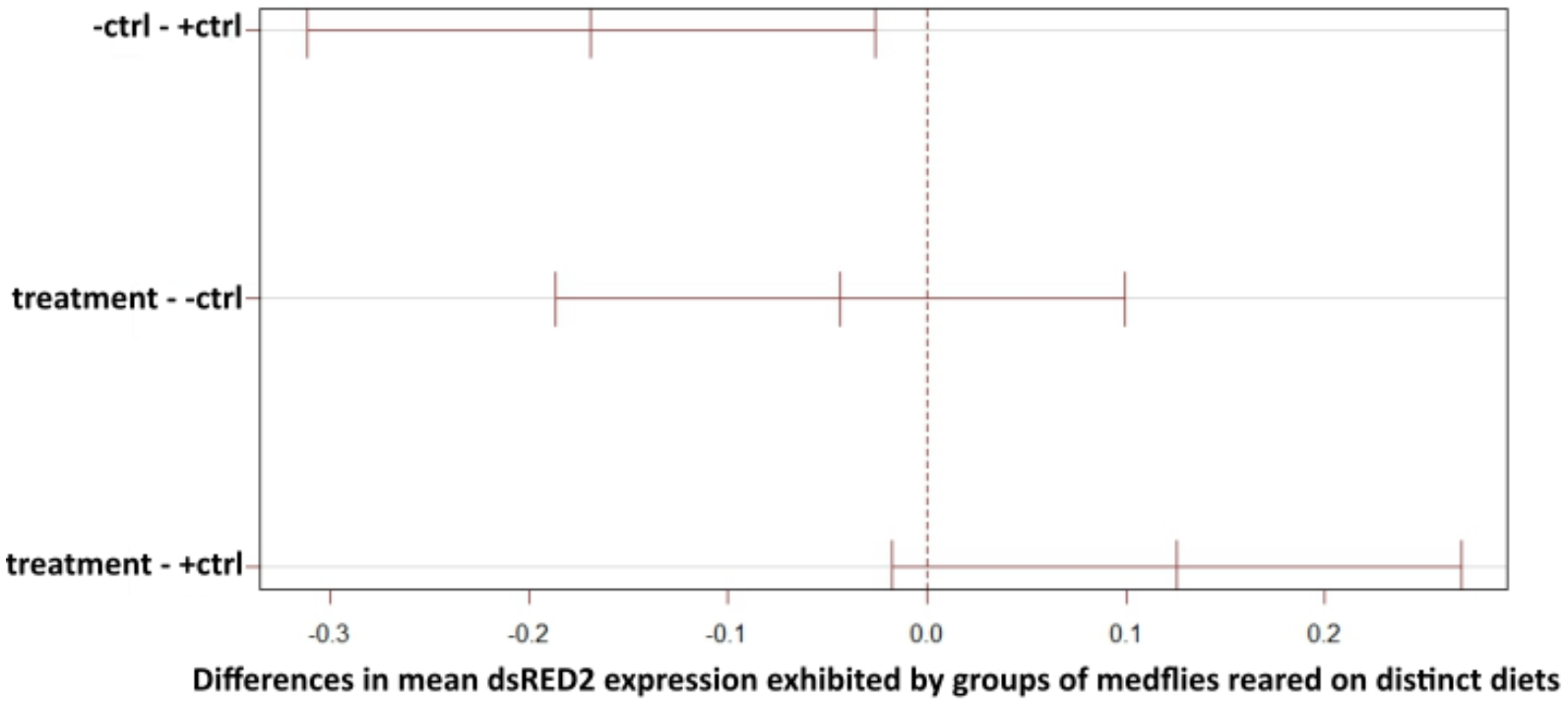

Figure 2.5 Post-hoc Tukey comparison of the mean dsRED2 expression exhibited by flies reared on either HT115 treatment, HT115 +ve-control or -ve-control diets. Calculated at a 95% confidence interval there was a significant difference in dsRED2 expression between flies subjected to the -ve-control and +ve-control feeding regimes. Contrary to the predicted trend, dsRED2 expression was not significantly different between the treatment regime and controls.

2.4) Silencing of dsRED2 via liposomally vectored and naked dsRNA

2.4.1) eRNAi of dsRED2 in OX3864A larvae via liposomally vectored and naked dsRNA

Second instar (L2) OX3864A larvae were supplied with diet that had been supplemented with dsRED2 dsRNA either as naked molecules or vectored in liposomes diluted at 10:1 or 20:1 in molecular water. It was predicted that exposure to environmental dsRNA would provoke eRNAi in medfly larvae and that this effect could be measured via RT-qPCR of the dsRED2 gene (Shen et al., 2019; Xu et al., 2019; Zhang et al., 2019). Every 24hrs, three groups of larvae (n=3) were extracted from the discrete feeding regimes and flash frozen in liquid nitrogen for subsequent RT-qPCR analysis. Relative dsRED2 expression was not normally distributed between groups of larvae and was therefore transformed using the cube root function (x^1/3). The transformed data were normally distributed (Shap.Wilk, W=0.96, p>0.05) with homogenous variance (Bartlett's K^2= 2.4, df = 3, p-value>0.05) and were utilised for parametric analysis (Figure 2.6). RT-qPCR data were analysed with an ANCOVA model that included an interaction between feeding condition and time as this was judged to be superior to a model that controlled for additive effects of feeding condition and time (Akaike Information Criterion = 66.3 vs 69.1). Correcting for the interactive covariate effects of time, there was no evidence that dsRED2 expression varied significantly between larval groups subjected to the different feeding conditions (ANCOVA, F (3,16) =0.256, p<0.05)

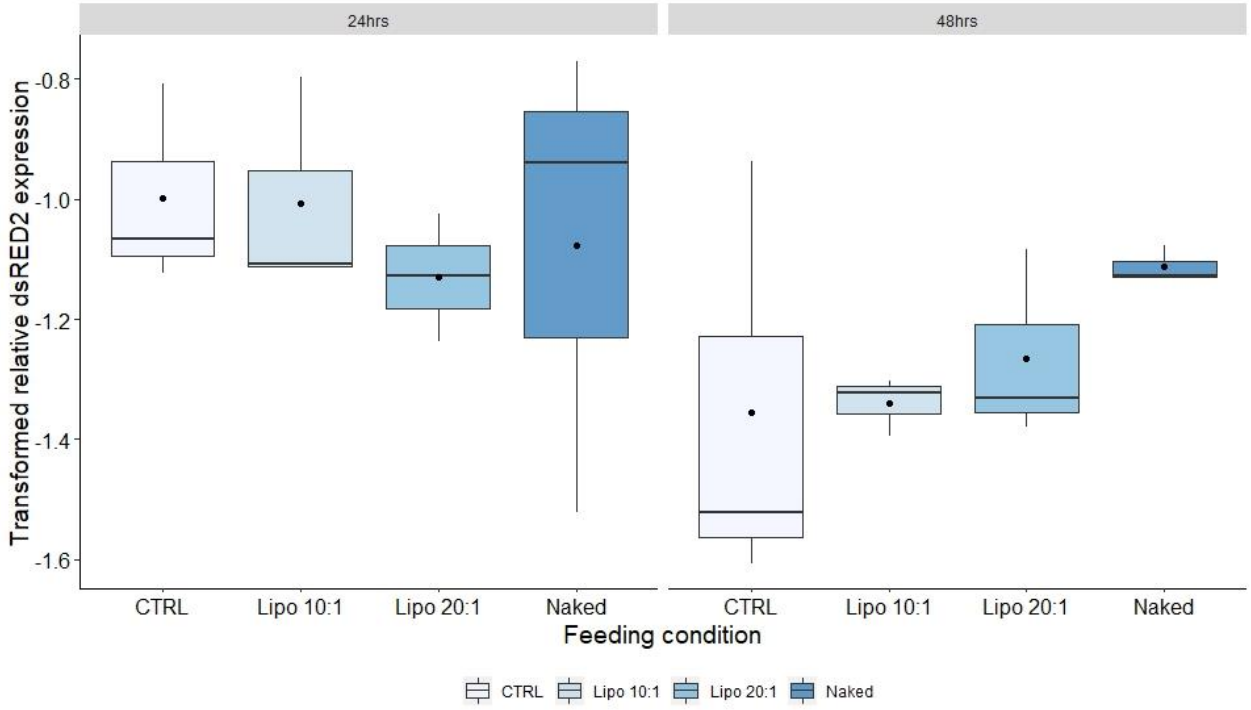

Figure 2.6 Relative expression of dsRED2 by OX3864A larvae that were exposed to a standard control diet or a treatment diet supplemented with either naked dsRED2 dsRNA or dsRNA vectored in liposomes ('Lipo') that had been diluted 10:1 or 20:1 in molecular water. Discrete feeding conditions are represented on the x-axis. The x-axis is faceted according to the period that flies were exposed to a given feeding condition. Cube root (x^1/3) relative expression of the dsRED2 gene is represented on the y-axis. The upper and lower hinges of boxplots represent the first and third quartiles of gene expression exhibited by three groups of larvae (n=3) extracted from feeding regimes at a particular time point. Median gene expression is represented by a horizontal line and mean signal is represented by a dot. Controlling for the interacting covariate effects of time, there was no significant difference in dsRED2 expression between larvae reared on the different diet treatments (ANCOVA, $F(3,16) = 0.256$, $p < 0.05$).

2.4.2) eRNAi of dsRED2 in OX3864A adults via liposomally-vectored and naked dsRNA

Individual medflies were supplied with diet that had been supplemented with dsRED2 dsRNA either as naked molecules or vectored in liposomes diluted 10:1 or 20:1 with molecular water. It was predicted that exposure to environmental dsRED2 dsRNA would provoke eRNAi in adult medflies and that this effect could be measured via RT-qPCR of the dsRED2 gene (Gu, Li, et al., 2019; Kyre et al., 2019; Tariq et al., 2019). Every 24hrs, three flies were extracted from the discrete feeding regimes and flash frozen in liquid nitrogen for subsequent RT-qPCR analysis. Relative dsRED2 expression was not normally distributed among flies and was therefore transformed (transformation = -1 * x ^ -1) using the rcompanion package (Mangiafico, 2016). The transformed data were normally distributed (Shap.Wilk, $W=0.93$, $p>0.05$) with homogenous variance (Bartlett's $K^2= 6.69$, $df = 3$, $p>0.05$) and were utilised for parametric analysis (Figure 2.7). RT-qPCR data were analysed with an ANCOVA model that included an interaction between feeding condition and time as this was judged to be superior to a model that controlled for additive effects of feeding condition and time (Akaike Information Criterion = 14.4 vs 17.7) Correcting for the interacting covariate effects of time, there were significant differences in dsRED2 expression between adults that were subjected to discrete feeding conditions (ANCOVA, $F(3,16) =5.29$, $p<0.01$), but dsRED2 expression did not differ significantly due to time (ANCOVA, $F(1,16) =2.77$, $p>0.05$). A post-hoc Tukey test revealed that dsRED2 expression was significantly different in flies reared on dsRNA vectored in liposomes (diluted 10:1) relative to flies reared on standard diet (Figure 2.8).

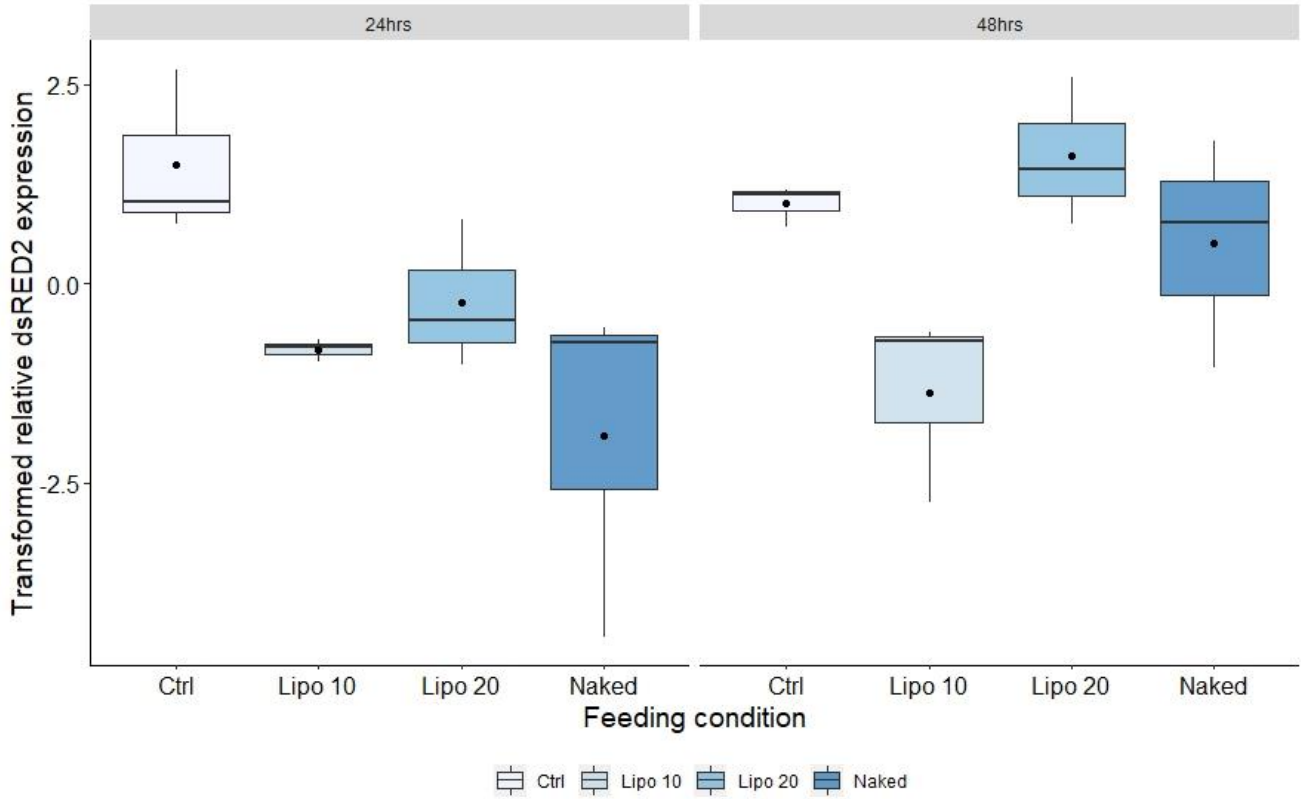

Figure 2.7 Relative expression of dsRED2 by OX3864A medflies that were individually exposed to a standard control diet or a treatment diet supplemented with naked dsRED2 dsRNA or dsRNA vectored in liposomes ('Lipo') that had been diluted 10:1 or 20:1 in molecular water. Discrete feeding conditions are represented on the x-axis. The x-axis is faceted according to the period that flies were exposed to a given feeding condition. Transformed relative expression of the dsRED2 gene is represented on the y-axis. The upper and lower hinges of boxplots represent the first and third quartiles of gene expression exhibited by three flies extracted from feeding regimes at a particular time point. Median gene expression is represented by a horizontal line and mean signal is represented by a dot. Controlling for the interacting covariate effects of time, there were significant differences in dsRED2 expression between flies reared on the different diet treatments (ANCOVA, $F(3,16) = 5.29$, $p < 0.01$).

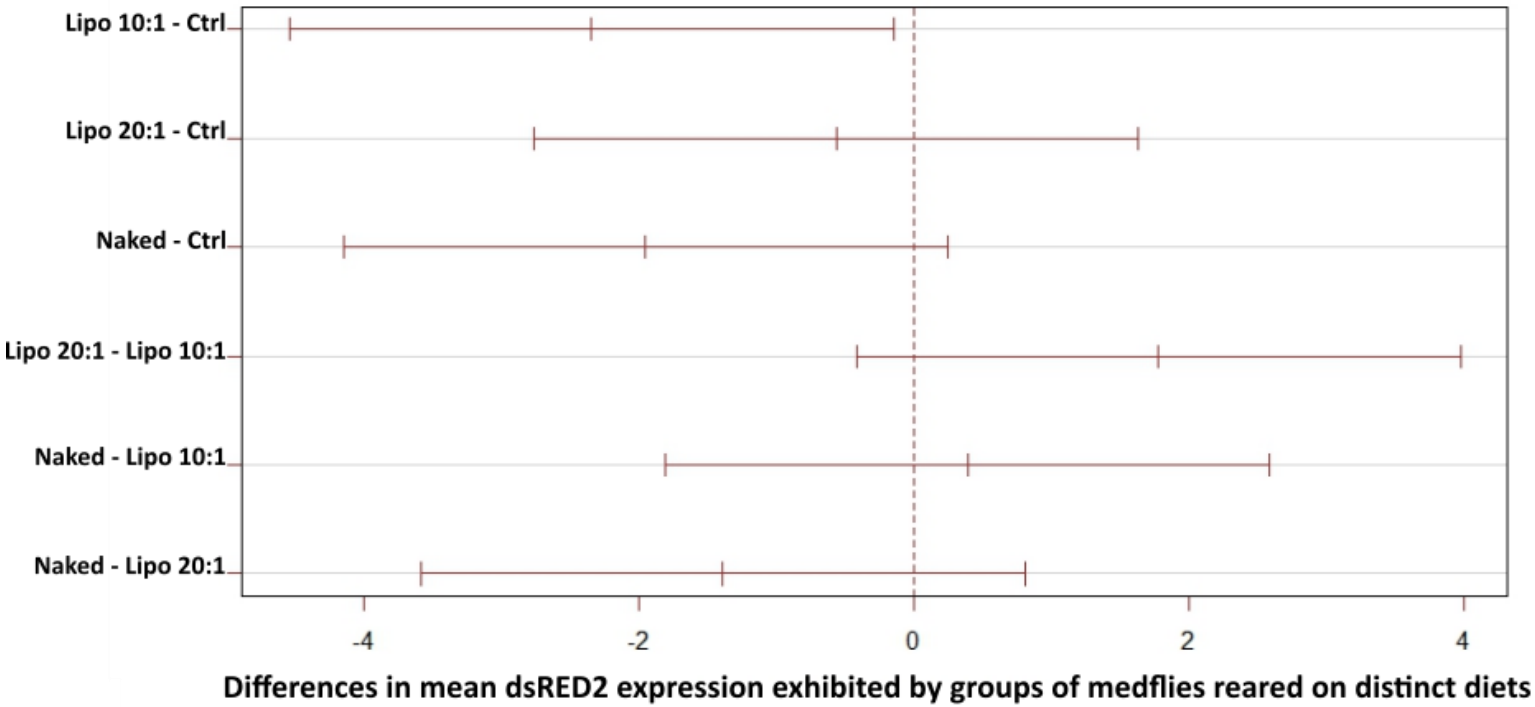

Figure 2.8 Post-hoc Tukey pairwise comparisons of mean dsRED2 expression exhibited by flies reared on diet supplemented either with naked dsRED2 dsRNA, dsRNA vectored in liposomes that had been diluted 10:1 or 20:1 in molecular water and flies reared on a standard control diet. Calculated at a 95% confidence interval, there was a significant difference in dsRED2 expression between flies reared on diet supplemented with dsRNA conjugated to liposomes that had been diluted 10:1 and flies reared on control diet. There were no significant differences in dsRED2 expression between flies reared on any of the remaining dietary regimes.

2.5) Discussion

Having reviewed studies that had implemented environmental RNA interference (eRNAi) in various insect models, I hypothesised that medflies suitable for use with the SIT could be produced with eRNAi technology (Darrington et al., 2017). My primary goal was to elucidate methods of dsRNA delivery that would provoke an eRNAi response from the medfly, which I investigated by subjecting OX3864A (Fu et al., 2007; Gong et al., 2005) medflies to various feeding regimes designed to elicit eRNAi of the fluorescent dsRED2 gene.

I fed OX3864A medflies (Fu et al., 2007; Gong et al., 2005) with HT115 *E. coli* bacteria (Kamath et al., 2001) that had been engineered to express dsRED2 dsRNA. I had predicted that consumption of dsRED expressing bacteria would silence expression of dsRED2 (Ganbaatar et al., 2017; Lopez et al., 2019; Taracena et al., 2015; Whyard et al., 2015), which I measured via fluorescence quantification (Waters, 2009) and RT-qPCR (Gu, Zhao, et al., 2019; Kyre et al., 2019; Shen et al., 2019; Tariq et al., 2019; Zhang et al., 2019). However, flies exposed to dsRED2 expressing HT115 bacteria did not exhibit a reduction in dsRED2 expression or fluorescence, despite evidence that they had been consuming the effector cells. Dissections of medfly guts revealed that consumed bacteria frequently accumulated in the anterior midgut within the peritrophic matrix. The peritrophic matrix is a lattice of protein, carbohydrate and chitin that protects the absorptive epithelial layer of the gut (Terra, 1990). There is evidence from several insect models, that invading microbes are contained by the peritrophic matrix limiting their potential for infection (Kuraishi et al., 2011; Rodgers et al., 2017; Weiss et al., 2014). It is plausible therefore that the medfly is eRNAi competent but that HT115 bacteria had been contained by the peritrophic matrix preventing access of dsRNA to the epithelium. To test this, one could produce peritrophic matrix-deficient flies as in Kuraishi et al. (2011), which should, if the peritrophic matrix is obviating eRNAi, be sensitive to environmental HT115 bacteria.

There is evidence that: 1) the response of insects to eRNAi can vary within species at different stages of life history (Coleman et al., 2015; Tian et al., 2015), and 2) eRNAi

recalcitrant species can be rendered competent if dsRNA effectors are vectored within liposomes (Taning et al., 2016; Whyard et al., 2009). To investigate this in the medfly, I exposed OX3864A adults and larvae to naked dsRED2 dsRNA molecules and to molecules that had been vectored in liposomes. As for the HT115 assay, I predicted that consumption of dsRED2 dsRNA would silence expression of the dsRED2 gene and I measured this potential effect with RT-qPCR (Gu, Zhao, et al., 2019; Kyre et al., 2019; Shen et al., 2019; Tariq et al., 2019; Zhang et al., 2019).

There was no evidence that consumption of either naked or liposomally-vectored dsRNA by OX3864A larvae, had effectively silenced expression of the dsRED2 gene. However, in these tests, the concentration of dsRED2 dsRNA within OX3864A diet was lower than had been fed to *Drosophila* larvae by Taning et al. (2016). It is possible therefore that medfly larvae could initiate eRNAi if dsRNA was supplied at a higher concentration, although this possibility is reduced by the finding that similar and lower concentrations of dsRNA to those used here in my experiments have elicited eRNAi from insect larvae of various other species (Sharath Chandra et al., 2018; Whyard et al., 2015). In addition, it is possible that residual RNAses contained in the larval diet had digested dsRNA, neutralising eRNAi. However, this seems unlikely as the diet had been boiled before effector molecules were added and no special measures were taken to ensure the absence of RNAses in diet that successfully elicited eRNAi from *Drosophila* larvae (Taning et al., 2016). Ideally, samples of diet from each regime could have been run on a gel to demonstrate that they contained constructs of the expected length, as it should be possible to extract and purify dsRNA from larval food with a gel extraction kit. Future repeats of this experiment could investigate whether larval diet could be melted in an autoclave, and if so, then dsRNAs could be added to the mixture as it cooled.

As larvae had not initiated eRNAi in response to dietary dsRNA, I increased the concentration of dsRNA fed to adults by a factor of ten. Adult flies fed dsRED2 dsRNA vectored in liposomes that had been diluted 10:1 in molecular water exhibited significantly lower expression of dsRED2 than flies that had been reared on standard diet. Flies did not exhibit reduced levels of dsRED2 expression subsequent to

exposure to naked dsRNA or dsRNA vectored in liposomes that had been diluted 20:1 in molecular water. I investigated various liposomal dilutions as it has been suggested that dilution factor can drastically affect dsRNA delivery (Dr. Wim Reidt, ThermoFisher, pers. comm.). It is plausible therefore that a 10:1 liposomal dilution can effectively elicit eRNAi from the medfly while a 20:1 dilution is ineffective. Future work should focus on liposomes that have been diluted 10:1 but perhaps focusing on a different target gene. dsRED2 was selected as the eRNAi target for these tests as it had been hypothesised that knockdown could be easily quantified via loss of the fluorescent phenotype. However, the ultimate goal of this study was to develop genetic tools for insect control, so follow up experiments should target genes that are predicted to cause mortality (Knorr et al., 2018; Niu et al., 2019; Shen et al., 2019; Tariq et al., 2019). Not only would such a system be directly applicable for insect control but mortality provides a more easily quantifiable phenotype for such tests.

In conclusion, I demonstrated that OX3864A adults did not express eRNAi when fed HT115 bacteria. Furthermore, OX3864A larvae did not express eRNAi when fed naked dsRNA or dsRNA vectored within liposomes, although these results may have been overturned if the concentration of dsRNA within the larval diet had been increased. However, adults appeared to express eRNAi when fed dsRNA vectored in liposomes that had been diluted 10:1. Further investigations into eRNAi of the medfly should focus on this technique and upon logistical and operational considerations of this potential method for medfly control.

2.6) Supplementary materials

2.1) RT-qPCR probes and primers

1) dsRED2f (5'-gctccaaggtgtacgtgaacg-3'), dsRED2r-(5'-tgcaagttcatcacgcgc-3'), dsRED2 probe (5' HEX–agaagctgtccttccccgagggct-BHQ1 3').

2) L27f (5'-ccgttattgatttggtgcaa-3'), L27r (5'-gctgtttaggcaaatgtcca-3'), L27 probe (5' TAMRA-cggcccaacaacttgtagtaaccga-BHQ2 3').

3) Rpl13af (5'-acgtttgtccagtgaagtcg-3'), Rpl13ar (5'-tagccttacgtttccgttcc-3'), Rpl13a probe (5' FAM-tcttaacgacatcctggtaatgccagc-BHQ2 3').

2.2) Estimating noise introduced to eRNAi gene expression analyses by dsRNA effector molecules

It was expected that samples of OX3864A RNA collected during eRNAi experiments would contain some residual dsRED2 dsRNA effectors. Although the Revertaid CDNA synthesis kit (ThermoFisher) utilises oligo(dT)18 primers designed to bind only the poly-A tail of mRNA, this system may not be 100% efficient (Professor Saskia Hogenhout, pers. comm.). It was therefore hypothesised that residual dsRED2 dsRNA contained in eRNAi samples had been reverse transcribed, introducing noise to the subsequent RT-qPCR analyses. To test if dsRNA was susceptible to reverse transcription, samples of *in vitro* synthesised dsRNA and RNA extracted from HT115 bacteria were subjected to RT-qPCR. The Revertaid protocol includes a 65˚C relaxation step during which the complementary strands of dsRNA would separate, making them susceptible to reverse transcription. CDNA synthesis was therefore performed both with and without a 65˚C relaxing step, to elucidate whether this stage of the protocol was a potential source of noise in gene expression data.

2.2.1) Testing if dsRNA extracted from HT115 bacteria was susceptible to reverse transcription with a Revertaid CDNA kit

HT115 bacteria were cultured, induced and nucleic acids were extracted, according to the HT115 eRNAi protocol. As for the eRNAi assay, 500ng of RNA extracted from

HT115 bacteria was processed with a Revertaid kit (ThermoFisher). RT-qPCR of bacterial CDNA that had been generated both with and without a 65˚C relaxing step was carried out according to the standard eRNAi protocol. HT115 RNA produced a measurable dsRED2 signal, both when reverse transcribed at 42˚C and 65˚C but the medfly reference genes (Rpl13a and L27) did not amplify (Figure 2.9). CDNA generated without a 65˚C relaxing step transmitted a stronger dsRED2 signal (a lower cycling threshold (Ct)) than CDNA treated at 65˚C. However, there was less variation in the dsRED2 signal transmitted by replicates of CDNA produced with a 65˚C relaxing step. These data serve as evidence that the relative expression of dsRED2 measured from OX3864A RNA may have been confounded by an aberrant dsRED2 signal originating from residual HT115 RNA. It is noteworthy however that the quantity of HT115 RNA analysed here was far greater than would have been present in the OX3864A eRNAi samples, meaning that the RT-qPCR analyses may not have been confounded by noise.

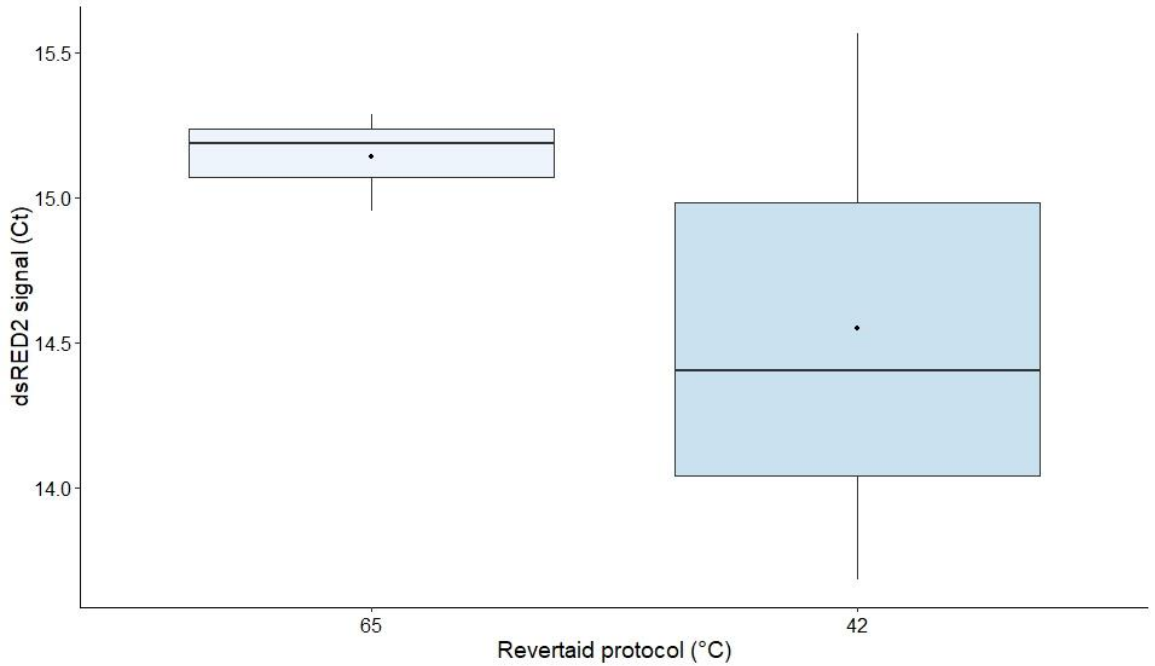

Figure 2.9 The dsRED2 signal that was detected via RT-qPCR of RNA extracted from HT115 bacteria. The cycling threshold (Ct) at which a dsRED2 signal was detected is represented on the y-axis. Samples of HT115 RNA treated with distinct versions of the Revertaid protocol are represented on the x-axis. The upper and lower hinges of boxplots represent the first and third quartiles of gene expression exhibited by three samples of HT115 RNA. Median dsRED2 signal is represented by a horizontal line and mean signal is represented by a dot. HT115 RNA is susceptible to reverse transcription with a Revertaid kit even in the absence of a 65˚C relaxing step. There was less variation in dsRED2 signal obtained from biological replicates of HT115 RNA that were treated with a 65˚C relaxing step, than RNA that was not exposed to this step.

2.2.2) Testing if *in vitro* synthesised dsRNA was susceptible to reverse transcription with a Revertaid CDNA kit

Serial dilutions of *in vitro* synthesised dsRNA (20ng, 4ng, 0.8ng and 0.16ng) were processed with a Revertaid kit, both with and without a 65˚C relaxing step. RT-qPCR of CDNA that had been generated both with and without a 65˚C relaxing step was carried out according to the standard eRNAi protocol. dsRED2 dsRNA produced a measurable signal both when treated at 42˚C and 65˚C but the medfly reference genes (Rpl13a and L27) did not amplify (Figure 2.10). When dsRNA had been treated with a 65˚C relaxing step, cycling threshold (Ct) and dsRNA quantity exhibited a consistent, inverse linear relationship. dsRNA that had not been treated with a 65˚C relaxing step produced a detectable dsRED2 signal but the relationship between dsRNA quantity and Ct was not absolutely linear. These data serve as evidence that the relative expression of dsRED2 measured from OX3864A RNA may have been confounded by a dsRED2 signal that originated from dsRNA effectors.

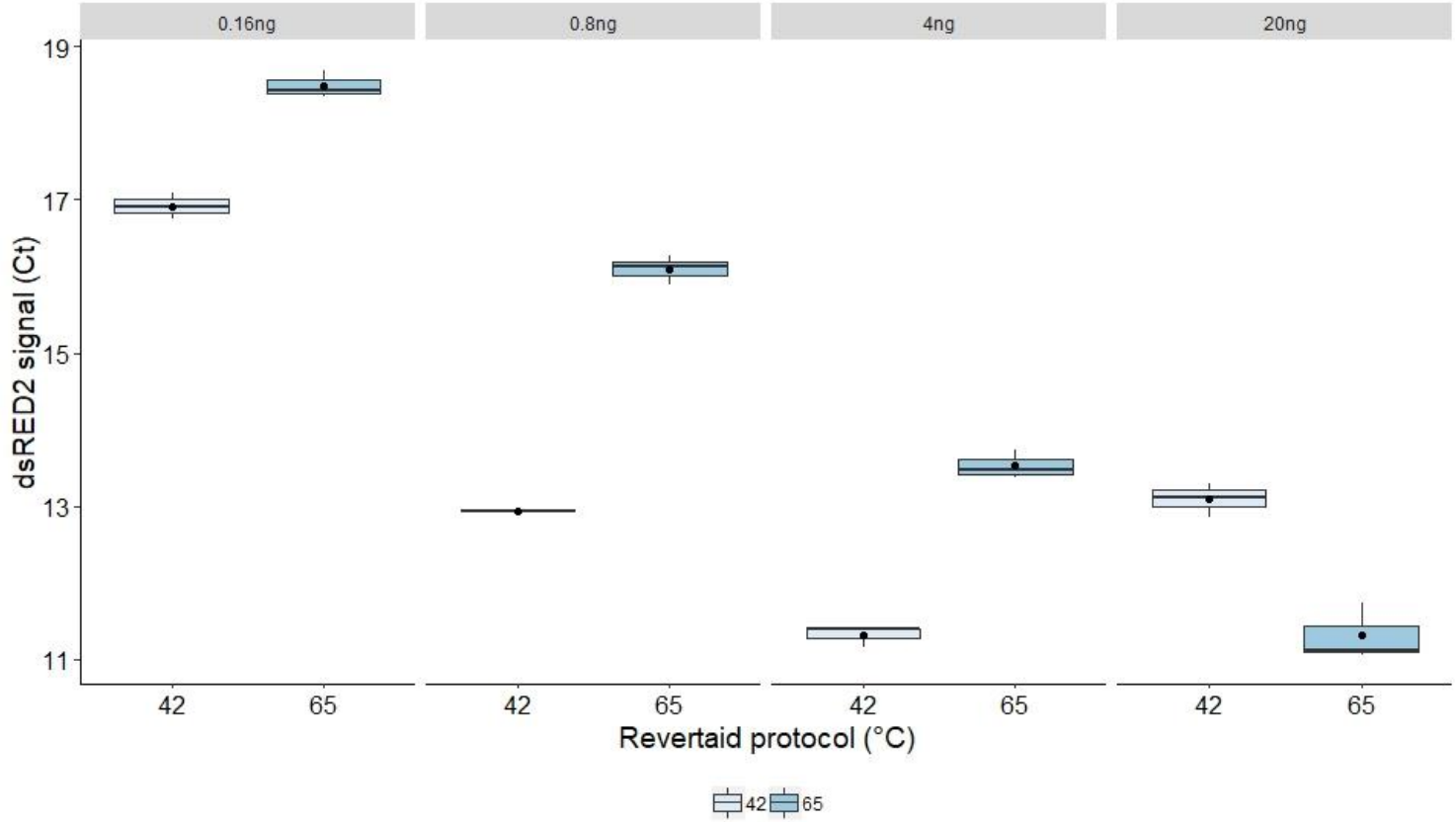

Figure 2.10 The dsRED2 signal that was detected via RT-qPCR of dsRED2 dsRNA. The cycling threshold (Ct) at which a dsRED2 signal was detected is represented on the y-axis. Discrete Revertaid protocols are represented on the x-axis. The x-axis is faceted according to the quantity of dsRNA that was subjected to reverse transcription. The upper and lower hinges of boxplots represent the first and third quartiles of gene expression exhibited by three samples of dsRNA. Median dsRED2 signal is represented by a horizontal line and mean signal is represented by a dot. dsRNA is susceptible to reverse transcription with a Revertaid kit even in the absence of a 65˚C relaxing step. The dsRED2 signal is more consistent when dsRNA is treated with a 65˚C relaxing step.

2.3) Feeding wild-type Toliman flies with dsRED2 dsRNA to estimate noise introduced to eRNAi gene expression analyses

When analysed according to the eRNAi gene expression protocol, it was established that a dsRED2 signal could be detected from both *in vitro* synthesised dsRED2 dsRNA and from RNA extracted from HT115 bacteria. Although these materials were capable of generating noise it was possible that this had not confounded the eRNAi gene expression data, as they had only been present in OX3864A RNA in trace amounts. It was hypotheised that any erroneous dsRED2 signalling introduced to OX3864A RNA by ingested dsRED2 dsRNA could be quantified if the eRNAi assays were repeated in wild-type Toliman medflies. This hypothesis relied on the fundamental assumption that a dsRED2 signal detected in Toliman RNA must be emanating from ingested dsRNA, as Toliman flies do not possess an endogenous dsRED2 gene. The HT115 and larval eRNAi assays were therefore repeated in both OX3864A and Toliman flies to gain an estimate of noise caused by dsRRED2 dsRNA effectors.

2.3.1) Feeding wild-type medflies with HT115 bacteria to quantify dsRED2 noise generated by effector molecules during eRNAi experiments

Wild-type Toliman flies and OX3864A strain medflies were supplied with dsRED2 expressing HT115 bacteria according to the HT115 eRNAi feeding protocol. After 96 hours of feeding, three groups of flies (n=3) representing each medfly strain were subjected to RT-qPCR analysis of the dsRED2 gene. It was predicted that residual dsRNA contained in HT115 RNA would generate a measurable signal of dsRED2 expression in the Toliman samples. After four days of feeding, RNA extracted from Toliman flies did not generate a detectable dsRED2 signal. Therefore, the quantities of residual dsRNA present in OX3864A RNA subsequent to the HT115 eRNAi assay, were not likely to have been sufficient to transmit a dsRED2 signal that confounded the overall gene expression analysis.

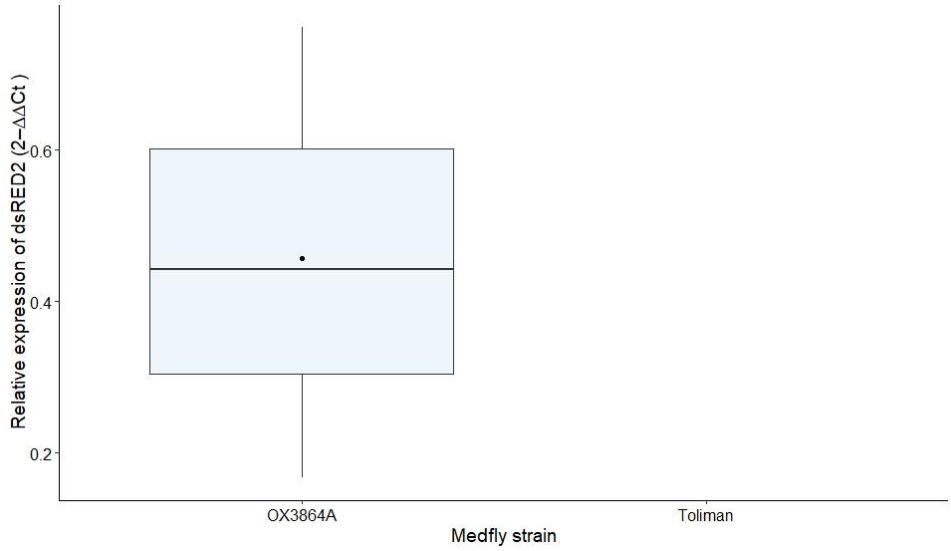

Figure 2.11 Relative expression of dsRED2 exhibited by OX3864A and Toliman medflies that had been supplied with diet supplemented with HT115 bacteria for four days. Medfly strains are represented on the x-axis. Relative dsRED2 expression (2–ΔΔCt) is represented on the y-axis. The upper and lower hinges of boxplots represent the first and third quartiles of dsRED2 expression exhibited by three groups of flies (n=3). Median dsRED2 signal is represented by a horizontal line and mean signal is represented by a dot. Wild-type Toliman medflies that had been exposed to dsRED2 expressing HT115 bacteria for four days did not transmit a detectable signal of dsRED2 expression

2.3.2) Feeding wild-type larvae with naked dsRED2 dsRNA and dsRNA vectored in liposomes to quantify noise generated during eRNAi experiments

Both wild-type and transgenic OX3864A medfly larvae were exposed to diet containing dsRED2 dsRNA for two days. As for the eRNAi assay, larvae were supplied with either naked dsRNA or dsRNA that had been vectored in two discrete dilutions of lipofectamine liposomes (ThermoFisher). After 48 hours of feeding, three groups of larvae (n=3) representing each medfly strain were subjected to RT-qPCR analysis of the dsRED2 gene. It was predicted that residual dsRNA contained in Toliman RNA would generate a measurable signal of dsRED2 expression. However, Toliman RNA did not generate a detectable dsRED2 signal. Therefore, dsRNA effectors present in OX3864A RNA subsequent to the larval eRNAi assay, were not likely to have been sufficient to transmit a confounding dsRED2 signal.

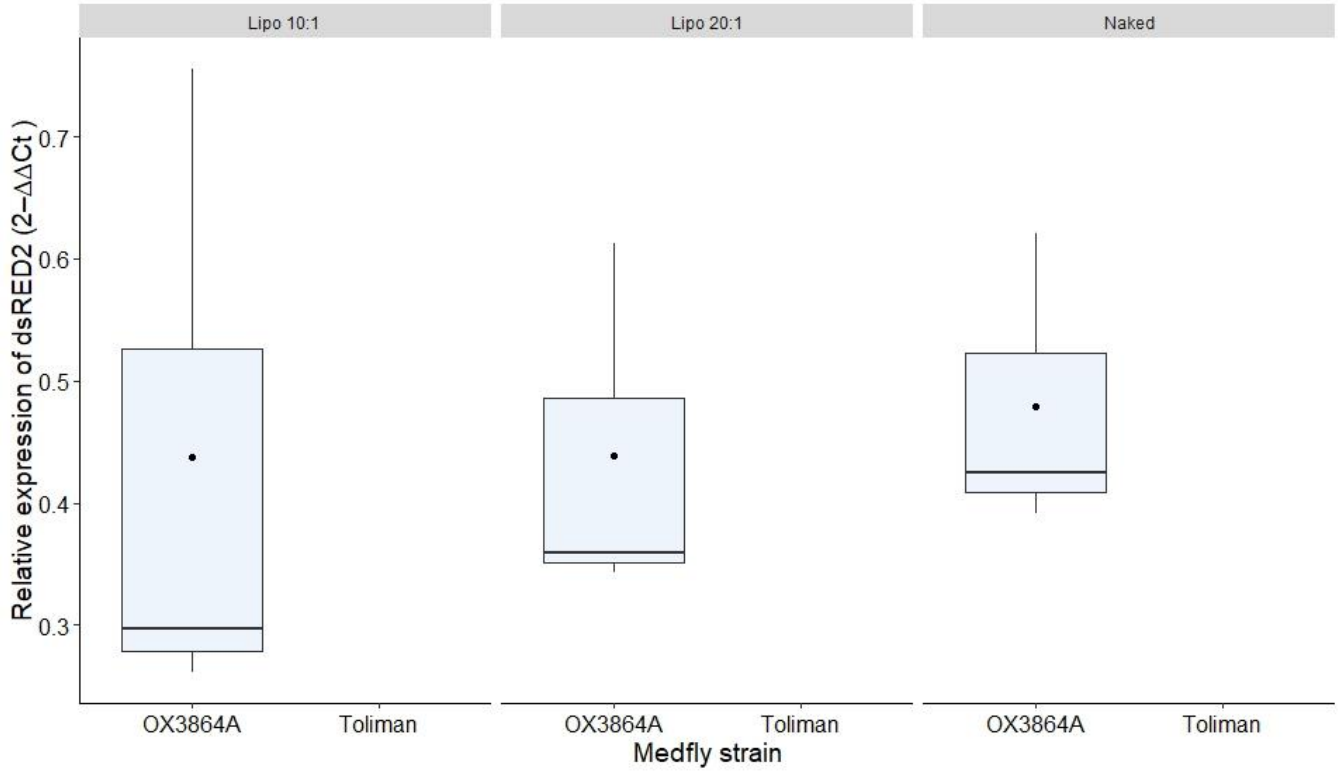

Figure 2.12 Relative expression of dsRED2 exhibited by OX3864A and Toliman medflies that had been supplied with diet supplemented with naked dsRNA and dsRNA vectored in discrete dilutions of liposomes. Medfly strains are represented on the x-axis. Relative dsRED2 expression (2–ΔΔCt) is represented on the y-axis. The upper and lower hinges of boxplots represent the first and third quartiles of dsRED2 expression exhibited by three groups of larvae (n=3). Median dsRED2 signal is represented by a horizontal line and mean signal is represented by a dot. Wild-type Toliman medflies that had been exposed to naked or liposomally vectored dsRED2 dsRNA for 48hrs did not transmit a detectable signal of dsRED2 expression.

2.4) The corrected volumes of FEEBs added to DAILYs over the course of HT115 eRNAi experiments

Cultures of treatment (dsRED2) and +ve-control (GFP) broth (feeding broth inoculums; FEEBs) were prepared at the beginning of the experiment to provide a continuous pool of bacteria to those regimes. Each day during the experiment, daily feeding broths (DAILYs) to be administered to the treatment and +ve-control regimes were prepared by inoculating 10ml of broth with a volume of the stored FEEBs. The OD600 of dsRED2 expressing bacteria on day 1 of the RT-qPCR assay was used to standardise the volumes of FEEBs added to remaining DAILYs in all feeding regimes and experimental conditions.

Table 2.1) The volumes of HT115 FBIs added to DFBs in eRNAi experiments.

FBI	Experiment	Day	OD600	Volume FBI added to DFB (µl)
dsRED2	RT-qPCR	1	1.144	100
dsRED2	RT-qPCR	2	1.224	93.46
dsRED2	RT-qPCR	3	1.193	95.89
dsRED2	RT-qPCR	4	1.171	97.69
GFP	RT-qPCR	1	1.204	95.02
GFP	RT-qPCR	2	1.19	96.13
GFP	RT-qPCR	3	1.183	96.7
GFP	RT-qPCR	4	1.184	96.62
dsRED2	Fluorescence quantification	1	1.128	101.42
dsRED2	Fluorescence quantification	2	1.155	99.05
dsRED2	Fluorescence quantification	3	1.158	98.79
dsRED2	Fluorescence quantification	4	1.064	107.52

2.5) Nucleotide sequences of *in vitro* synthesised dsRNAs used for eRNAi experiments

dsRED2 (612bp) -

atggcctcctccgagaacgtcatcaccgagttcatgcgcttcaaggtgcgcatggagggcaccgtgaacggccacgagttcg
agatcgagggcgagggcgagggccgcccctacgagggccacaacaccgtgaagctgaaggtgaccaagggcggccccctg
cccttcgcctgggacatcctgtcccccagttccagtacggctccaaggtgtacgtgaagcaccccgccgacatccccgacta
caagaagctgtccttccccgagggcttcaagtgggagcgcgtgatgaacttcgaggacggcggcgtggcgaccgtgacccag
gactcctccctgcaggacggctgcttcatctacaaggtgaagttcatcggcgtgaacttcccctccgacggccccgtgatgcag
aagaagaccatgggctgggaggcctccaccgagcgcctgtacccccgcgacggcgtgctgaagggcgagacccacaaggc
cctgaagctgaaggacggcggccactacctggtggagttcaagtccatctacatggccaagaagcccgtgcagctgcccggc
tactactacgtggacgccaagctggacatcacctcccacaacgaggactacaccatcgtggagcagtacgagcgcaccgag
ggccgccaccacctgttcctg

GFP (710bp) –

atgagtaaaggagaagaacttttcactggagttgtcccaattcttgttgaattagatggtgatgttaatgggcacaaattttctg
tcagtggagagggtgaaggtgatgcaacatacggaaaacttacccttaaatttatttgcactactggaaaactacctgttccat
ggccaacacttgtcactactttctcttatggtgttcaatgcttttcccgttatccggatcatatgaaacggcatgactttttcaag
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tcgagtacaactataactcacacaatgtatacatcacggcagacaaacaaaagaatggaatcaaagctaacttcaaaattcg
ccacaacattgaagatggatccgttcaactagcagaccattatcaacaaaatactccaattggcgatggccctgtccttttacc
agacaaccattacctgtcgacacaatctgccctttcgaaagatcccaacgaaaagcgtgaccacatggtccttcttgagtttgt
aactgctgctgggattacacatggcatggatgagctctacaaataa

2.6) HT115 expression vectors

HT115 bacteria were engineered at the *Caenorhabditis* Genetics Centre, Minnesota University and transformed with appropriate expression vectors at Oxitec Ltd.

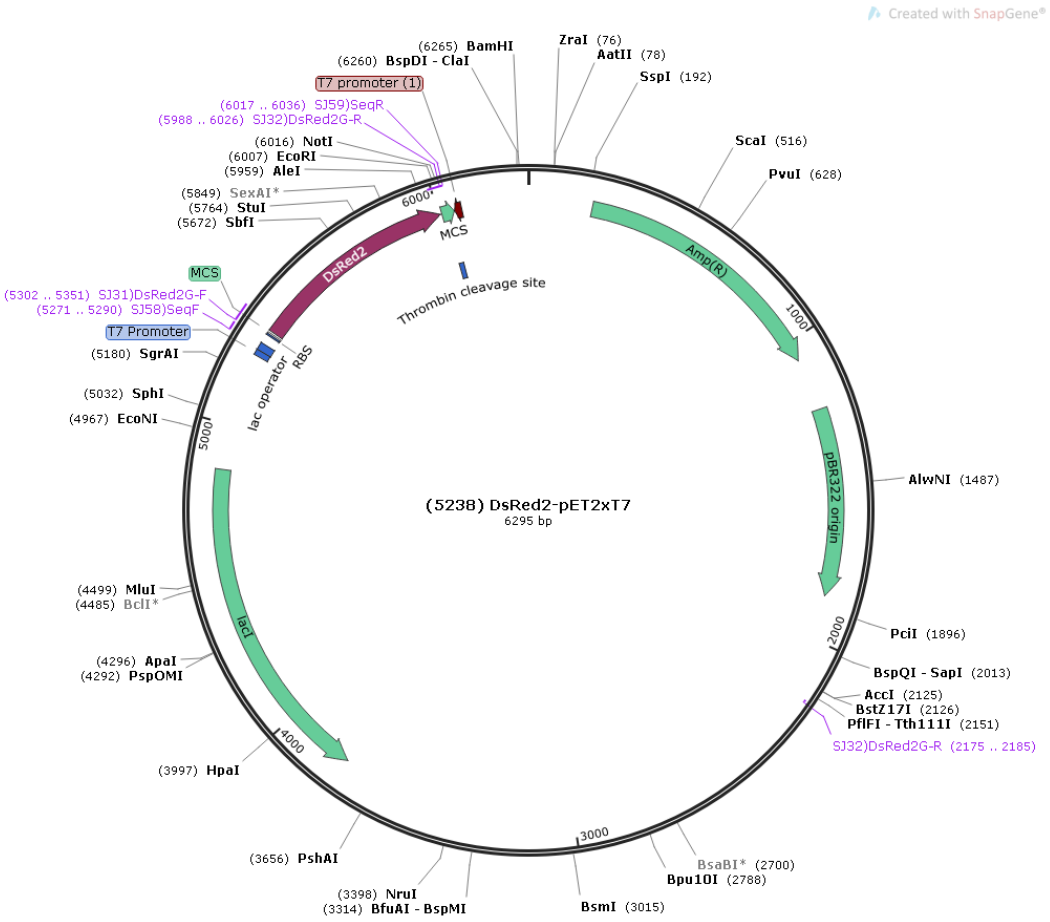

Figure 2.13 Map of the dsRED2 expression vector. The pET2xT7 dsRED2 expression vector was constructed in house at Oxitec Ltd.

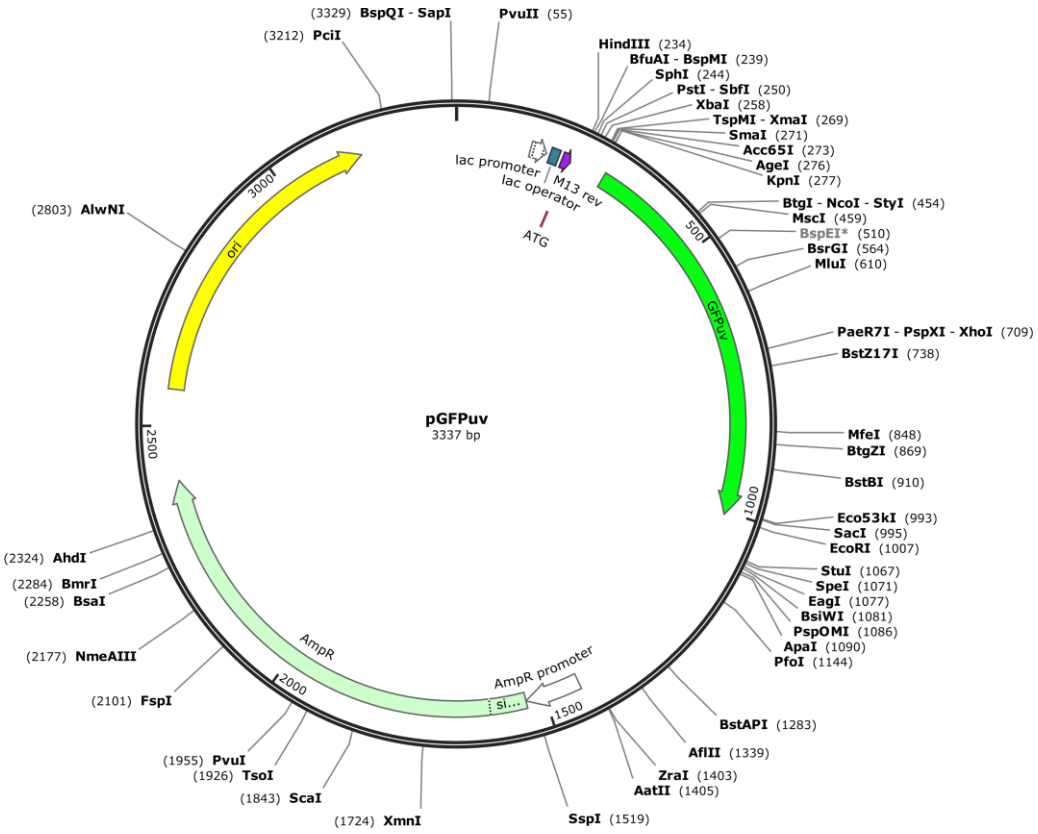

Figure 2.14 Map of the GFP expression vector. The pET6xHN GFP expression vector was purchased from Takara Clontech (Clontech, 2019).

2.7 References

Ahmed, M. (2015). *Status of citrus ipm in the Southern Mediterranean basin, Morocco, North Africa*.

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

Argov, Y., & Gazit, Y. (2008). Biological control of the Mediterranean fruit fly in Israel: Introduction and establishment of natural enemies. *Biological Control, 46*(3), 502-507. doi:10.1016/j.biocontrol.2008.04.021

Asadi, R., Elaini, R., Lacroix, R., Ant, T., Collado, A., Finnegan, L., . . . Koukidou, M. (2019). Preventative releases of self-limiting *Ceratitis capitata* provide pest suppression and protect fruit quality in outdoor netted cages. *International Journal of Pest Management*, 1-12. doi:10.1080/09670874.2019.1601293

Barry, J. D., Blessinger, T., & Morse, J. G. (2004). Recapture of sterile Mediterranean fruit flies *(Diptera: Tephritidae)* in California's preventative release program. *Journal of Economic Entomology, 97*(5), 1554-1562. doi:10.1603/0022-0493-97.5.1554

Baum, J. A., & Roberts, J. K. (2014). Chapter five - progress towards RNAi-mediated insect pest management. In S. D. Tarlochan & S. G. Sarjeet (Eds.), *Advances in insect physiology* (Vol. Volume 47, pp. 249-295): Academic Press.

Behar, A., Jurkevitch, E., & Yuval, B. (2008). Bringing back the fruit into fruit fly–bacteria interactions. *Molecular Ecology, 17*(5), 1375-1386. doi:10.1111/j.1365-294X.2008.03674.x

Bento, F. M. M., Marques, R. N., Campana, F. B., Demétrio, C. G. B., Leandro, R. A., Parra, J. R. P., & Figueira, A. (2019). Gene silencing by RNAi via oral delivery of dsRNA by bacteria in the South American tomato pinworm, *Tuta absoluta*. *Pest Management Science*. doi:10.1002/ps.5513

Briceño, R. D., & Eberhard, W. G. (2003). Decisions during courtship by male and female medflies (*Diptera, Tephritidae*): Correlated changes in male behavior and female acceptance criteria in mass-reared flies. *Florida Entomologist, 85*(1), 14-31.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., . . . Wittwer, C. T. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry, 55*(4), 611-622. doi:10.1373/clinchem.2008.112797

CABI. (2019). Invasive species compendium. Retrieved from https://www.cabi.org/isc/datasheet/12367

Caccia, S., Astarita, F., Barra, E., Di Lelio, I., Varricchio, P., & Pennacchio, F. (2019). Enhancement of *Bacillus thuringiensis* toxicity by feeding *Spodoptera littoralis* larvae with bacteria expressing immune suppressive dsRNA. *Journal of Pest Science*. doi:10.1007/s10340-019-01140-6

Carmell, M. A., Xuan, Z., Zhang, M. Q., & Hannon, G. J. (2002). The argonaute family: Tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. *Genes and Development, 16*(21), 2733-2742. doi:10.1101/gad.1026102

Clontech, T. (2019). Takara. Retrieved from https://go.takarabio.com/EU

Coleman, A. D., Wouters, R. H. M., Mugford, S. T., & Hogenhout, S. A. (2015). Persistence and transgenerational effect of plant-mediated RNAi in aphids. *Journal of Experimental Botany, 66*(2), 541-548. doi:10.1093/jxb/eru450

Couso-Ferrer, F., Arouri, R., Beroiz, B., Perera, N., Cervera, A., Navarro-Llopis, V., . . . Ortego, F. (2011). Cross-resistance to insecticides in a malathion-resistant strain of *Ceratitis capitata (Diptera: Tephritidae)*. *Journal of Economic Entomology, 104*(4), 1349-1356. doi:10.1603/ec11082

Darrington, M., Dalmay, T., Morrison, N. I., & Chapman, T. (2017). Implementing the sterile insect technique with RNA interference – a review. *Entomologia experimentalis et applicata, 164*(3), 155-175. doi:10.1111/eea.12575

Ding, S.-W. (2010). RNA-based antiviral immunity. *Nature Reviews Immunology, 10*(9), 632-644. doi:10.1038/nri2824

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., & Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature, 411*(6836), 494-498. doi:10.1038/35078107

Enkerlin, W., & Mumford, J. (1997). Economic evaluation of three alternative methods for control of the Mediterranean fruit fly (*Diptera: Tephritidae*) in Israel, palestinian territories, and Jordan. *Journal of Economic Entomology, 90*(5), 1066-1072. doi:10.1093/jee/90.5.1066

Enkerlin, W. R., Gutiérrez Ruelas, J. M., Pantaleon, R., Soto Litera, C., Villaseñor Cortés, A., Zavala López, J. L., . . . Hendrichs, J. (2017). The Moscamed regional programme: Review of a success story of area-wide sterile insect technique application. *Entomologia experimentalis et applicata, 164*(3), 188-203. doi:10.1111/eea.12611

Fire, A., Xu, S. Q., Montgomery, M. K., Kostas, S. A., Driver, S. E., & Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature, 391*(6669), 806-811. doi:10.1038/35888

Fu, G., Stainton, K., J Epton, M., Gong, P., Jin, L., Condon, G., . . . Alphey, L. (2007). Female-specific insect lethality engineered using alternative splicing. *25*, 353-357. doi:10.1038/nbt1283

Gabrieli, P., Scolari, F., Di Cosimo, A., Savini, G., Fumagalli, M., Gomulski, L. M., . . . Gasperi, G. (2016). Sperm-less males modulate female behaviour in *Ceratitis capitata (Diptera: Tephritidae)*. *Insect Biochem Mol Biol, 79*, 13-26. doi:10.1016/j.ibmb.2016.10.002

Ganbaatar, O., Cao, B., Zhang, Y., Bao, D., Bao, W., & Wuriyanghan, H. (2017). Knockdown of *Mythimna separata* chitinase genes via bacterial expression and oral delivery of RNAi effectors. *BMC Biotechnol, 17*(1), 9. doi:10.1186/s12896-017-0328-7

Gasperi, G., Guglielmino, C. R., Malacrida, A. R., & Milani, R. (1991). Genetic variability and gene flow in geographical populations of *Ceratitis capitata* (wied.) (medfly). *Heredity, 67*(3), 347-356. doi:10.1038/hdy.1991.98

Genolution. (2019). Genolution inc. Retrieved from http://genolution.cafe24.com/default/

Gong, P., Epton, M. J., Fu, G. L., Scaife, S., Hiscox, A., Condon, K. C., . . . Alphey, L. (2005). A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly. *Nature Biotechnology, 23*(4), 453-456. doi:10.1038/nbt1071

Gonzalez, J., & Troncoso, P. (2007, 2007//). *The fruit fly exclusion programme in Chile*. Paper presented at the Area-Wide Control of Insect Pests, Dordrecht.

Gu, X., Li, Z., Su, Y., Zhao, Y., & Liu, L. (2019). Imaginal disc growth factor 4 regulates development and temperature adaptation in *Bactrocera dorsalis*. *Scientific Reports, 9*(1), 931. doi:10.1038/s41598-018-37414-9

Gu, X., Zhao, Y., Su, Y., Wu, J., Wang, Z., Hu, J., . . . Li, Z. (2019). A transcriptional and functional analysis of heat hardening in two invasive fruit fly species, *Bactrocera dorsalis* and *Bactrocera correcta*. *Evolutionary Applications, 12*(6), 1147-1163. doi:10.1111/eva.12793

Guerfali, M. M., Parker, A., Fadhl, S., Hemdane, H., Raies, A., & Chevrier, C. (2011). Fitness and reproductive potential of irradiated mass-reared Mediterranean fruit fly males *Ceratitis capitata* (*Diptera: Tephritidae*): Lowering radiation doses. *Florida Entomologist, 94*(4), 1042-1050.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., & Vandesompele, J. (2007). Qbase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biology, 8*(2), R19-R19. doi:10.1186/gb-2007-8-2-r19

Hooper, G. H. S. (1972). Sterilization of the Mediterranean fruit fly with gamma radiation: Effect on male competitiveness and change in fertility of females alternately mated with irradiated and untreated males. *Journal of Economic Entomology, 65*(1), 1.

Huvenne, H., & Smagghe, G. (2010). Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: A review. *Journal of Insect Physiology, 56*(3), 227-235. doi:10.1016/j.jinsphys.2009.10.004

Kamath, R. S., Martinez-Campos, M., Zipperlen, P., Fraser, A. G., & Ahringer, J. (2001). Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. *Genome Biology, 2*(1).

Knipling, E. F. (1955). Possibilities of insect control or eradication through the use of sexually sterile males. *Journal of Economic Entomology, 48*(4), 459.

Knorr, E., Fishilevich, E., Tenbusch, L., Frey, M. L. F., Rangasamy, M., Billion, A., . . . Narva, K. E. (2018). Gene silencing in *Tribolium castaneum* as a tool for the targeted identification of candidate RNAi targets in crop pests. *Scientific Reports, 8*(1), 2061. doi:10.1038/s41598-018-20416-y

Kuraishi, T., Binggeli, O., Opota, O., Buchon, N., & Lemaitre, B. (2011). Genetic evidence for a protective role of the peritrophic matrix against intestinal bacterial infection in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences, 108*(38), 15966. doi:10.1073/pnas.1105994108

Kyre, B. R., Rodrigues, T. B., & Rieske, L. K. (2019). RNA interference and validation of reference genes for gene expression analyses using qPCR in southern pine beetle, *Dendroctonus frontalis*. *Scientific Reports, 9*(1), 5640. doi:10.1038/s41598-019-42072-6

Leftwich, P. T., Koukidou, M., Rempoulakis, P., Gong, H.-F., Zacharopoulou, A., Fu, G., . . . Alphey, L. (2014). Genetic elimination of field-cage populations of Mediterranean fruit flies. *Proceedings of the Royal Society B-Biological Sciences, 281*(1792). doi:10.1098/rspb.2014.1372

Li, J., Li, X., Bai, R., Shi, Y., Tang, Q., An, S., . . . Yan, F. (2015). RNA interference of the p450 cyp6cm1 gene has different efficacy in b and q biotypes of *Bemisia tabaci*. *Pest Management Science, 71*(8), 1175-1181. doi:10.1002/ps.3903

Li, X., Zhang, M., & Zhang, H. (2011). RNA interference of four genes in adult *Bactrocera dorsalis* by feeding their dsRNAs. *Plos One, 6*(3). doi:10.1371/journal.pone.0017788

Liquido, N. J., Shinoda, L. A., & Cunningham, R. T. (1991). *Host plants of the Mediterranean fruit fly (Diptera: Tephritidae): An annotated world review*: Entomological Society of America.

Lopez, S. B. G., Guimarães-Ribeiro, V., Rodriguez, J. V. G., Dorand, F. A. P. S., Salles, T. S., Sá-Guimarães, T. E., . . . Moreira, M. F. (2019). RNAi-based bioinsecticide for *Aedes* mosquito control. *Scientific Reports, 9*(1), 4038. doi:10.1038/s41598-019-39666-5

Lysandrou, M. (2009). Fruit flies in the Mediterranean and arab world: How serious a threat are they and how can we minimize their impact. *Arab Journal of Plant Protection, 27*(2), 236-239.

Malacrida, A. R., Gomulski, L. M., Bonizzoni, M., Bertin, S., Gasperi, G., & Guglielmino, C. R. (2006). Globalization and fruitfly invasion and expansion: The medfly paradigm. *Genetica, 131*(1), 1. doi:10.1007/s10709-006-9117-2

Mangan, R. L., & Moreno, D. S. (2014). Development of bait stations for fruit fly population suppression. *Journal of Economic Entomology, 100*(2), 440-450. doi:10.1093/jee/100.2.440

Mangel, M., & Ludwig, D. (1992). Definition and evaluation of the fitness of behavioral and developmental programs. *Annual Review of Ecology and Systematics, 23*, 507-536.

Mangiafico, S. S. (2016). Summary and analysis of extension program evaluation in r.

Maniataki, E., & Mourelatos, Z. (2005). A human, atp-independent, RISC assembly machine fueled by pre-miRNA. *Genes & Development, 19*(24), 2979-2990. doi:10.1101/gad.1384005

Manoukis, N. C. (2016). To catch a fly: Landing and capture of *Ceratitis capitata* in a jackson trap with and without an insecticide. *Plos One, 11*(2), e0149869. doi:10.1371/journal.pone.0149869

Marec, F., Neven, L. G., Robinson, A. S., Vreysen, M., Goldsmith, M. R., Nagaraju, J., & Franz, G. (2005). Development of genetic sexing strains in *Lepidoptera*: From traditional to transgenic approaches. *J Econ Entomol, 98*(2), 248-259. doi:10.1603/0022-0493-98.2.248

Mazih, A., Rachid, E., H, D., S, S., M, E., M, A., . . . K, L. (2016). *Integrated pest management if key insect pests in organic citrus orchard: Emphasising Mediterranean fruit fly and California red scale*.

Nash, W., Mohorianu, I., & Chapman, T. (2018). Mate choice and gene expression signatures associated with nutritional adaptation in the medfly (*Ceratitis capitata*). *bioRxiv*, 362210. doi:10.1101/362210

Niu, J., Yang, W. J., Tian, Y., Fan, J. Y., Ye, C., Shang, F., . . . Wang, J. J. (2019). Topical dsRNA delivery induces gene silencing and mortality in the pea aphid. *Pest Manag Sci*. doi:10.1002/ps.5457

Ordax, M., Piquer-Salcedo, J. E., Santander, R. D., Sabater-Muñoz, B., Biosca, E. G., López, M. M., & Marco-Noales, E. (2015). Medfly *Ceratitis capitata* as potential vector for fire blight pathogen *Erwinia amylovora*: Survival and transmission. *Plos One, 10*(5), e0127560. doi:10.1371/journal.pone.0127560

Oxitec. (2019). Oxitec ltd. Retrieved from https://www.oxitec.com/

Pane, A., Salvemini, M., Bovi, P. D., Polito, C., & Saccone, G. (2002). The transformer gene in *Ceratitis capitata* provides a genetic basis for selecting and remembering the sexual fate. *Development, 129*(15), 3715-3725.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research, 29*(9), e45-e45. doi:10.1093/nar/29.9.e45

Rand, T. A., Petersen, S., Du, F. H., & Wang, X. D. (2005). Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Cell, 123*(4), 621-629. doi:10.1016/j.cell.2005.10.020

Rendon, P., McInnis, D., Lance, D., & Stewart, J. (2004). Medfly (*Diptera: Tephritidae*) genetic sexing: Large-scale field comparison of males-only and bisexual sterile fly releases in Guatemala. *J Econ Entomol, 97*(5), 1547-1553. doi:10.1603/0022-0493-97.5.1547

Rodgers, F. H., Gendrin, M., Wyer, C. A. S., & Christophides, G. K. (2017). Microbiota-induced peritrophic matrix regulates midgut homeostasis and prevents systemic infection of malaria vector mosquitoes. *PLoS Pathogens, 13*(5), e1006391. doi:10.1371/journal.ppat.1006391

Salvemini, M., Robertson, M., Aronson, B., Atkinson, P., Polito, L. C., & Saccone, G. (2009). *Ceratitis capitata* transformer-2 gene is required to establish and maintain the autoregulation of cctra, the master gene for female sex determination. *International Journal of Developmental Biology, 53*(1), 109-120. doi:10.1387/ijdb.082681ms

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., . . . Cardona, A. (2012). Fiji: An open-source platform for biological-image analysis. *Nat Methods, 9*(7), 676-682. doi:10.1038/nmeth.2019

Sharath Chandra, G., Asokan, R., Manamohan, M., Ellango, R., Sharma, H. C., Akbar, S. M. D., & Krishna Kumar, N. K. (2018). Double-stranded RNA-mediated suppression of trypsin-like serine protease (t-sp) triggers over-expression of another t-sp isoform in *Helicoverpa armigera*. *Applied Biochemistry and Biotechnology, 184*(2), 746-761. doi:10.1007/s12010-017-2584-3

Shen, G.-M., Chen, W., Li, C.-Z., Ou, S.-Y., & He, L. (2019). RNAi targeting ecdysone receptor blocks the larva to adult development of *Tetranychus cinnabarinus*. *Pesticide Biochemistry and Physiology*. doi:10.1016/j.pestbp.2019.05.020

Shimomura, O. (2005). The discovery of aequorin and green fluorescent protein. *Journal of Microscopy, 217*(1), 3-15. doi:10.1111/j.0022-2720.2005.01441.x

Siebert, J. B., & Cooper, T. (1995). If medfly infestation triggered a trade ban: Embargo on California produce would cause revenue, job loss. *California Agriculture, 49*(4), 7-12. doi:10.3733/ca.v049n04p7

Taning, C. N. T., Christiaens, O., Berkvens, N., Casteels, H., Maes, M., & Smagghe, G. (2016). Oral RNAi to control *Drosophila suzukii*: Laboratory testing against larval and adult stages. *Journal of Pest Science, 89*(3), 803-814. doi:10.1007/s10340-016-0736-9

Taracena, M. L., Oliveira, P. L., Almendares, O., Umaña, C., Lowenberger, C., Dotson, E. M., . . . Pennington, P. M. (2015). Genetically modifying the insect gut microbiota to control chagas disease vectors through systemic RNAi. *Plos Neglected Tropical Diseases, 9*(2). doi:10.1371/journal.pntd.0003358

Tariq, K., Ali, A., Davies, T. G. E., Naz, E., Naz, L., Sohail, S., . . . Ullah, F. (2019). RNA interference-mediated knockdown of voltage-gated sodium channel (mpnav) gene causes mortality in peach-potato aphid, *Myzus persicae*. *Scientific Reports, 9*(1), 5291. doi:10.1038/s41598-019-41832-8

Team, R. C. (2016). R: A language and environment for statistical computing: R Foundation for Statistical Computing, Vienna, Austria. Retrieved from www.R-project.org

Terra, W. R. (1990). Evolution of digestive systems of insects. *Annual Review of Entomology, 35*(1), 181-200. doi:10.1146/annurev.en.35.010190.001145

Thomas, D. D., Donnelly, C. A., Wood, R. J., & Alphey, L. S. (2000). Insect population control using a dominant, repressible, lethal genetic system. *Science, 287*(5462), 2474.

Tian, G., Cheng, L., Qi, X., Ge, Z., Niu, C., Zhang, X., & Jin, S. (2015). Transgenic cotton plants expressing double-stranded RNAs target hmg-coa reductase (hmgr) gene inhibits the growth, development and survival of cotton bollworms. *International Journal of Biological Sciences, 11*(11), 1296-1305. doi:10.7150/ijbs.12463

Timmons, L., Court, D. L., & Fire, A. (2001). Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene, 263*(1-2), 103-112. doi:10.1016/s0378-1119(00)00579-5

Toledo, J., Rull, J., Oropeza, A., Hernandez, E., & Liedo, P. (2004). Irradiation of *Anastrepha obliqua (Diptera: Tephritidae)* revisited: Optimizing sterility induction. *J Econ Entomol, 97*(2), 383-389.

Vision, G. (2019). GXcam hichrome-s camera. Retrieved from https://www.gtvision.co.uk/

Wang, X.-F., & Ye, D. (2010). On nonparametric comparison of images and regression surfaces. *Journal of statistical planning and inference, 140*(10), 2875-2884. doi:10.1016/j.jspi.2010.03.011

Waters, J. C. (2009). Accuracy and precision in quantitative fluorescence microscopy. *J Cell Biol, 185*(7), 1135-1148. doi:10.1083/jcb.200903097

Weiss, B. L., Savage, A. F., Griffith, B. C., Wu, Y., & Aksoy, S. (2014). The peritrophic matrix mediates differential infection outcomes in the tsetse fly gut following challenge with commensal, pathogenic, and parasitic microbes. *The Journal of Immunology, 193*(2), 773. doi:10.4049/jimmunol.1400163

White, I. M., & Elson-Harris, M. M. (1992). *Fruit flies of economic significance: Their identification and bionomics*. Wallingford: CAB International.

Whyard, S., Erdelyan, C. N., Partridge, A. L., Singh, A. D., Beebe, N. W., & Capina, R. (2015). Silencing the buzz: A new approach to population suppression of mosquitoes by feeding larvae double-stranded RNAs. *Parasites & vectors, 8*(1), 716-716. doi:10.1186/s13071-015-0716-6

Whyard, S., Singh, A. D., & Wong, S. (2009). Ingested double-stranded RNAs can act as species-specific insecticides. *Insect Biochemistry and Molecular Biology, 39*(11), 824-832. doi:10.1016/j.ibmb.2009.09.007

Wickham, H. (2017). Tidyverse: Easily install and load the 'tidyverse'. Retrieved from https://cran.r-project.org/web/packages/tidyverse/index.html

Xu, Q.-Y., Deng, P., Mu, L.-L., Fu, K.-Y., Guo, W.-C., & Li, G.-Q. (2019). Silencing taiman impairs larval development in *Leptinotarsa decemlineata*. *Pesticide Biochemistry and Physiology*. doi:10.1016/j.pestbp.2019.06.013

Zhang, D.-W., Wang, H.-J., Jin, X., Pan, B.-Y., Zeng, B.-P., Xiao, Z.-J., . . . Tang, B. (2019). Knockdown of glycogen phosphorylase and glycogen synthase influences expression of chitin synthesis genes of rice brown planthopper *Nilaparvata lugens*. *Journal of Asia-Pacific Entomology, 22*(3), 786-794. doi:10.1016/j.aspen.2019.06.006

Chapter 3 – Electroporation of Mediterranean Fruit Fly (*Ceratitis capitata*) eggs with double stranded RNA to establish pseudomale populations for application of the Sterile Insect Technique

3.1) Abstract

The Sterile Insect Technique (SIT) relies upon the production of large numbers of sterile male insects for release. RNA interference (RNAi) targeting the *transformer-2 gene* (*tra2*) in early-stage Mediterranean Fruit Fly embryos induces a "pseudomale" phenotype in genetically female XX individuals. As pseudomales are phenotypically male, perform male-specific mating rituals and remain fertile, I hypothesised that they could assist in SIT control by allowing the production of 100% 'male' cohorts for subsequent sterilisation. However, successful targeting of genes by RNAi in medfly embryos has only been reported via microinjection, which is low throughput and would not support production of insects in the numbers required for SIT. The aim here was to investigate whether electrical transfection (electroporation) of double stranded RNA could provide an alternative method for successful RNAi in medfly eggs. If successful, such electroporation could facilitate high throughput treatment of eggs at population sizes suitable for SIT. I electroporated both chorionated and dechorionated eggs, using three different electroporation systems. None of the treatments or conditions tested demonstrated successful RNAi induction via electroporation. However, given its success in other insects, this method still offers the potential for medfly control, if electroporation treatment conditions can be tested further and optimised.

3.2) Introduction

RNA interference (RNAi) (Fire et al., 1998) is a conserved eukaryotic anti-viral response (Ding, 2010). Eukaryotic cells recognise intracellular molecules of double-stranded RNA (dsRNA) as a signal of viral infection, and process them into 21bp small interfering RNAs (siRNA) by using Dicer enzymes (Elbashir et al., 2001). siRNAs are subsequently bound to an Argonaut protein (Carmell et al., 2002), forming an RNA-induced silencing complex (RISC). In the final step of the RNAi pathway, RISCs degrade cellular mRNAs that have nucleotide sequence homologous to the siRNA component (Maniataki et al., 2005; Rand et al., 2005). This process prevents the translation of mRNAs that have been encoded by parasitic viral dsRNA, protecting the cell from the effects of viral infection.

The RNAi pathway has been used extensively for genetic research, due to two fundamental traits: 1) The RNAi pathway can be artificially induced if dsRNA is deliberately introduced into a eukaryotic cell (Fire et al., 1998), 2) Once initiated, the RNAi pathway can be employed to silence endogenous genes (Fire et al., 1998). The combination of these two factors has facilitated quick and definitive loss of function analyses in a wide range of eukaryotes (Kleinhammer et al., 2010; Lu et al., 2004; Yan et al., 2012), including insects (Cao et al., 2015; Hu et al., 2016; Yang et al., 2014).

RNAi has been employed to induce mortality in insects (Cao et al., 2015; Hu et al., 2016; Yang et al., 2014), create beneficial phenotypes for insect control (Liu et al., 2015; Salvemini et al., 2009) and prevent pesticide resistance in insect pests (Bona et al., 2016; Figueira-Mansur et al., 2013; Sandoval-Mojica et al., 2016). RNAi has therefore been widely viewed as a potentially important means of insect pest control (Reviewed in: (Baum et al., 2014; Darrington et al., 2017; Huvenne et al., 2010; Yu et al., 2013)).

Many insect species will initiate RNAi in response to dsRNA encountered in their environment (eRNAi) (Baum et al., 2014; Darrington et al., 2017). Such eRNAi 'competent' insects are good candidates for control, as dsRNA designed to elicit a desired response can be administered topically (Toprak et al., 2013; Whyard et al., 2015) or in food (Coleman et al., 2015; Li et al., 2015). However, some insects (including important

pest species) are recalcitrant to eRNAi (Baum et al., 2014; Darrington et al., 2017) for reasons that are not yet fully known.

In chapter 2, I attempted to induce eRNAi in adults and larvae of the agricultural pest *Ceratitis capitata* (Mediterranean Fruit Fly; medfly) by using various feeding techniques. The medfly was refractory to eRNAi by this method, which may indicate it lacks the genes that are required for transport of environmental dsRNAs to the cytoplasm (Darrington et al., 2017). However, as RNAi can be induced in medfly eggs via direct microinjection of dsRNA (Pane et al., 2002; Salvemini et al., 2009), I hypothesised that RNAi remains a viable method of control for the medfly if eggs can be made competent for eRNAi.

Electroporation is the application of precise electrical pulses to cell membranes (Chen et al., 2006) and is considered one of the more reliable systems of transfection for animal embryos (reviewed in (Gosse et al., 2017)). Electroporation of cell membranes causes local polarisation which in turn causes phospholipids to rapidly change conformation (Weaver, 2003). As the phospholipids are rearranged, aqueous pores are formed that allow passage of charged molecules such as dsRNA to the cytoplasm (Weaver, 2003). In the context of electroporation, the cell membrane can be thought of as a barrier separating two conducting solutions that is made permeable through polarisation with electric current (Tien et al., 2003). When electroporation is successfully applied, the membrane potential returns to its resting state when electrical pulsing has ceased and the pores close (Weaver, 2003).

Several studies have demonstrated that *Dipteran* and *Lepidopteran* embryos can be successfully electroporated with plasmid vectors (Kamdar et al., 1995; Leopold et al., 1996; Thomas, 2003). Currently, transfection of insect embryos with dsRNA by electroporation (Elec-eRNAi) has not been reported. However, Ando et al. (2013) have reported successful Elec-eRNAi of adult *Lepidopterans* and both Karim et al. (2010) and Ruiz et al. (2015) report successful Elec-eRNAi of arachnid embryos. In the following study, I tested three different systems of electroporation to evaluate whether medfly eggs could be made eRNAi competent via Elec-eRNAi and thus whether this technique had potential application for control of adult flies.

Investigations into Elec-eRNAi were carried out using BTX830, Intracel TSS20 and Nucleofector 2b devices. The Nucleofector 2b device operates by means of pre-set nucleofection programs about which pulsing parameters are not known, while the BTX830 and Intracel TSS20 devices allow pulsing parameters to be set manually. Hence BTX830 and Intracel devices were utilised to establish the specific pulsing parameters that induce eggs to become transfectable.

OX3864A medflies are genetically modified having been developed for use with Oxitec's self-limiting pest control (Asadi et al., 2019; Thomas et al., 2000) and ubiquitously express a fluorescent dsRED2 marker (Gong et al., 2005). In a preliminary experiment, I treated the eggs of OX3864A medflies with Elec-eRNAi targeting the dsRED2 gene that is carried by this strain. I assessed the effect of Elec-eRNAi in OX3864A eggs via quantification of dsRED2 expression in L1 larvae. The hypothesis tested was that successful induction would lead to a reduction in dsRED2 expression.

I then carried out a set of Elec-eRNAi experiments targeting the *transformer-2* (*tra2*) gene in eggs of wild type Toliman strain medflies. When RNAi of *tra2* is applied to early stage medfly (Salvemini et al., 2009), oriental fruit fly (Liu et al., 2015) or red flour beetle embryos (Shukla et al., 2012), genetic females become XX pseudomales. Pseudomale medflies are indistinguishable from genetic males and perform typical male behaviours, including mating rituals (Salvemini et al., 2009). I hypothesised that Elec-eRNAi of *tra2* targeting groups of medfly eggs would produce large, male only populations, that if sterilised would be amenable to the Sterile Insect Technique (SIT) (Knipling, 1955). The effect of Elec-eRNAi of *tra2* in eggs was measured via the sex ratio of adult flies that developed from treatment. The expectation was that cohorts would become severely male biased if the transfection of *tra2* was highly effective. However, if the effect of Elec-eRNAi was weak, sex-ratio would be less skewed. Therefore, in populations that exhibited a slight male bias, the DNA of phenotypic males was subjected to PCR targeting the Y-chromosome to elucidate the presence of cryptic pseudomales (Salvemini et al., 2009).

Medfly eggs are encased in a proteinaceous outer chorion (Mouzaki et al., 1991) (Figure 3.1) that protects them against environmental insults such as microbial invasion

(Marchini et al., 1997). I therefore tested a second hypothesis, that the egg chorion might act as a barrier to dsRNA effectors which would attenuate Elec-eRNAi even if the egg membrane had been successfully permeabilised. To test this, medfly eggs were electroporated with dsRNA in both chorionated and dechorionated conditions.

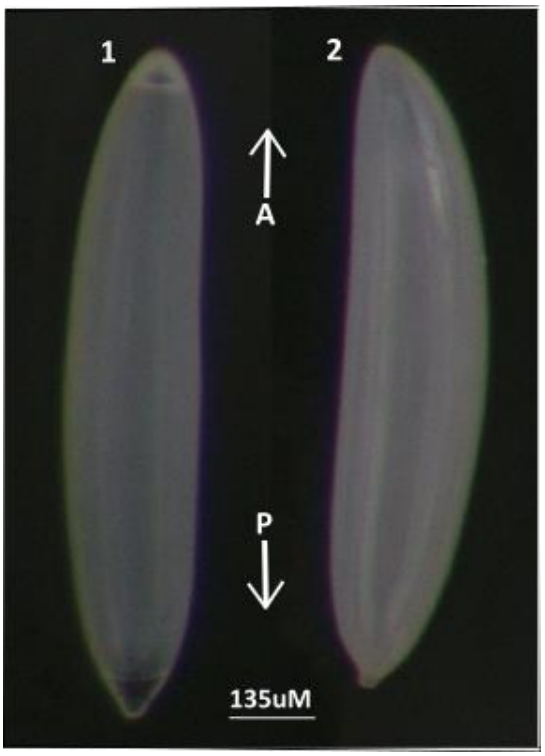

Figure 3.1 Chorionated and dechorionated medfly eggs: Two medfly eggs are positioned with anterior poles at the top of the image. 1) dechorionated egg - visibly clear tips on anterior and posterior poles of membrane, 2) chorionated egg – egg is covered with opaque chorion obscuring the clear membrane at the anterior and posterior poles.

3.3) Methods

3.1) Medfly strains treated with Elec-eRNAi

The tests of Elec-eRNAi were conducted using the eggs of OX3864A medflies (supplied by Oxitec Ltd) and eggs of the Toliman wild type medfly strain. The OX3864A strain has been genetically modified to ubiquitously express a dsRED2 fluorescent marker (Gong et al., 2005).

3.2.1) Fly husbandry - larvae

OX3864A and Toliman larvae were reared on equivalent diet, but OX3864A diet contained tetracycline (Sigma) to block expression of toxic tetracycline-repressible activator variant (tTAV) protein (Gong et al., 2005). Larvae were reared in a controlled environment: 25^{o}C ambient temperature, 51% relative humidity and 12L: 12D photoperiod. Approximately 500 eggs which had been laid within a 24hr period, were placed on 100ml of larval diet (Agar 12.5g/L; Sucrose 73.5g/L; Maize 67g/L; Brewer's yeast 47.5g/L; 10% Nipagin 25ml/L; Propionic acid 2ml/L; (Tetracycline 0.1g/L in OX3864A diet)) in 300ml plastic bottles. Seven days after egg collection when larvae had reached the L3 stage, bottles were laid in 2cm of play-sand in plastic containers (170mm x 130mm x 50mm), with a mesh lid. L3 stage larvae dispersed naturally from the bottles to pupate in the sand. 16 days after egg collection, pupae were collected from sand to be placed in adult cages.

3.2.2) Fly husbandry - adults

Adults were reared in a controlled environment: 25^{o}C ambient temperature, 51% relative humidity and 12L: 12D photoperiod. For both Toliman and OX3864A medfly strains, 100 pupae were placed in plastic cages (120mm x 120mm x 135mm) with 3g of solid diet (3:1, sucrose: hydrolysed yeast) and allowed to eclose. Flies were supplied with ddH_2O *ad libitum*. 0.1g/l of tetracycline (Sigma) was added the ddH_2O of OX3864A flies to block expression of toxic tetracycline-repressible activator variant (tTAV) protein (Gong et al., 2005).

3.3.1) Assaying the effect of transfection buffer on medfly eggs development

Groups of <1hr old Toliman eggs (n = 300) were placed in either ddH_2O or transfection buffer (200mM HEPES; 137mM NaCl; 5mM KCl; 6mM D-Glucose; 7mM Na_2HPO_4) for 30 mins, then washed with ddH_2O for 30 secs. Eggs were transferred to filter paper and hatched eggs were counted after 44 hrs.

3.3.2) Assaying the toxic effect of electricity to medfly eggs

The following procedure was carried out with groups of eggs at 6, 8 and 9 days post-eclosion (DPE), commencing when the first fly within the cage eclosed. OX3864A eggs <1hr old were collected from a single adult cage and transferred to a 2ml Eppendorf tube containing 1ml of molecular H_2O (ThermoFisher). Once the eggs settled, a 100µl pipette that had 5mm cut from the end was used to draw up 12.5µl from the settled aggregate. The 12.5µl egg aliquot was transferred to an aluminium cuvette (Lonza) containing 80ul of transfection buffer (200mM HEPES; 137mM NaCl; 5mM KCl; 6mM D-Glucose; 7mM Na_2HPO_4) and placed in a Nucleofector 2b device (Lonza). Eggs were exposed to one of seven nucleofection programs that were selected by Lonza for use with the Lonza cell line optimisation protocol (COP), or a no-pulse control. The COP was designed to optimise electrical transfection of novel cell types (i.e. cells for which conditions for successful transfection are not yet known) and includes nucleofection programs which encompass a wide range of pulsing parameters. Electroporated eggs were transferred to larval diet (agar 12.5g/L; sucrose 73.5g/L; maize 67g/L; brewer's yeast 47.5g/L; 10% nipagin 25ml/L; propionic acid 2ml/L; tetracycline 0.1g/L) and the number of hatched vs unhatched eggs was assessed after 96 hours.

3.4) Resuspension of dsRNAs in transfection buffer for Elec-eRNAi

In vitro synthesised dsRED2 and Cctra2 dsRNA suspended in H_2O were purchased from Genolution, Korea (Genolution, 2019) and supplied by Oxitec Ltd (Oxitec, 2019) (see supplementary materials 3.2). To precipitate dsRNA from H_2O, 750µl aliquots of dsRNA solution were added to 2ml Eppendorf tubes. 750µl of 100% isopropanol was added to the tube, then 150µl of 3M sodium acetate. The solution was briefly mixed, before being centrifuged at 10000 RPM for 5 mins. The supernatant was removed, then 1.5ml of

transfection buffer (200mM HEPES; 137mM NaCl; 5mM KCl; 6mM D-Glucose; 7mM Na_2HPO_4) was added to the tube and the pellet was resuspended by vortex. RNA concentration and purity were measured by Nanodrop (ThermoFisher).

3.5) Transfection of chorionated OX3864A strain medfly eggs with dsRED2 dsRNA using a Nucleofector 2b device

Prior to use, embryo collection cages and brushes were sterilised with 10 mins exposure to UV radiation (Labcaire PCR workstation) and wiped down with RNAseZap (ThermoFisher). OX3864A eggs no more than one hour old, laid at 6 days post eclosion, were washed for 30 secs with molecular H_2O (ThermoFisher) in an embryo collection cage. A brush was then used to transfer eggs to a 2ml Eppendorf tube containing molecular H_2O (ThermoFisher). A 100µl pipette that had 5mm cut from the end was used to draw a volume of 12.5µl of eggs which were transferred to an aluminium cuvette (Lonza) containing 80ul of dsRED2 transfection buffer (3.2mg/ml dsRED2 dsRNA in 200mM HEPES; 137mM NaCl; 5mM KCl; 6mM D-Glucose; 7mM Na_2HPO_4). Prior to use the cuvette had been cleaned with 800µl of 0.2M HCl. The cuvette was placed in a Nucleofector 2b device (Lonza) and exposed to one of six nucleofection programs or a no-pulse control. Specific nucleofection programs used in this study were informed by a previous assay of the toxicity of electrical pulses to medfly eggs (protocol 3.3.2). Eggs were washed from the cuvette onto filter paper and allowed to progress to the larval stage when expression of the target gene was measured with RT-qPCR.

3.6) Quantification of dsRED2 expression by OX3864A larvae that developed from eggs which had been electroporated with dsRED2 dsRNA

3.6.1) Extraction of nucleic acids and digestion of genomic DNA

Larvae began to emerge on the third day after electroporation and were collected over a 24hr period. Upon emergence, groups of five larvae were placed in 2ml Eppendorf tubes and flash frozen in liquid nitrogen. Larvae were pulverised using a micropestle that had been sterilised with 10 mins exposure to UV radiation (Labcaire PCR workstation) and wiped down with RNAseZap (ThermoFisher). Eppendorfs were returned to liquid nitrogen and fly matter was re-pulverised three times. RNA extraction was carried out

on pulverised fly material using a mirVana kit (ThermoFisher) according to the manufacturer's instructions. To digest residual genomic DNA, samples were treated with Turbo DNAase (ThermoFisher) according to the manufacturer's instructions.

3.6.2) CDNA synthesis

CDNA was synthesised using a Revertaid first strand synthesis kit (ThermoFisher). 0.5µg of RNA was incubated with 1µl of OligodT (10µM) in molecular water (ThermoFisher) (11.5µl total volume) at 65˚C for 5 mins. The remaining Revertaid components (4µl 5X reaction buffer; 0.5µl Ribolock; 2µl 10mM dNTP mix; 1µl Revertaid) were then added to the suspension and incubated for a further 60 mins at 42˚C, then 5 mins at 70˚C.

3.6.3) Quantitative reverse transcriptase PCR (RT-qPCR)

RT-qPCR was carried out on a StepOnePlus thermocycler (Applied Biosystems) with Taqman gene expression mastermix (Applied Biosystems). Primers and probes for the dsRED2 target and two reference genes (Rpl13a and L27) (Nash et al., 2018) were purchased from Eurofins Genomics (see supplementary materials 3.1). Each RT-qPCR well contained 1µl of CDNA (25ng total CDNA assuming 100% recovery during reverse transcription), 10µl of primers (1.6µl of each primer at 15.63µM), 12.5µl of 2X reaction mix and 1.25µl of fluorescent probes (0.416µl of each probe at 15.63µM). Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001) and analysed using the tidyverse packages in R (Team, 2016).

3.7) Transfection of chorionated wild-type medfly eggs with Cctra2 using a Nucleofector 2b device

Prior to use, embryo collection cages and brushes were sterilised with 10 mins exposure to UV radiation (Labcaire PCR workstation) and wiped down with RNAseZap (ThermoFisher). Toliman eggs no more than one hour old, laid at 6 days post eclosion, were washed with molecular H_2O (ThermoFisher) for 30 secs in an embryo collection cage. A brush was then used to transfer eggs to a 2ml Eppendorf tube containing molecular H_2O (ThermoFisher). A 100µl pipette that had 5mm cut from the end was used to draw a volume of 12.5µl of eggs which were transferred to an aluminium cuvette

(Lonza) containing 80µl of Cctra2 transfection buffer (3.8mg/ml Cctra2 dsRNA in 200mM HEPES; 137mM NaCl; 5mM KCl; 6mM D-Glucose; 7mM Na_2HPO_4). Prior to use the cuvette had been cleaned with 800µl of 0.2M HCl. The cuvette was placed in a Nucleofector 2b device (Lonza) and exposed to one of eight nucleofection programs or a no-pulse control. Specific nucleofection programs used in this experiment were defined following communication with Lonza nucleofection specialists, regarding results from an earlier assay that targeted dsRED2 with Elec-eRNAi. Eggs were washed from cuvettes onto filter paper and reared to adulthood.

3.8) Transfection of chorionated wild-type medfly eggs with Cctra2 dsRNA using a BTX 830 square wave electroporator

Prior to use, embryo collection cages, microslides (BTX) and brushes were sterilised with 10 mins exposure to UV radiation (Labcaire PCR workstation) and wiped down with RNAseZap (ThermoFisher). Eggs no more than one hour old laid at 6 days post eclosion, were washed with molecular H_2O (ThermoFisher) for 30 secs in an embryo collection cage. A brush was then used to transfer eggs to a 2ml Eppendorf tube containing molecular H_2O (ThermoFisher). A 100µl pipette that had 5mm cut from the end was used to draw 12.5µl of eggs, which were transferred to a 450-1 microslide (BTX) (Figure 3.5). Excess water was absorbed with filter paper. Microslides were attached to a BTX830 device (BTX) and the electroporation reservoir was flooded with 40µl of Cctra2 transfection buffer (3.8mg/ml dsRED2 dsRNA in 200mM HEPES; 137mM NaCl; 5mM KCl; 6mM D-Glucose; 7mM Na_2HPO_4). Eggs were electroporated with standardised pulsing parameters (10 pulses of 50ms width at 1 second intervals) at either 0, 100, 125 or 150V. Following electroporation, eggs were washed into filter paper and reared to adulthood.

3.9.1) Dechorionation of wild-type medfly eggs for transfection with Cctra2

Prior to use, embryo collection cages, cover slips, and brushes were sterilised with 10 mins exposure to UV radiation (Labcaire PCR workstation) and wiped down with RNAseZap (ThermoFisher). Toliman eggs no more than one hour old, laid at 6 days post eclosion were collected in an embryo collection cage for treatment. Eggs were dechorionated in the cage, by vigorous washing in 1% sodium hypochlorite for 1.5 mins.

Dechorionated eggs were immediately washed under a continuous flow of ddH_2O for 30 secs to remove residual sodium hypochlorite, then blotted on filter paper and placed in a reservoir of clean ddH_2O. A group of dechorionated eggs was inspected with a Leica M80 stereo microscope to assess if the procedure had been successful (Figure 3.1).

3.9.2) Transfection of dechorionated Toliman medfly eggs with Cctra2 and dsRED2 dsRNA using an Intracel TSS20 ovodyne electroporator

Groups of dechorionated eggs were transferred from embryo collection cages to a 22mm cover slip using a sterile brush. Egg groups were electroporated on the cover slip with platinum electrodes at 0, 5, 12.5 or 20V, in a 15µl drop of Cctra2 transfection buffer (3.8mg/ml dsRED2 dsRNA in 200mM HEPES; 137mM NaCl; 5mM KCl; 6mM D-Glucose; 7mM Na_2HPO_4) or dsRED2 transfection buffer (3.2mg/ml dsRED2 dsRNA in 200mM HEPES; 137mM NaCl; 5mM KCl; 6mM D-Glucose; 7mM Na_2HPO_4). At all voltages, pulsing parameters were consistently 10 pulses of 50ms width at 1 second intervals. Post electroporation, excess fluid was absorbed with filter paper and the eggs were covered with a drop of blended oil (7:1, halocarbon oil 700 (Sigma): paraffin oil (Sigma)) to prevent desiccation. Cover slips were moved to petri dish containing larval diet (Agar 12.5g/L; Sucrose 73.5g/L; Maize 67g/L; Brewer's yeast 47.5g/L; 10% Nipagin 25ml/L; Propionic acid 2ml/L). As larvae hatched, they dispersed from cover slips to larval diet and were reared to adulthood.

3.10) Extraction of DNA for identification of pseudomale flies by multiplex PCR

Single adult files were placed in 2ml Eppendorf tubes and placed in liquid nitrogen. Fly matter was pulverised inside Eppendorf tubes with a micropestle that had been sterilised with 10 mins exposure to UV radiation (Labcaire PCR workstation) and wiped down with 1% sodium hypochlorite (Sigma). Eppendorfs were returned to liquid nitrogen and fly matter was re-pulverised three times. 50µl of DNA extraction buffer (10mM Tris-HCL (pH 8.2); 1mM EDTA; 25mM NaCl; 200µg/ml proteinase K) was added to the pulverised material and incubated at room temperature for 25mins, then 95˚C for two mins. The sample was centrifuged at 12000 RPM for 3 mins and the supernatant was used for PCR analysis. DNA purity and concentration were measured by Nanodrop (ThermoFisher).

3.11) Multiplex PCR for identification of pseudomale medflies

Multiplex PCR with Y-chromosome specific primers and Rpl13a control primers was carried out to detect the presence of cryptic pseudomales within populations of phenotypically male flies (Salvemini et al., 2009). Y-chromosome specific (CcY) primers were designed to amplify only male DNA as in Gabrieli et al. (2010), and Rpl13a acted as a positive control that would amplify both male and pseudomale DNA (Nash et al., 2018). 50µl PCR reaction mixtures were set up as follows: 5µl of TopTaq PCR buffer (Qiagen), 1µl of 10mM DNTP mix, 5µl of 10X CoralLoad (Qiagen), 1µl of each primer (0.5µM), 0.25µl of TopTaq enzyme (Qiagen), 1µl of medfly DNA (30ng/µl), 33.75µl of molecular water (ThermoFisher). Cycling parameters were: 1) 94^{o}C for 2 mins, 2) 35 cycles of 94^{o}C for 30 secs, 55^{o}C for 1 min and 72^{o}C for 45 secs, 3)72^{o}C for 5 mins.

3.4) Results

3.1) Evaluating the base line effect of transfection buffer on medfly eggs in preparation for subsequent Elec-eRNAi analyses

Groups of Toliman medfly eggs (n=300) were submerged in either transfection buffer or ddH_2O for 30 mins to assess if exposure to transfection buffer itself would inhibit medfly egg development. ddH_2O exposure served as a negative control in this assay, as medfly eggs are regularly exposed to ddH_2O as part of the typical rearing process without consequences for development. Three replicates of eggs were exposed to both solutions and the number of hatched eggs in each group was assessed after 44 hours. The mean number of hatched eggs was 100 following exposure to ddH_2O and 102 following exposure to transfection buffer. As the mean hatch rates of eggs treated with both solutions were comparable, transfection buffer was considered non-toxic and used in subsequent Elec-eRNAi analyses.

3.2) Evaluating the base line effect of electricity on medfly eggs in preparation for subsequent Elec-eRNAi analyses

To test the hypothesis that electricity would inhibit medfly egg development, groups of eggs collected from a single cage of OX3864A medflies were electroporated with a Nucleofector 2b device (Lonza). Eggs <1hr old were collected on three separate days (DPE) and treated with one of seven nucleofection programs in compliance with the Lonza cell optimisation protocol (COP). The COP was designed to optimise electrical transfection of novel cell types (i.e. cells for which reports of successful transfection do not exist) and includes nucleofection programs which encompass a wide range of pulsing parameters. Each nucleofection program included in the COP represents a unique set of electrical treatment conditions but the specific pulsing parameters associated with each program are undisclosed by Lonza. The percentage of hatched eggs vs unhatched eggs was quantified 96 hours after treatment and compared to a no-pulse control (Figure 3.2). The rate of egg survival was not normally distributed between electrical treatment groups (Shap.Wilk, $W=0.82$, $p<0.0001$) and could not be transformed to normality. The data were therefore analysed using non-parametric Kruskal-Wallis tests. Egg survival was not

significantly different between groups of eggs treated with discrete nucleofection programs (Kruskal-Wallis chi-squared = 8.72, df = 7, $p>0.05$). However, egg survival was low for all groups of eggs that were treated with nucleofection program D-023, which was excluded from subsequent Elec-eRNAi analyses. Survival following electrical treatment was significantly different between groups of eggs that were collected on different DPE's (Kruskal-Wallis chi-squared = 6.66, df = 2, $p<0.05$). Eggs collected at 8 and 9DPE were less viable for electroporation than eggs collected at 6DPE. Therefore, all subsequent Elec-eRNAi experiments were carried out using eggs that were collected at 6DPE.

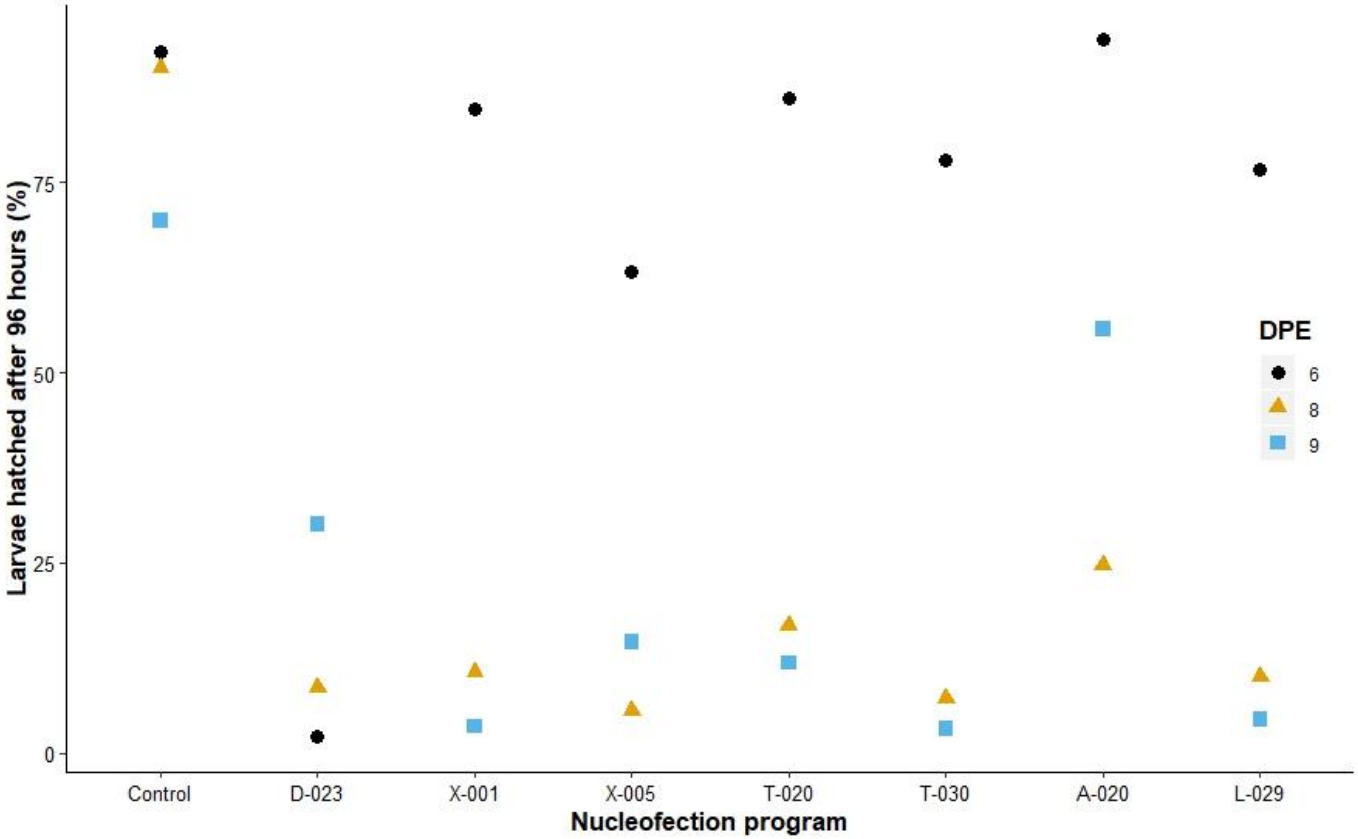

Figure 3.2 Survival rates of OX3864A eggs that were electrically treated with seven discrete nucleofection programs or a no-pulse control: The percentage of hatched eggs 96hrs after electrical treatment is represented on the Y-axis. Specific nucleofection programs and a no-pulse control are represented on the x-axis. Eggs were collected from a single cage of OX3864A medflies at 6, 8 and 9 days post-eclosion (DPE) (commencing when the first fly in the population had eclosed). DPE significantly affected egg survival (Kruskal-Wallis chi-squared = 6.66, df = 2, $p<0.05$), but nucleofection treatment program did not significantly affect egg survival (Kruskal-Wallis chi-squared = 8.72, df = 7, $p>0.05$).

3.3) Transfection of chorionated OX3864A eggs with dsRED2 dsRNA for Elec-eRNAi of the dsRED2 gene

OX3864A eggs were electroporated in dsRED2 transfection buffer with a Nucleofector 2b device (Lonza) to induce Elec-eRNAi of the dsRED2 gene. The effect of Elec-eRNAi on eggs was measured in resulting larvae, via relative expression of the dsRED2 gene using the 2–ΔΔCt method (Pfaffl, 2001). Elec-eRNAi of eggs was tested with six nucleofection programs that each represented a unique set of electrical treatment conditions. Nucleofection programs were selected according to the Lonza cell optimisation protocol (COP). However, program D-023 which forms part of the standard COP was omitted, as the initial investigation described above showed that this setting resulted in high egg mortality. 64 hours after electroporation, larvae from each electrical treatment group began to hatch and were flash frozen in groups of five. Three replicates of larvae from each electrical treatment group were assessed for relative dsRED2 expression. Larvae treated with program X-005 appeared to express less dsRED2 than larvae treated with other nucleofection programs (Figure 3.3) but dsRED2 expression was not significantly different between treatment groups (ANOVA, $F(6,14) = 0.96$, $p > 0.05$).

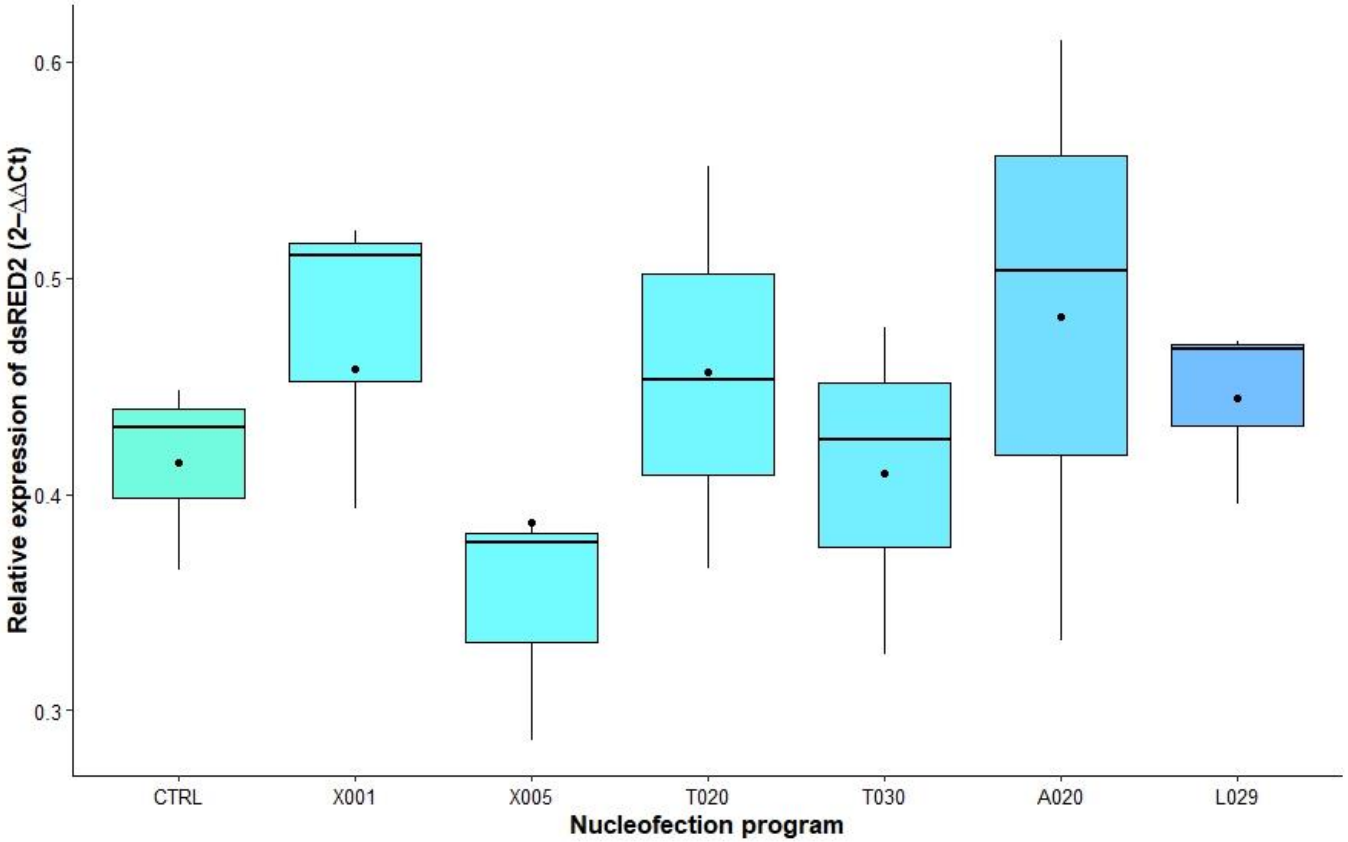

Figure 3.3 The relative expression of dsRED2 in larvae that developed from eggs electroporated with dsRED2 dsRNA: Relative dsRED2 expression (2–ΔΔCt (Pfaffl, 2001)) is represented on the y-axis. Six nucleofection programs and a no-pulse control (CTRL) are represented on the x-axis. The upper and lower hinges of boxplots represent the first and third quartiles of dsRED2 expression calculated from three replicates of larvae (n=5) 64 hours after egg treatment. Horizontal boxplot lines represent median dsRED2 expression and mean expression is represented by a dot. The specific electroporation treatment conditions imposed on eggs did not significantly affect the expression of dsRED2 in developing larvae (ANOVA, $F_{(6,14)} = 0.96$, $p > 0.05$).

3.3.1) Transfection of chorionated Toliman eggs with Cctra2 dsRNA using a Nucleofector 2b device for induction of the pseudomale phenotype

The results of the Elec-eRNAi experiment which targeted the dsRED2 gene in medfly eggs were shared with Lonza nucleofection specialists. Based on those data, Lonza recommended eight nucleofection programs for use in subsequent Elec-eRNAi experiments. To induce the pseudomale phenotype in genetically female flies, medfly eggs were submerged in Cctra2 transfection buffer and treated with electricity using a Nucleofector 2b device. The sex ratio of adult populations that developed from treated eggs was measured to assess whether Elec-eRNAi of Cctra2 had been effective at the egg stage (Salvemini et al., 2009). All nucleofection programs were tested with four replicates of eggs, other than X-007 and L029 which were tested with three replicates due to low egg availability (Figure 3.4). The sex ratio of adult flies was normally distributed between treatment groups (Shap.Wilk, $W=0.96$, $p>0.05$) but the variance between groups was heteroscedastic (Bartlett's $K^2= 21.4$, $df = 8$, p-value<0.01). As the data could not be transformed to create homogenous variances, differences in sex ratio were assessed with a non-parametric Kruskal-Wallis model. The sex ratios of groups of flies that were treated with different nucleofection programs were not significantly different (Kruskal-Wallis chi-squared = 9.22, $df = 8$, $p>0.05$). However, three of the four populations of flies that developed from eggs which had been treated with program X-005 had a slight male bias (Table 3.2). Although not statistically significant this was an interesting tend, as in an earlier Elec-eRNAi experiment, program X-005 had produced larvae with slightly reduced expression of a target gene (Figure 3.3). To investigate whether populations of flies treated as eggs with program X-005 contained any pseudomales, DNA extracted from phenotypically male flies was tested for the presence of a Y-chromosome (Figure 3.8).

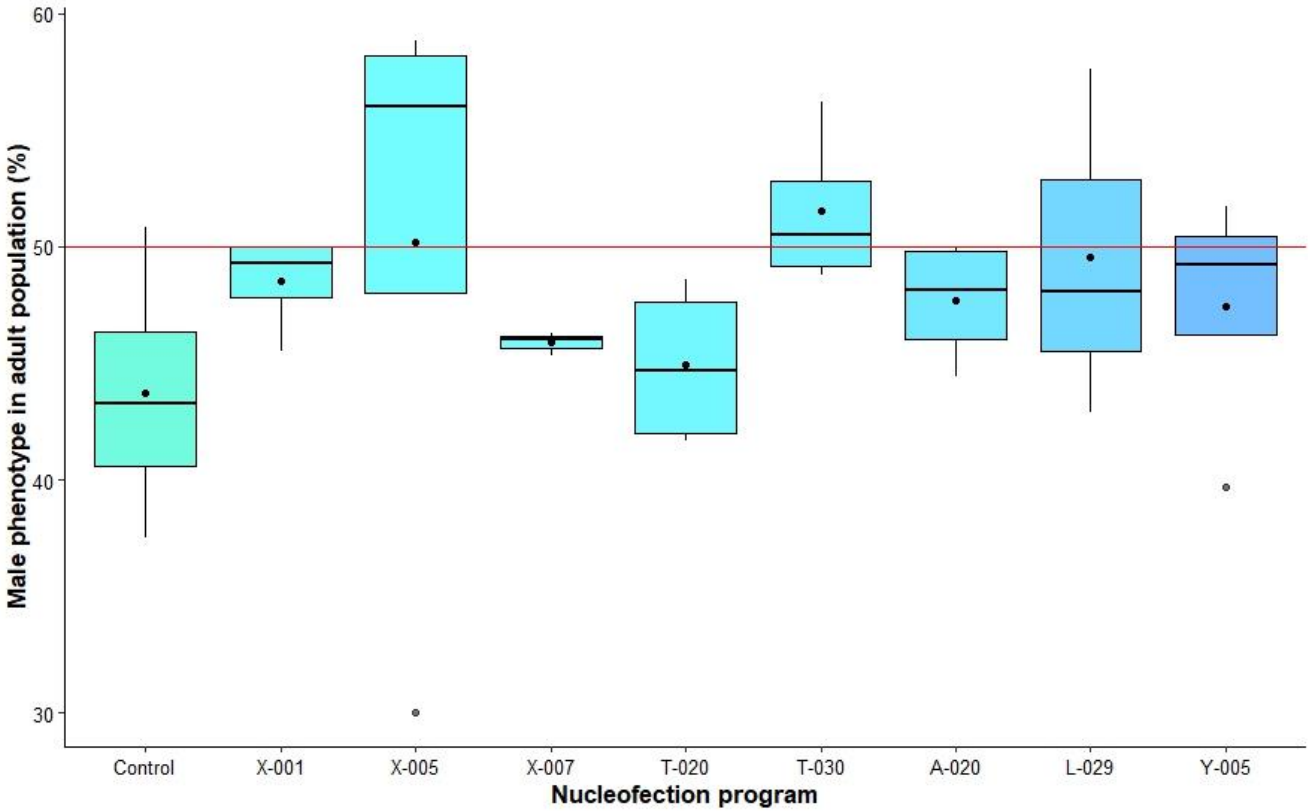

Figure 3.4 The percentage of phenotypically male adult flies in populations that developed from eggs which were electroporated with Cctra2 dsRNA using a Nucleofector 2b device: The percentage of male flies in adult populations treated with Elec-eRNAi of Cctra2 as eggs is represented on the y-axis. Specific nucleofection programs and a no-pulse control are represented on the x-axis. The first and third quartiles of male ratio in analysed populations are represented by upper and lower boxplot hinges. All but two boxplots represent four treatment groups of flies (X-007 and L-029 represent three treatment groups). Horizontal boxplot lines represent the median male percentage in treated populations and mean male percentage is represented by a dot. 50% male phenotype is defined by a red line. Treatment of eggs with different nucleofection programs did not significantly affect the sex ratio of adult flies (Kruskal-Wallis chi-squared = 9.22, df = 8, p>0.05).

3.3.2 Transfection of chorionated Toliman eggs with Cctra2 dsRNA using a BTX830 electroporator for induction of the pseudomale phenotype

Successful Elec-eRNAi of Cctra2 in medfly eggs is expected to produce a male biased sex ratio in adult fly populations (Salvemini et al., 2009). To test this hypothesis, chorionated Toliman eggs were electroporated in Cctra2 transfection buffer using a BTX830 device (BTX). The advantage of the BTX830 system over the Nucleofector 2b is that it allows manual specification of electrical pulsing parameters, whereas the Nucleofector 2b is operated by means of pre-set nucleofection programs in which pulsing parameters are not known. Therefore, successful transfection of eggs with a BTX830 device would establish if eggs were transfectable in response to specific pulsing parameters, while the Nucleofector 2b could only establish if eggs were transfectable *per se*. Electroporation of eggs was carried out at 0, 100, 125 or 150 V. Four discrete BTX microslides (Figure 3.5) were used to treat a single group of eggs at each voltage. Universal pulsing parameters of 10 pulses of 50ms width at 1 second intervals were used for all voltage conditions. The sex ratios of fly populations that developed from different electroporation groups were normally distributed (Shap.Wilk, W=0.97, p>0.05) and the variance between groups was homoscedastic (Bartlett's K^2= 14.8, df = 15, p-value>0.05). The effect of different voltages on the sex ratios fly populations were analysed with ANCOVA models that included both additive and interactive slide effects, but an ANOVA model which removed slide effects completely was found to be a better fit (Akaike Information Criterion = 51.47 Vs 53.42). The sex ratios of groups of adult flies that were electroporated at the egg stage with Cctra2 at different voltages were not significantly different from 50:50 (ANOVA, F (3,12) =2.75, p>0.05).

Figure 3.5 BTX microslide (model 450-1) used in conjunction with a BTX830 electroporator for transfection of medfly eggs with Cctra2 dsRNA. Model 450-1 microslides are composed of two 0.5mm stainless steel wire electrodes (marked by an arrow) set at a gap of 1mm. The space between the electrodes creates a 40µl electroporation reservoir.

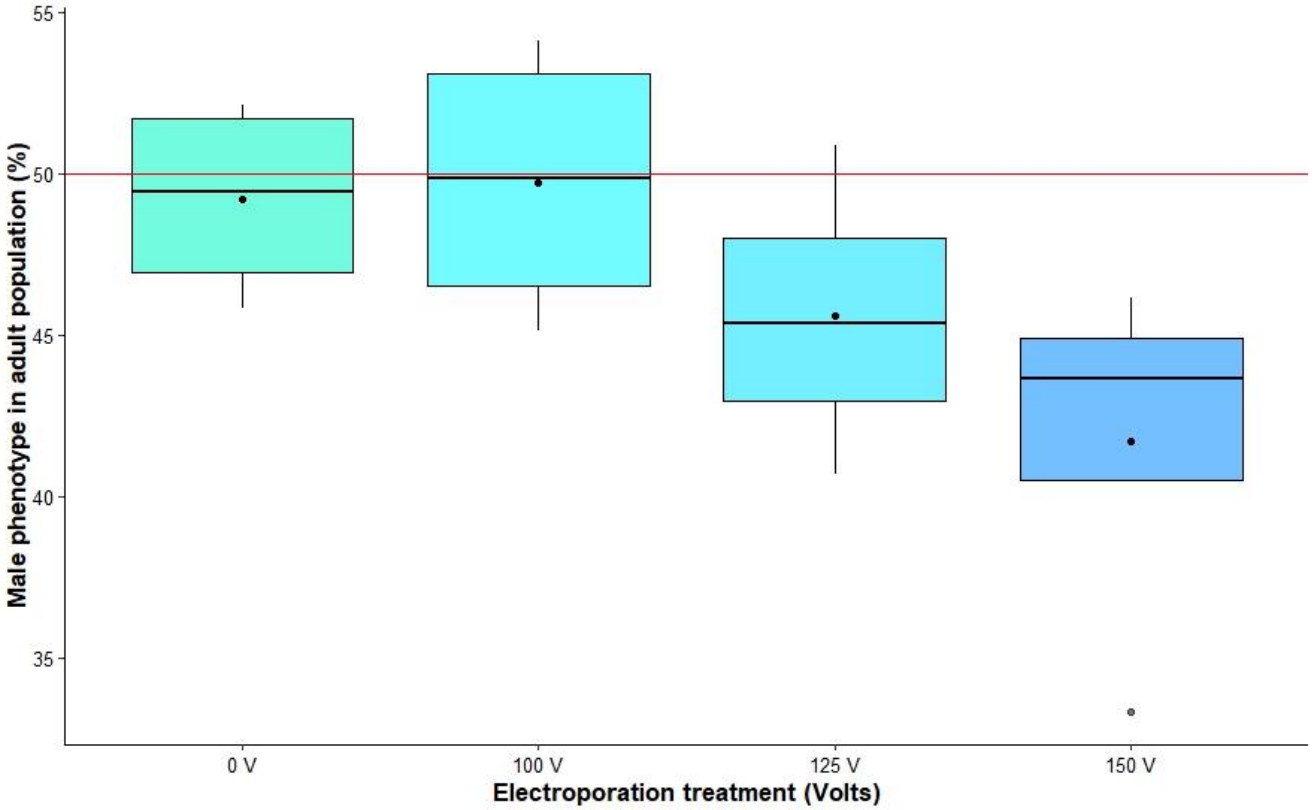

Figure 3.6 The percentage of phenotypically male adult flies in populations that developed from chorionated eggs that were electroporated with Cctra2 dsRNA using a BTX830 device: The percentage of male flies in adult populations electroporated with Cctra2 as eggs is represented on the y-axis. Voltage conditions are represented on the x-axis. The upper and lower hinges of boxplots represent the first and third quartiles of the male ratio in analysed populations. All boxplots represent four populations of flies treated with discrete microslides. Horizontal boxplot lines represent median male percentage and mean male percentage is represented by a dot. 50% male sex ratio is defined by a red line. The sex ratio of adult fly populations was not significantly different between groups that were electroporated with Cctra2 at different voltages as eggs (ANOVA, $F(3,12) = 2.75$, $p > 0.05$).

3.3.3 The base line effect of different electrical voltages on medfly eggs

In a preliminary experiment the base line effect of electricity *per se* on medfly eggs was tested with a Nucleofector 2b device. Egg survival was not found to differ significantly between groups of eggs exposed to various nucleofection programs (Figure 3.2). When eggs were subsequently treated with a BTX830 at different voltages, it was noted that treatment of eggs at 150V produced with fewer individuals than treatment at lower voltages (Table 3.1). The number of individuals surviving treatment was normally distributed between all treatment groups (Shap.Wilk, W=0.94, p>0.05) and the variance between groups was homoscedastic (Bartlett's K^2= 7.33, df = 3, p-value>0.05). The effect of different voltages on the survival of fly populations were analysed with ANCOVA models that included both additive and interactive slide effects, but an ANOVA model which removed slide effects completely was found to be a better fit (Akaike Information Criterion = 75.99 Vs 78.29). The survival of eggs was found to be significantly different between groups treated at different voltages (ANOVA, F (1,14) =39.6, p<0.001).

Table 3.1 The number of surviving adult flies in populations that were treated with different voltages at the egg stage: Eggs were treated at either 0, 100, 125 or 150V and allowed to progress to the adult stage. There were fewer surviving adults in the 150V treatment than at lower voltages. There was a significant difference in egg survival between voltage treatment groups (ANOVA, F (1,14) =39.6, p<0.001).

Voltage	Slide 1	Slide 2	Slide 3	Slide 4	Mean
0	48	59	56	64	56.75
100	27	30	33	41	32.75
125	28	43	43	25	34.75
150	10	7	6	9	8

3.3.4 Transfection of dechorionated Toliman eggs with Cctra2 dsRNA using a an Intracel TSS20 ovodyne electroporator for induction of the pseudomale phenotype

Successful Elec-eRNAi of medfly eggs with Cctra2 dsRNA should produce a phenotypically male biased sex ratio in adult fly populations. However, the medfly egg chorion presents a physical barrier to dsRNA effectors (Mouzaki et al., 1991) that may prohibit access to the egg membrane and could therefore attenuate Elec-eRNAi. To test this hypothesis, dechorionated Toliman eggs were electroporated in Cctra2 transfection buffer and dsRED2 transfection buffer using an Intracel TSS20 ovodyne electroporator (Figure 3.7). Eggs were treated with dsRED2 dsRNA as a positive control in this assay as it was plausible that Elec-eRNAi of medfly eggs *per se* might cause adult populations to become male biased. Electroporation of dechorionated eggs was carried out at 0, 5, 12.5 and 20V with standardised pulsing parameters (10 pulses of 50ms width at 1 second intervals). Dechorionated eggs were treated with lower voltages than chorionated eggs as the Intracel system did not support treatment with voltages greater than 20V, and at this point the BTX830 system was no longer available. Electroporation of dechorionated eggs with Cctra2 dsRNA at different voltages did not produce significant differences in the sex ratio of adult populations (ANOVA, $F(1,10) = 0.05$, $p > 0.05$). Electroporation of dechorionated eggs with dsRED2 dsRNA did not produce adult populations with significantly different sex ratios (ANOVA, $F(1,10) = 0.18$, $p > 0.05$).

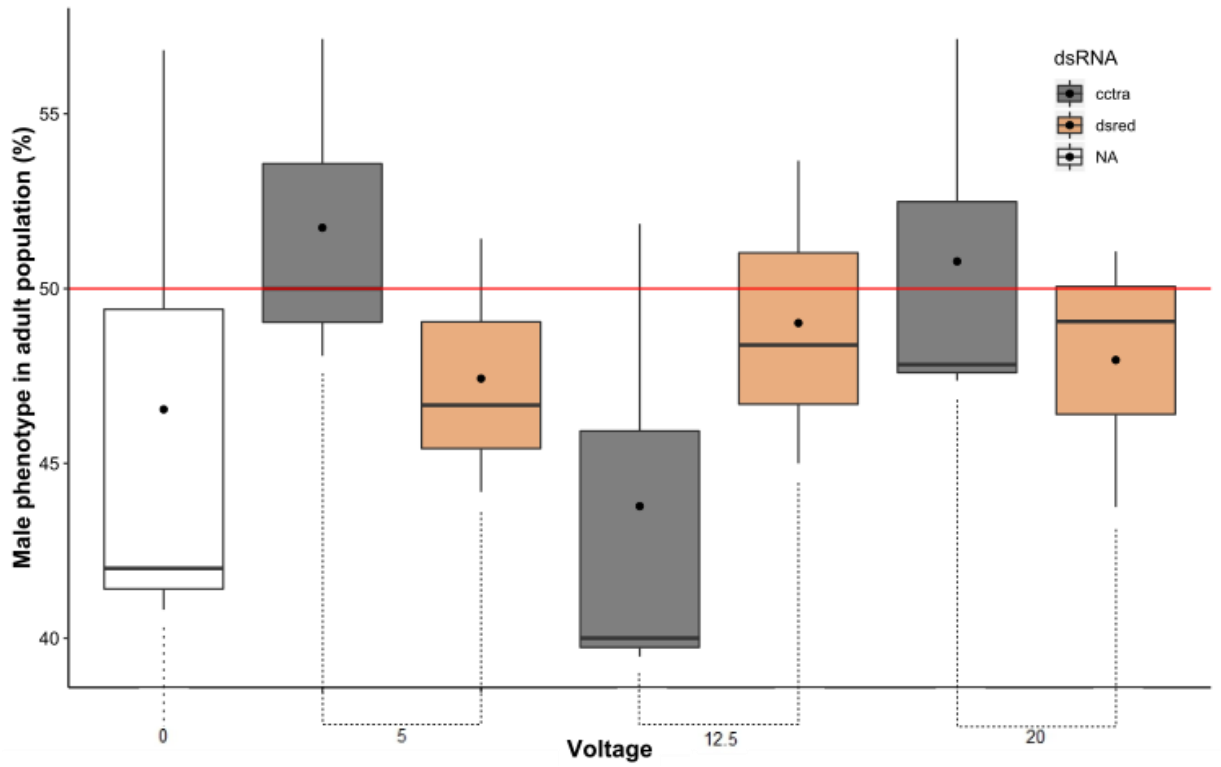

Figure 3.7 The percentage of phenotypically male adult flies in populations that developed from dechorionated eggs that were electroporated with Cctra2 or dsRED2 dsRNA using a BTX830 device: The percentage of male flies in adult populations that were treated with Elec-eRNAi of two discrete genes as eggs is represented on the y-axis. Voltage conditions are represented on the x-axis. The upper and lower hinges of boxplots represent the first and third quartiles of the male ratio in analysed populations. Horizontal boxplot lines represent median male percentage and mean male percentage is represented by a dot. 50% male sex ratio is defined by a red line. Electroporation of dechorionated eggs with Cctra2 dsRNA (ANOVA, $F(1,10) = 0.05$, $p > 0.05$), or dsRED2 dsRNA (ANOVA, $F(1,10) = 0.18$, $p > 0.05$) at different voltages did not produce significantly male biased fly populations.

3.4) Y-Specific PCR to elucidate cryptic pseudomales in populations of phenotypically male flies that were treated with nucleofection program X-005 as eggs

Three of four replicates of eggs that were electroporated with Cctra2 dsRNA using nucleofection program X-005 produced adult populations with a slight male skew (Table 3.2). To elucidate if these populations contained cryptic pseudomales, ten phenotypically male flies from each population were analysed with multiplex PCR. The PCR targets were a 727bp Y-chromosome specific amplicon (Gabrieli et al., 2010) and a 51bp amplicon targeting a medfly ribosomal gene (Rpl13a) (Nash et al., 2018). Pseudomale/female DNA analysed in this manner is Y-chromosome negative and Rpl13a positive, while male DNA is both Y-positive and Rpl13a positive (Figure 3.8). All DNA extracted from phenotypically male flies from replicates one and two amplified both regions of DNA, indicating that there were no pseudomales present in those populations. However, individuals 25 and 29 from replicate 3 amplified Rpl13a but not the Y-specific amplicon, indicating that they might be pseudomales (Figure 3.8A). The DNA of individuals 25 and 29 was therefore subjected to a further round of PCR, during which they both amplified the Y-specific region (Figure 3.8B), demonstrating that they were in fact genetic males. It seems therefore that this assay was not 100% consistent as it produced some type 1 errors, but these errors were detected with repeated rounds of PCR. To be certain that replicate three did not contain any pseudomales, DNA extracted from all 18 phenotypic males was subjected to Y-specific PCR analysis. All 18 individuals amplified the 727bp Y-specific region which established their status as genetic males.

Table 3.2 The sex ratios of populations of adult flies that developed from eggs electroporated with Cctra2 dsRNA using nucleofection program X-005: Four replicates of eggs were electroporated with nucleofection program X-005 in Cctra2 transfection buffer. The sex-ratio skews male in replicates 1-3 but skews drastically female in replicate 4.

Replicate	Males	Females	Total	Males (%)
1	30	21	51	58.8
2	27	23	50	54.0
3	18	13	31	58.1
4	10	23	33	30.3

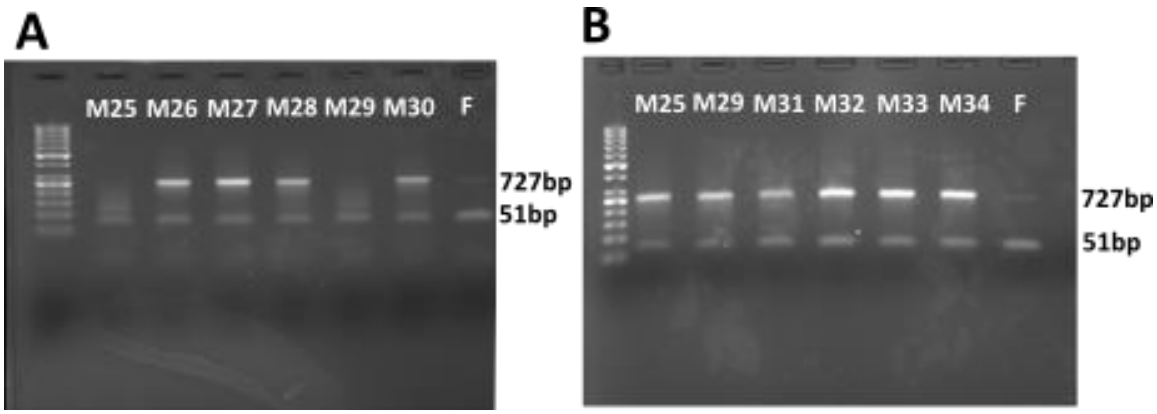

Figure 3.8 Gel images of PCR reactions performed of the DNA of flies that developed from eggs electroporated with Cctra2 dsRNA using nucleofection program X-005: PCR reactions were multiplexed targeting a 727bp Y-specific region (Gabrieli et al., 2010) and a 51bp Rpl13a positive control (Nash et al., 2018). All gels contain six lanes of DNA extracted from phenotypically male flies (M's) and a single lane of female DNA (F). Pseudomale/female DNA analysed in this manner should be Y-chromosome negative and Rpl13a positive, while genetic males should be Y positive and Rpl13a positive. A) Males 25 and 29 both failed to amplify Y-specific DNA but did amplify Rpl13a indicating that they might be pseudomales. All remaining male flies amplified both regions of DNA which is typical of genetic males. As predicted the single female fly only amplified Rpl13a and not Y-specific DNA. B) PCR of M25 and M29 was repeated and produced both Y-specific and Rpl13a amplicons, suggesting that the first PCR runs had produced false positives. All remaining male flies amplified both regions of DNA indicating that they were genetic males. As predicted the single female fly only amplified Rpl13a and not Y-specific DNA.

3.5) Discussion

In this study, I demonstrated that medfly eggs are capable of surviving exposure to electric current and may therefore be amenable to Elec-eRNAi if conditions are optimised. Seven discrete electrical treatment conditions applied with a Nucleofector 2b device did not significantly affected egg survival, although one set of treatment conditions (program D-023) did appear to be detrimental to egg development. However, this analysis did little to inform about the specific electrical limits at which eggs can survive, as the pulsing parameters associated with nucleofection program D-023 are not publicly available. However, in a subsequent experiment in which I was able to manually set pulsing parameters and voltages imposed on eggs, I found that adult populations that developed from eggs treated with 150V contained roughly 80% fewer individuals than populations treated with 125V or 100V. Therefore, it seems that 150V represents an upper limit for trials investigating Elec-eRNAi of medfly eggs.

I then electroporated the eggs of OX3864A medflies (Gong et al., 2005) with dsRNA designed to silence an endogenously expressed dsRED2 marker gene. Eggs were treated with the Nucleofector 2b system then allowed to progress for 64 hours to the L1 larval stage. After 64 hours, dsRED2 expression was assessed with RT-qPCR using the 2–ΔΔCt method (Pfaffl, 2001). Nucleofection of eggs with dsRED2 dsRNA did not significantly affect the expression of dsRED2 in larvae but one treatment group (nucleofection program X-005) did exhibit a slight reduction in dsRED2 expression. As the effect of RNAi is transient (Ding, 2010), it was plausible that dsRED2 expression had been significantly reduced in eggs at the time of treatment but had almost returned to typical levels when tested 64 hours later. It is difficult to be certain whether Elec-eRNAi of dsRED2 in medfly eggs would be detectable after 64 hours, as the latency of RNAi induced gene knockdown varies greatly between insect species (Darrington et al., 2017). Therefore, if the effect of Elec-eRNAi in medfly eggs is to be measured with RT-qPCR then it is recommended that the eggs themselves are subjected to analysis (Lü et al., 2018). Alternatively, eggs could be transfected with expression vectors designed to continuously synthesise siRNA, inducing a permanent gene knockdown effect (Katahira et al., 2003).

I attempted to transfect medfly eggs with dsRNA designed to silence the *transformer 2* gene (*tra2*), as this would produce pseudomale flies that could be used for the sterile insect technique (SIT) (Darrington et al., 2017). RNAi of *tra2* in oocytes has been demonstrated to induce the pseudomale phenotype in the medfly (Salvemini et al., 2009), oriental fruit fly (Liu et al., 2015) and the red flour beetle (Shukla et al., 2012). I quantified the sex ratio of fly populations that developed from eggs treated with eight nucleofection programs in buffer containing Cctra2 dsRNA. The electrical treatment conditions imposed on eggs did not significantly affect the sex ratio of adult fly populations. However, three of four groups of eggs that were treated with program X-005 developed into medfly populations that exhibited a slight male bias (Figure 3.4). This was an interesting result, as Elec-eRNAi of eggs with program X-005 had produced larvae that exhibited a noticeable but insignificant reduction in the expression of dsRED2. To definitively elucidate whether phenotypic males in the X-005 Cctra2 treatment groups were in fact cryptic pseudomales, I analysed the DNA of individuals in those groups with PCR (Salvemini et al., 2009). I found that all phenotypic males in the X-005 treatment groups amplified a Y-specific DNA sequence, which identified them as genetic males and indicated that Elec-eRNAi of Cctra2 with the Nucleofector 2b had been ineffective.

I subsequently electroporated chorionated eggs with a BTX830 device, to transfect Cctra2 dsRNA and induce the pseudomale phenotype (Salvemini et al., 2009). I used identical pulsing parameters in this experiment to those reported in Ruiz et al. (2015), as those authors were able to successfully transfect tick eggs with dsRNA but I varied voltage conditions from 0 to 150V. There was no difference in the sex ratio of adult populations that developed from different electrical treatment groups, but survival was significantly lower for eggs treated with 150V than eggs treated at lower voltages.

Finally, I tested whether the egg chorion (Mouzaki et al., 1991) was attenuating Elec-eRNAi in the Nucleofector 2b and BTX830 systems, by electroporating dechorionated eggs with an Intracel device. As with the previous two experiments I attempted to transfect eggs with Cctra2 to induce the pseudomale phenotype, and the effect was measured via the sex ratios of adult populations (Salvemini et al., 2009). The limitations of the Intracel machine meant that I was not able to treat dechorionated eggs at the same

voltages as in the BTX830 trials. However, the voltages used here have been reported to efficiently electroporate chick (Kinal et al., 2017; Naito et al., 2000) and mouse (Miao et al., 2019; Qin et al., 2015) embryos. Electroporation of dechorionated eggs with the Intracel device did not produce adult populations with the predicted male skew, indicating that Elec-eRNAi of Cctra2 had also been ineffective.

In conclusion, I tested Elec-eRNAi of two gene targets in the eggs of two medfly strains using Nucleofector 2b, BTX830 and Intracel electroporators. My primary goal was to transfect medfly eggs with Cctra2 dsRNA, as this would produce pseudomale flies (Salvemini et al., 2009) that would be amenable to the Sterile Insect Technique (Darrington et al., 2017). I was not able to demonstrate effective Elec-eRNAi using any electroporation device but Elec-eRNAi remains a viable system of control for the medfly, as my trials were not exhaustive. The pulsing parameters and buffers used in my experiments were chosen based on examples from the literature (Ruiz et al., 2015; Zeitelhofer et al., 2007) and applied consistently. However, these variables can greatly affect the effect of electroporation (Gosse et al., 2017). It is known that medfly eggs are RNAi competent when microinjected with dsRNA (Salvemini et al., 2009), so further tests of Elec-eRNAi on medfly eggs may provide positive results if transfection buffers and pulsing parameters are varied.

3.6) Supplementary materials

3.1) Probes and primers used for dsRED2 RT-qPCR analyses

1) dsRED2f (5'-gctccaaggtgtacgtgaacg-3'), dsRED2r-(5'-tgcaagttcatcacgcgc-3'), dsRED2 probe (5' HEX–agaagctgtccttccccgagggct-BHQ1 3').

2) L27f (5'-ccgttattgatttggtgcaa-3'), L27r (5'-gctgtttaggcaaatgtcca-3'), L27 probe (5' TAMRA-cggcccaacaacttgtagtaaccga-BHQ2 3').

3) Rpl13af (5'-acgtttgtccagtgaagtcg-3'), Rpl13ar (5'-tagccttacgtttccgttcc-3'), Rpl13a probe (5' FAM-tcttaacgacatcctggtaatgccagc-BHQ2 3').

3.2) dsRNA nucleotide sequences

dsRED(612bp) -

atggcctcctccgagaacgtcatcaccgagttcatgcgcttcaaggtgcgcatggagggcaccgtgaacggccacgagttcg
agatcgagggcgagggcgagggccgcccctacgagggccacaacaccgtgaagctgaaggtgaccaagggcggccccctg
cccttcgcctgggacatcctgtccccccagttccagtacggctccaaggtgtacgtgaagcaccccgccgacatccccgacta
caagaagctgtccttccccgagggcttcaagtgggagcgcgtgatgaacttcgaggacggcggcgtggcgaccgtgacccag
gactcctccctgcaggacggctgcttcatctacaaggtgaagttcatcggcgtgaacttcccctccgacggccccgtgatgcag
aagaagaccatgggctgggaggcctccaccgagcgcctgtacccccgcgacggcgtgctgaagggcgagacccacaaggc
cctgaagctgaaggacggcggccactacctggtggagttcaagtccatctacatggccaagaagcccgtgcagctgcccggc
tactactacgtggacgccaagctggacatcacctcccacaacgaggactacaccatcgtggagcagtacgagcgcaccgag
ggccgccaccacctgttcctg

GFP(710bp) –

atgagtaaaggagaagaacttttcactggagttgtcccaattcttgttgaattagatggtgatgttaatgggcacaaattttctg
tcagtggagagggtgaaggtgatgcaacatacggaaaacttacccttaaatttatttgcactactggaaaactacctgttccat
ggccaacacttgtcactactttctcttatggtgttcaatgcttttcccgttatccggatcatatgaaacggcatgacttttcaag
agtgccatgcccgaaggttatgtacaggaacgcactatatctttcaaagatgacgggaactacaagacgcgtgctgaagtca
agtttgaaggtgataccttgttaatcgtatcgagttaaaaggtattgattttaaagaagatggaaacattctcggacacaaac
tcgagtacaactataactcacacaatgtatacatcacggcagacaaacaaaagaatggaatcaaagctaacttcaaaattcg
ccacaacattgaagatggatccgttcaactagcagaccattatcaacaaaatactccaattggcgatggccctgtccttttacc

agacaaccattacctgtcgacacaatctgccctttcgaaagatcccaacgaaaagcgtgaccacatggtccttcttgagtttgt
aactgctgctgggattacacatggcatggatgagctctacaaataa

3.7) References

Ando, T., & Fujiwara, H. (2013). Electroporation-mediated somatic transgenesis for rapid functional analysis in insects. *Development, 140*(2), 454. doi:10.1242/dev.085241

Asadi, R., Elaini, R., Lacroix, R., Ant, T., Collado, A., Finnegan, L., . . . Koukidou, M. (2019). Preventative releases of self-limiting *Ceratitis capitata* provide pest suppression and protect fruit quality in outdoor netted cages. *International Journal of Pest Management*, 1-12. doi:10.1080/09670874.2019.1601293

Baum, J. A., & Roberts, J. K. (2014). Chapter five - progress towards RNAi-mediated insect pest management. In S. D. Tarlochan & S. G. Sarjeet (Eds.), *Advances in insect physiology* (Vol. Volume 47, pp. 249-295): Academic Press.

Bona, A. C., Chitolina, R. F., Fermino, M. L., de Castro Poncio, L., Weiss, A., Lima, J. B., . . . Maori, E. (2016). Larval application of sodium channel homologous dsRNA restores pyrethroid insecticide susceptibility in a resistant adult mosquito population. *Parasit Vectors, 9*(1), 397. doi:10.1186/s13071-016-1634-y

Cao, J., Liu, Y., Yang, Y., Zhang, H., Li, Z., Yang, Q., . . . Liu, X. (2015). Molecular characterization and functional analysis of the ultraspiracle (usp) in the oriental fruit moth *Grapholita molesta (Lepidoptera: Olethreutidae). Comparative Biochemistry and Physiology Part - B: Biochemistry and Molecular Biology, 190*, 54-62. doi:10.1016/j.cbpb.2015.08.008

Carmell, M. A., Xuan, Z., Zhang, M. Q., & Hannon, G. J. (2002). The argonaute family: Tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. *Genes and Development, 16*(21), 2733-2742. doi:10.1101/gad.1026102

Chen, C., Smye, S. W., Robinson, M. P., & Evans, J. A. (2006). Membrane electroporation theories: A review. *Med Biol Eng Comput, 44*(1-2), 5-14. doi:10.1007/s11517-005-0020-2

Coleman, A. D., Wouters, R. H. M., Mugford, S. T., & Hogenhout, S. A. (2015). Persistence and transgenerational effect of plant-mediated RNAi in aphids. *Journal of Experimental Botany, 66*(2), 541-548. doi:10.1093/jxb/eru450

Darrington, M., Dalmay, T., Morrison, N. I., & Chapman, T. (2017). Implementing the sterile insect technique with RNA interference – a review. *Entomologia experimentalis et applicata, 164*(3), 155-175. doi:10.1111/eea.12575

Ding, S.-W. (2010). RNA-based antiviral immunity. *Nature Reviews Immunology, 10*(9), 632-644.
doi:10.1038/nri2824

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., & Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature, 411*(6836), 494-498.
doi:10.1038/35078107

Figueira-Mansur, J., Ferreira-Pereira, A., Mansur, J. F., Franco, T. A., Alvarenga, E. S. L., Sorgine, M. H. F., . . . Moreira, M. F. (2013). Silencing of p-glycoprotein increases mortality in temephos-treated *Aedes aegypti* larvae. *Insect Molecular Biology, 22*(6), 648-658.
doi:10.1111/imb.12052

Fire, A., Xu, S. Q., Montgomery, M. K., Kostas, S. A., Driver, S. E., & Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature, 391*(6669), 806-811.
doi:10.1038/35888

Gabrieli, P., Falaguerra, A., Siciliano, P., Gomulski, L. M., Scolari, F., Zacharopoulou, A., . . . Gasperi, G. (2010). Sex and the single embryo: Early deveopment in the Mediterranean fruit fly, *Ceratitis capitata*. *Bmc Developmental Biology, 10*.
doi:10.1186/1471-213x-10-12

Gong, P., Epton, M. J., Fu, G. L., Scaife, S., Hiscox, A., Condon, K. C., . . . Alphey, L. (2005). A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly. *Nature Biotechnology, 23*(4), 453-456.
doi:10.1038/nbt1071

Gosse, C., Zhao, X., Migeotte, I., Suárez-Boomgaard, D., Hue, I., Degrelle, S., . . . Mazari, E. (2017). The use of electroporation in developmental biology. In D. Miklavcic (Ed.), *Handbook of electroporation* (pp. 1-35). Cham: Springer International Publishing.

Hu, X., Richtman, N. M., Zhao, J. Z., Duncan, K. E., Niu, X., Procyk, L. A., . . . Wu, G. (2016). Discovery of midgut genes for the RNA interference control of corn rootworm. *Scientific Reports, 6*.
doi:10.1038/srep30542

Huvenne, H., & Smagghe, G. (2010). Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: A review. *Journal of Insect Physiology, 56*(3), 227-235.
doi:10.1016/j.jinsphys.2009.10.004

Kamdar, K. P., Wagner, T. N., & Finnerty, V. (1995). Electroporation of *Drosophila* embryos. *Methods Mol Biol, 48*, 239-243.
doi:10.1385/0-89603-304-x:239

Karim, S., Troiano, E., & Mather, T. N. (2010). Functional genomics tool: Gene silencing in *Ixodes scapularis* eggs and nymphs by electroporated dsRNA. *BMC Biotechnol, 10*, 1. doi:10.1186/1472-6750-10-1

Katahira, T., & Nakamura, H. (2003). Gene silencing in chick embryos with a vector-based small interfering RNA system. *Dev Growth Differ, 45*(4), 361-367.

Kinal, A., Koz, owska, I., akota, P., Wawrzy, ska, M., & Kijewska, E. (2017). Efficiency of chicken blastoderm cell transfection combining two nonviral methods: Electroporation and synthetic carriers. *Folia Biologica, 65*(1), 32-40. doi:10.3409/fb65_1.33

Kleinhammer, A., Wurst, W., & Kuhn, R. (2010). Gene knockdown in the mouse through RNAi. *Methods Enzymol, 477*, 387-414. doi:10.1016/s0076-6879(10)77020-8

Knipling, E. F. (1955). Possibilities of insect control or eradication through the use of sexually sterile males. *Journal of Economic Entomology, 48*(4), 459.

Leopold, R. A., Hughes, K. J., & DeVault, J. D. (1996). Using electroporation and a slot cuvette to deliver plasmid DNA to insect embryos. *Genetic Analysis: Biomolecular Engineering, 12*(5), 197-200. doi:10.1016/S1050-3862(96)80006-4

Li, X., Dong, X., Zou, C., & Zhang, H. (2015). Endocytic pathway mediates refractoriness of insect *Bactrocera dorsalis* to RNA interference. *Scientific Reports, 5*. doi:10.1038/srep08700

Liu, G., Wu, Q., Li, J., Zhang, G., & Wan, F. (2015). RNAi-mediated knock-down of transformer and transformer 2 to generate male-only progeny in the oriental fruit fly, *Bactrocera dorsalis* (hendel). *Plos One, 10*(6). doi:10.1371/journal.pone.0128892

Lü, J., Chen, S., Guo, M., Ye, C., Qiu, B., Wu, J., . . . Pan, H. (2018). Selection and validation of reference genes for RT-qPCR analysis of the ladybird beetle *Henosepilachna vigintioctomaculata*. *Frontiers in Physiology, 9*(1614). doi:10.3389/fphys.2018.01614

Lu, S. F., Shi, R., Tsao, C. C., Yi, X. P., Li, L. G., & Chiang, V. L. (2004). RNA silencing in plants by the expression of siRNA duplexes. *Nucleic Acids Research, 32*(21). doi:10.1093/nar/gnh170

Maniataki, E., & Mourelatos, Z. (2005). A human, atp-independent, RISC assembly machine fueled by pre-miRNA. *Genes & Development, 19*(24), 2979-2990. doi:10.1101/gad.1384005

Marchini, D., Marri, L., Rosetto, M., Manetti, A. G. O., & Dallai, R. (1997). Presence of antibacterial peptides on the laid egg chorion of the medfly *Ceratitis capitata*. *Biochemical and Biophysical Research Communications, 240*(3), 657-663. doi:10.1006/bbrc.1997.7694

Miao, D., Giassetti, M. I., Ciccarelli, M., Lopez-Biladeau, B., & Oatley, J. M. (2019). Simplified pipelines for genetic engineering of mammalian embryos by CRISPR-cas9 electroporation. *Biology of Reproduction*. doi:10.1093/biolre/ioz075

Mouzaki, D. G., & Margaritis, L. H. (1991). Choriogenesis in the medfly *Ceratitis capitata* (wiedermann) (*Diptera : Tephritidae*). *International Journal of Insect Morphology and Embryology, 20*(1), 51-68. doi:10.1016/0020-7322(91)90027-7

Naito, M., Sano, A., Tagami, T., Harumi, T., & Matsubara, Y. (2000). Efficient transfection of chicken blastoderms in vivo by lipofection and electroporation using green fluorescent protein gene as a marker. *日本畜産学会報, 71*(4), 377-385. doi:10.2508/chikusan.71.377

Nash, W., Mohorianu, I., & Chapman, T. (2018). Mate choice and gene expression signatures associated with nutritional adaptation in the medfly (*Ceratitis capitata*). *bioRxiv*, 362210. doi:10.1101/362210

Pane, A., Salvemini, M., Bovi, P. D., Polito, C., & Saccone, G. (2002). The transformer gene in *Ceratitis capitata* provides a genetic basis for selecting and remembering the sexual fate. *Development, 129*(15), 3715-3725.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research, 29*(9), e45-e45. doi:10.1093/nar/29.9.e45

Qin, W., Dion, S. L., Kutny, P. M., Zhang, Y., Cheng, A. W., Jillette, N. L., . . . Wang, H. (2015). Efficient CRISPR/cas9-mediated genome editing in mice by zygote electroporation of nuclease. *Genetics, 200*(2), 423-430. doi:10.1534/genetics.115.176594

Rand, T. A., Petersen, S., Du, F. H., & Wang, X. D. (2005). Argonaute2 cleaves the anti-guide strand of siRNA during RSIC activation. *Cell, 123*(4), 621-629. doi:10.1016/j.cell.2005.10.020

Ruiz, N., De Abreu, L. A., Parizi, L. F., Kim, T. K., Mulenga, A., Braz, G. R. C., . . . Logullo, C. (2015). Non-invasive delivery of dsRNA into de-waxed tick eggs by electroporation. *Plos One, 10*(6). doi:10.1371/journal.pone.0130008

Salvemini, M., Robertson, M., Aronson, B., Atkinson, P., Polito, L. C., & Saccone, G. (2009). *Ceratitis capitata* transformer-2 gene is required to establish and maintain the autoregulation of cctra, the master gene for female sex determination. *International Journal of Developmental Biology, 53*(1), 109-120. doi:10.1387/ijdb.082681ms

Sandoval-Mojica, A. F., & Scharf, M. E. (2016). Silencing gut genes associated with the peritrophic matrix of *Reticulitermes flavipes (Blattodea: Rhinotermitidae)* increases susceptibility to termiticides. *Insect Molecular Biology*. doi:10.1111/imb.12259

Shukla, J. N., & Palli, S. R. (2012). Sex determination in beetles: Production of all male progeny by parental RNAi knockdown of transformer. *Scientific Reports, 2*. doi:10.1038/srep00602

Team, R. C. (2016). R: A language and environment for statistical computing: R Foundation for Statistical Computing, Vienna, Austria. Retrieved from www.R-project.org

Thomas, D. D., Donnelly, C. A., Wood, R. J., & Alphey, L. S. (2000). Insect population control using a dominant, repressible, lethal genetic system. *Science, 287*(5462), 2474.

Thomas, J.-l. (2003). Electroporation, an alternative to biolistics for transfection of *Bombyx mori* embryos and larval tissues. *Journal of insect science (Online), 3*, 17-17.

Tien, H. T., & Ottova, A. (2003). The bilayer lipid membrane (blm) under electrical fields. *IEEE Transactions on Dielectrics and Electrical Insulation, 10*(5), 717-727. doi:10.1109/TDEI.2003.1237323

Toprak, U., Baldwin, D., Erlandson, M., Gillott, C., Harris, S., & Hegedus, D. D. (2013). *In vitro* and *in vivo* application of RNA interference for targeting genes involved in peritrophic matrix synthesis in a lepidopteran system. *Insect Science, 20*(1), 92-100. doi:10.1111/j.1744-7917.2012.01562.x

Weaver, J. C. (2003). Electroporation of biological membranes from multicellular to nano scales. *IEEE Transactions on Dielectrics and Electrical Insulation, 10*(5), 754-768. doi:10.1109/TDEI.2003.1237325

Whyard, S., Erdelyan, C. N., Partridge, A. L., Singh, A. D., Beebe, N. W., & Capina, R. (2015). Silencing the buzz: A new approach to population suppression of mosquitoes by feeding larvae double-stranded RNAs. *Parasites & vectors, 8*(1), 716-716. doi:10.1186/s13071-015-0716-6

Yan, P., Shen, W., Gao, X., Li, X., Zhou, P., & Duan, J. (2012). High-throughput construction of intron-containing hairpin RNA vectors for RNAi in plants. *Plos One, 7*(5), e38186. doi:10.1371/journal.pone.0038186

Yang, J., & Han, Z.-j. (2014). Efficiency of different methods for dsRNA delivery in cotton bollworm (*Helicoverpa armigera*). *Journal of Integrative Agriculture, 13*(1), 115-123. doi:10.1016/S2095-3119(13)60511-0

Yu, N., Christiaens, O., Liu, J., Niu, J., Cappelle, K., Caccia, S., . . . Smagghe, G. (2013). Delivery of dsRNA for RNAi in insects: An overview and future directions. *Insect Science, 20*(1), 4-14. doi:10.1111/j.1744-7917.2012.01534.x

Zeitelhofer, M., Vessey, J. P., Xie, Y., Tübing, F., Thomas, S., Kiebler, M., & Dahm, R. (2007). High-efficiency transfection of mammalian neurons via nucleofection. *Nature Protocols, 2*, 1692. doi:10.1038/nprot.2007.226

Chapter 4 – Genome sequencing, characterisation and control potential of a *Klebsiella* bacterium isolated from the gut of the Mediterranean Fruit Fly (*Ceratitis capitata*; medfly)

4.1) Abstract

Symbioses between bacteria and their insect hosts can range from very loose associations through to obligate interdependence. Strong mutualistic relationships between microbes and hosts offer the potential for exploitation for insect control via paratransgenesis. For example, bacteria that have co-evolved interdependence can be modified to negatively affect the fitness of their host through paratransgenesis. In this chapter I characterised the 'Medkleb' bacterium to assess whether it might offer an appropriate platform for paratransgenic control. I first confirmed the taxonomic placement of Medkleb and then tested the hypothesis that it had developed a co-evolutionary relationship with the medfly. I did this by sequencing the Medkleb genome and interrogating it for signals of nascent interdependence with the medfly host. I confirmed that Medkleb was a strain *Klebsiella* bacteria which has been suggested to be a facultative mutualist of the medfly, with the capacity to exhibit vertical transmission. The Medkleb genome did exhibit some features consistent with a mutualistic relationship, like small genome size and the synthesis of pectinolytic enzymes. However, there is as yet no evidence that metabolites synthesised by Medkleb are directly transferred to the medfly host. I conclude that, though Medkleb's evolutionary relationship with the medfly requires further investigation, it does nevertheless offer a realistic platform for paratransgenic control of the medfly.

4.2) Introduction

All Eukaryotic organisms host bacteria, which can be located both internally and externally (McFall-Ngai et al., 2013). Some of the best studied associations between microbes and Eukaryotes are those that occur between insects and bacteria (McCutcheon et al., 2011; Moran et al., 2014; Moran et al., 2008). The majority of microbe-insect interactions occur at random through ecological chance and result in temporary associations. However, in some cases, bacteria and insects have remained persistently associated and have evolved to become co-dependent (Moran et al., 2008).

In the most extreme cases, associations between bacteria and their insect hosts can persist for hundreds of millions of years (Moran et al., 2005). In cases where the association between bacteria and insects has stabilised, various types of co-evolutionary relationships can develop (Moran et al., 2008). Heritable symbioses are one category of such co-evolutionary relationship and are defined by direct passage of bacteria from insects to progeny, usually via maternal transmission (Moran et al., 2008).

Heritable symbioses are subcategorised as either facultative or obligate, based on the ability of the bacteria to survive when separated from their host. Facultative symbionts can survive when isolated from their host, but obligate symbionts are host-dependent and die when separated from the host-niche (Moran et al., 2008). Over evolutionary time, facultative symbionts may lose genes that facilitate life in varied environments through purifying selection and Muller's ratchet (Moran et al., 2014). Progressive gene-loss can limit the bacteria's environmental range, eventually preventing survival outside the host-niche and the facultative symbiosis becomes obligate (Moran et al., 2008).

Host insects provide heritable symbionts with a fitness benefit in the form of a safe, stable niche but if the microbe provides the host with a fitness benefit in return, then the relationship is described as mutualistic (Moran et al., 2008). Mutualistic traits provided to insects by bacteria can include synthesis of: 1) nutrients (Sabree et al., 2013; Sinotte et al., 2018; Storelli et al., 2018), carotenoids (Sloan et al., 2012) and 3) molecules that provide protection from predators (Oliver et al., 2003; Sinotte et al., 2018). Hence a

facultative mutualist is a bacterium that is heritable but not obligate and provides its insect host with a fitness benefit (Moran et al., 2008).

The heritable symbiont group includes the "reproductive parasite" subgroup. Reproductive parasites enhance their own fitness by distorting the sex ratio of the host's offspring, an action that diminishes the fitness of the host themselves (Hurst et al., 2015). For example, via manipulation of epigenetic markers, male *Zyginidia pullulan* leafhopper embryos are feminised by maternally inherited *Wolbachia* bacteria (Negri et al., 2009). A female biased F1 generation benefits *Wolbachia,* which preferentially resides within the cells of the ovaries, and hence, is found in densities four orders of magnitude higher in female insects over males (Negri et al., 2009). However, facultative mutualists and reproductive parasites are not mutually exclusive categories, as symbionts can, simultaneously, selfishly manipulate host reproduction whilst providing them with a fitness benefit (Ballinger et al., 2017).

The 21st century has seen an explosion in research relating to bacterial symbionts of insects. The *Buchnera* symbionts of aphids are probably the most extensively studied and represent an example of an advanced obligate mutualism (Shigenobu et al., 2000; Tamas et al., 2002; van Ham et al., 2003). However, there is a burgeoning body of research into an ever increasing variety of symbionts of various phyla (Anbutsu et al., 2017; Ballinger et al., 2017; de Souza et al., 2009; Heine et al., 2018; Holmes et al., 2016; Weiss et al., 2016; Whitten et al., 2015).

Typically, *Diptera* host a diverse bacterial microbiome which they obtain primarily from their diet (Chandler et al., 2011; Woruba et al., 2019). However, it is common for the gut microbiomes of *Diptera* to include only a small, consistent group of bacteria (Chandler et al., 2011; Deguenon et al., 2019; Gould et al., 2018). For example, the gut microbiome of *Drosophila melanogaster* is consistently formed by a core of five species, *Lactobacillus plantarum, Lactobacillus brevis, Acetobacter pasteurianus, Acetobacter tropicalis and Acetobacter orientalis* (Gould et al., 2018). Furthermore, the gut microbiomes of *Diptera* are often consistent, regardless of the host's ecology or geographic origin (Ben Ami et al., 2010; Chandler et al., 2011; Liu et al., 2016). That certain bacteria reliably colonise the gut while others do not, suggests that the host may

select and retain potentially mutualistic species, which is supported by evidence that *Drosophila melanogaster* can actively regulate its gut microbiome (Bosco-Drayon et al., 2012; Lhocine et al., 2008; Lindberg et al., 2018).

Selection by a host of specific, beneficial gut bacteria is classic co-evolution between two organisms. However, bacteria within the gut microbiome can form part of a functional community by supporting the growth of different species. Hence, it may be a viable evolutionary strategy for a host to acquire a gut bacterium that provides only a slight fitness benefit, or no benefit at all, if that species supports the growth of another more beneficial species. Such higher order interactions frame the gut microbiome as a network of co-evolved of organisms, where evolutionary *quid pro quo* can be difficult to ascertain. For example, Gould et al. (2018) report that the five core members of *Drosophila melanogaster's* gut microbiome interact with one another to enhance the fecundity of the host. Fecundity increased when Gould et al. (2018) fed germ-free flies with any of these five bacteria in isolation but in some cases combining bacteria had as large an effect on fecundity as feeding any individual species.

The gut microbiomes of pest and non-pest fruit flies reflect the distinct life-history strategies of their hosts. As discussed above, the gut microbiome of *Drosophila melanogaster* is formed by five core species which metabolise lactic acid and acetic acid. The metabolic capacity of *Drosophila's* gut microbiome benefits larvae which hatch into rotten fruit. However, the *Tephritidae* family of "true fruit-fly" pests hatch in unripe fruit, and hence, the gut microbiomes of *Tephritidae* frequently contain pectinolytic bacteria that can assist the host in breaking down plant cell walls (Behar et al., 2008; Ben-Yosef et al., 2014; Liu et al., 2016).

In addition to host selection, gut bacteria will often exhibit traits that facilitate the colonisation of their target species. For example, Abraham et al. (2017) report that *Anaplasma phagocytophilum* induces its host (*Ixodes scapularis*) to produce an antifreeze protein that alters the composition of its pre-existing gut flora and that this facilitates the bacteria's passage across the gut barrier. This type of co-evolved intimacy between microbes and insects has the potential to be manipulated for novel modes of control. Paratransgenesis, which is the genetic manipulation of heritable bacteria for transfer of

desired traits to host organisms, is one such manipulation (Durvasula et al., 1997). For example, Whitten et al. (2015) genetically manipulated *Rhodococcus rhodnii* bacteria to deliver effector molecules that had been designed to reduce expression of the vitellin gene by its host *Rhodnius prolixus,* resulting in reduced fecundity for the insect.

To be applicable for insect control, bacteria that have co-evolved with insects may not even require genetic modification. For example, the Sterile Insect Technique (SIT) generally requires the irradiation of insects (Knipling, 1955) which has well documented fitness costs (Guerfali et al., 2011; Toledo et al., 2004). The reduced fitness of irradiated insects is a weakness of SIT, which requires the release of large numbers of competitive males to be effective. Potentially, a rejuvenating probiotic diet containing mutualistic bacteria can be fed to irradiated males to compensate for the fitness costs inflicted by irradiation (Jurkevitch, 2011). For example, Gavriel et al. (2011) found that irradiated male Mediterranean Fruit Flies (*Ceratitis capitata;* medfly) fed *Klebsiella oxytoca* bacteria were longer lived than flies fed a sterile diet.

Several studies have identified *Klebsiella* bacteria as a major component of the medfly microbiome (Aharon et al., 2013; Behar et al., 2008a; Ben Ami et al., 2010). Behar et al. (2008a) were able to demonstrate that *Klebsiella oxytoca* dominate the microbiome of medfly larvae, pupae and adult guts, in both wild populations and lab specimens. Behar et al. (2008a) suggest that during oviposition, medfly transmit *K. oxytoca* to fruit, the fruit is then degraded due to the pectinolytic activity of the bacteria, and both fruit and bacteria are subsequently consumed by developing larvae. Furthermore, they report that the nitrogen fixing *nifH* gene is expressed in larvae and that this signal is probably of bacterial origin (Behar et al., 2008a). In a later study, Ben Ami et al. (2010) used Amplified rDNA restriction analysis (ARDRA) to demonstrate that *Klebsiella* bacteria compose between 20-30% of the medfly gut microbiome. Consistent with Ben Ami et al. (2010), a study by Leftwich et al. (unpublished) also found that *K. oxytoca* forms between 20-30% of the medfly microbiome, regardless of the flies' geographical origin or diet. Ben Ami et al. (2010) irradiated medfly pupae then 16S sequenced the guts of adult flies and demonstrated that irradiation causes the bacterial community to shrink drastically in absolute size, being particularly toxic to *Klebsiella* bacteria. Reintroduction of *K. oxytoca*

to irradiated flies significantly reduced mating latency in comparison to males fed sterile diet (Ben Ami et al., 2010).

Although *K. oxytoca* has been demonstrated by several groups to be a core component of the medfly microbiome, the nature of the relationship between host and microbe is yet to be properly defined. Based on available literature, it is unclear whether *K. oxytoca* confers fitness to the medfly, or if it is heritable. Behar et al. (2008a) suggest that *K. oxytoca* is heritable and transmitted during oviposition. In support of this, these authors provide evidence that *K. oxytoca* is present on medfly eggs via denaturing gradient gel electrophoresis of 16S rDNA products. However, the data are equivocal as *K. oxytoca* was only found in 1 of 4 egg replicates and there was no treatment to prevent transmission of *K. oxytoca* during oviposition, to provide a negative control. There is as yet no direct evidence of the fitness benefits that might be conferred to the medfly by *K. oxytoca,* or indeed what *K. oxytoca* gains from its association with the medfly host. Behar et al. (2008a) report that *K. oxytoca* has the capacity for pectinolysis and possibly for nitrogen fixation, but there is currently no direct evidence of whether the medfly derives any resulting benefit. The best evidence of mutualism comes from Behar et al. (2008b), Ben Ami et al. (2010) and Gavriel et al. (2011). The Gavriel et al. (2011) study, for example, experimentally depleted the microbiome of male medflies with irradiation, then fed them either a diet either containing *K. oxytoca* (*pro*) or a sterile diet (*ster*). Gavriel et al. (2011) found that *pro* flies outcompeted *ster* flies for mating opportunities, and that females mated to *pro* flies were less inclined to re-mate than females mated to *ster* flies. However, the initial depletion of the microbiome in the Gavriel et al. (2011) study and related studies (Behar et al., 2008b; Ben Ami et al., 2010) was achieved by a toxic insult to the flies in the form of irradiation and antibiotics. Therefore, whether any fitness benefit experienced by the flies in these studies is representative of natural populations is yet to be determined.

The aim of this study was to sequence and analyse the genome of the putative *K. oxytoca* (hereafter "Medkleb") extracted from the gut of medflies from the Toliman wild type strain of medfly. Analysis of the full genome sequence allowed me to confirm the phylogenetic placement of Medkleb within other sequenced free-living and host-

associated *K. oxytoca,* as well as other *Klebsiella* species. Importantly, it allowed me to test the key hypothesis that Medkleb exhibits genomic features characteristic of a nascent evolutionary interdependence with its medfly host. To do this, I assembled and annotated the Medkleb genome sequence, then interrogated it for signatures of facultative transition. Initially, I investigated whether there had been a reduction in Medkleb's genome size or GC content (McCutcheon et al., 2011; Moran et al., 2014; Moran et al., 2008). I then conducted functional comparisons between Medkleb and conspecifics to reveal phenotypes that might potentially facilitate a mutualistic relationship with the medfly host, or alternatively indicate a restriction to the environments in which Medkleb can live. My analyses of the Medkleb genome sequence were complemented with a functional test for pectinolysis. Medkleb's pectinolytic ability was investigated to both uncover a potential function that might benefit its medfly host and also to confirm Medkleb's identify. The applied importance of this work is that evidence for an incipient mutualistic association could offer the potential for manipulation of Medkleb for control (Behar et al., 2008b; Behar et al., 2008b; Ben Ami et al., 2010; Gavriel et al., 2011).

4.3) Methods

4.1) DNA preparation for Medkleb genome sequencing

Medkleb clones were first streaked onto LB media (15g/L Agar; 5g/L NaCl; 5g/L yeast extract; 1.5g/L glucose; 10g/L tryptone) and incubated overnight at 25°C. All resulting bacteria were scraped into a 1.5ml microcentrifuge tube containing 1ml of 10% glycerol. The sample was vortexed for 30 secs then centrifuged at 12000 RPM for 10 mins. Glycerol was removed, and the pelleted bacteria were re-suspended in 2ml of SET buffer (65% v/v molecular grade H_2O (ThermoFisher); 20% v/v Tris (pH8); 5% v/v 5M NaCl; 5% v/v 10% SDS; 5% v/v 0.5M EDTA) before transfer to a 15ml falcon tube. 20µg of lysozyme (Sigma) and 0.4µg achromopeptidase (Sigma) suspended in 40µl of molecular grade water (ThermoFisher) and 0.02µg of RNase (Fermentas) were added. The sample was mixed gently then incubated at 37°C for 2hrs. 240µL of 10% sodium dodecyl sulphate and 56µL of proteinase K (20mg/ml) were added, before a second incubation at 56°C for 2hrs, with manual mixing every 30 mins. 800µL of 5M NaCl and 2ml of chloroform were added and the sample was mixed by hand for 10 mins, before centrifugation at 4000 RPM for 12 mins. The aqueous phase was then carefully transferred to a fresh tube. DNA was precipitated in 0.6 volume isopropanol, then transferred to a 1.5ml Eppendorf tube by pipette. DNA was washed once with 70% ethanol. Ethanol was removed, then 1ml of 70% ethanol was added, and DNA was left to incubate overnight at 4°C. Ethanol was removed again, and DNA was re-suspended in 200µL of molecular grade water (ThermoFisher).

4.2) Single molecule real time (SMRT/PacBio) Medkleb genome sequencing

DNA purity, concentration, and average fragment size were analysed using Nanodrop (ThermoFisher), Qubit v2.0 (Invitrogen) and Agilent Tapestation 4200 (Agilent) respectively. DNA was fragmented using a G-tube (Covaris), and SMRTbell library construction was carried out using a Template Prep Kit 1.0 (PacBio). The library was then size selected to >7kb using the BluePippin system (Sage Science). The library was quantified by Qubit (Invitrogen) and the average size of the library was analysed using the Agilent Tapestation 4200 (Agilent). Sequencing was carried out on a Pacific

Biosciences RSII instrument, using two RSII SMRTcells v3 (PacBio) and P6-C4 chemistry (PacBio). Each cell was sequenced using a 240-minute movie, using the Magbead OCPW v1 protocol (PacBio).

4.2.1) Medkleb genome assembly

The Medkleb genome was assembled according to the Hierarchical Genome-Assembly Process (HGAP.3) protocol (Chin et al., 2013) as follows. 1) Mapping – BLASR (Chaisson et al., 2012) was used to map reads >500bp with a read quality >0.8 to seed reads >6000bp. 2) Pre-assembly - The Directed Acyclic Graph Consensus (DAGCon) algorithm (Lee et al., 2002) was used to produce a consensus sequence based on BLASR mapping. DAGCon then trimmed the consensus, producing an error-corrected pre-assembled read. 3) *de novo* genome assembly - The overlap-layout-consensus assembler Celera Assembler v8.1 was used to process the pre-assembled read into a draft assembly. 4) Final consensus - the draft assembly was polished using the Quiver multiread consensus algorithm (Chin et al., 2013). 5) The final consensus sequence was then manually trimmed to circularise the genome and place the stop codon (TGA) of the dnaA gene at the 5' terminus.

4.3) Medkleb genome quality control

An estimated Quiver quality value (QV) for the Medkleb final consensus genome was provided by the Earlham Institute. Genome completeness was estimated with both benchmarking universal single copy orthologues (BUSCO) software 3.0.0 (Simao et al., 2015), and CheckM (Parks et al., 2015). The *Enterobacteriales* order and *Enterobacteriaceae* family were used as reference datasets for BUSCO and CheckM analyses respectively. Genome contamination was estimated with CheckM (Parks et al., 2015).

4.4) Medkleb contig classification

mlplasmids (Arredondo-Alonso et al., 2018) was used to classify contigs as either chromosomal or plasmid DNA. The *Klebsiella pneumoniae* support-vector machine (SVM) model was utilised for the analysis, with minimum posterior probability specified at 0.7 and minimum contig length at 1,000nt.

4.5) Medkleb gene calls, annotation and genome mapping

Coding sequences within the Medkleb chromosomal DNA and plasmids, were called with the Prodigal algorithm (Hyatt et al., 2010). Gene calls were then annotated with Classic-RAST (Overbeek et al., 2014). Ribosomal RNAs (rRNAS) and transfer RNAs (tRNAs) were called and annotated with Classic-RAST. Circular maps were created for the Medkleb chromosomal DNA and plasmids using DNAplotter (Carver et al., 2009).

4.6) Prediction of Medkleb's higher order metabolic functions and secondary metabolites

The higher order metabolic functions of genes were predicted using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) (Kanehisa et al., 2016). Secondary metabolites were predicted using antiSMASH 3.0 (Weber et al., 2015).

4.7) Identification of Medkleb's 16S sequence

The Medkleb genome was searched for regions homologous to the 16S sequence of *K. oxytoca* strain ATCC 13182 (NR_118853.1) with BLASTn (Altschul et al., 1997). The region with greatest homology to NR_118853.1 (nts 341370-342821) was then parsed with RNAmmer 1.2 (Lagesen et al., 2007) which predicts ribosomal genes.

4.8) Phylogenetic analyses of Medkleb sequences

4.8.1) Phylogenetic tree construction using 16S sequence data

Sixty-one 16S nucleotide sequences representing 60 *Klebsiella* strains*, Pseudomonas aeruginosa* strain JB2 and a putative Medkleb 16S sequence were used to create a phylogeny with the SILVA ACT web app (Pruesse et al., 2012). Where possible, non-redundant sequences were extracted from the SILVA rRNA database (Quast et al., 2013). All sequences were almost complete (>1400bp) and met the standard operating procedure for phylogenetic inference (SOPPI) quality criteria set out by Peplies et al. (2008). The tree was aligned and bootstraps were computed with the FastTree2 maximum likelihood program (Price et al., 2010) using the GTR evolutionary model and gamma distribution parameters (Yang, 1994). The resulting phylogeny was constructed with FigTree 1.4.3 (Rambaut et al., 2009).

4.8.2) Phylogenetic tree construction using Average Nucleotide Identity (ANI)

35 RefSeq whole genome entries extracted from the NCBI database (Marchler-Bauer et al., 2009), representing four *Klebsiella* species were used to calculate a phylogeny based on Average Nucleotide Identity (Konstantinidis et al., 2005). The ANI Calculator (Figueras et al., 2014) was used to compute the phylogeny using the BIONJ algorithm (Gascuel, 1997). The tree was constructed with FigTree 1.4.3 (Rambaut et al., 2009).

4.9) Analysis of synteny between Medkleb and closely related bacteria

To predict recently acquired Medkleb traits, the genome was aligned with three closely related bacteria. Genomes were manually re-ordered to place the dnaA stop codon at the 5' terminus. Synteny was then predicted with progressiveMauve (Darling et al., 2010).

4.10) Culture of bacteria for pectinolytic analyses

Bacteria were cultured in LB broth (5g/L NaCl; 5g/L yeast extract; 1.5g/L glucose; 10g/L tryptone) which had been stored in 50ml glass bottles and autoclaved prior to use. Each bottle was inoculated with a "loop" of bacteria and incubated in an orbital incubator (New Brunswick Scientific Innova 44) at 200RPM. Medkleb and *Erwinia carotovora* were incubated at 37°C and *Rhizobium leguminosarum* was incubated at 28°C until optical density was greater than 1.0 at 600nm.

4.11) DNA preparation for PCR of pehX and 16S

Bacterial DNA was extracted using a DNeasy blood and tissue kit (Qiagen) and microbe lysis buffer (MLB) (20 mg/ml of lysozyme (Sigma) and 5mg/ml of achromopeptidase (Sigma) in 20 mM Tris-HCl, 2 mM EDTA, 1.2% Triton X (pH 8.0)). 2ml of Medkleb, *Erwinia carotovora* and *Rhizobium leguminosarum* cultures were centrifuged at 13K RPM for 5 mins, before the supernatants were removed. Pellets were then homogenised with a clean pestle in liquid nitrogen. 180µl of MLB was added before the sample was vortexed and incubated at 37°C for two hours. Samples were vortexed every 30 mins during the two-hour incubation. Buffer AL and ethanol were mixed 1:1 (Buffer ALE) and warmed to 55°C. Each sample had 400µl of warm Buffer ALE added before being immediately

vortexed for 10-15 secs. Samples were transferred to a spin column and centrifuged at 8000RPM for 60 secs. 500µl of Buffer AW1 was added and the sample was centrifuged again at 8000RPM for 60 secs. 500µl of Buffer AW2 was added and the samples were centrifuged at 13 000 RPM for 4 mins. 35µl of warm Buffer AE (60°C) was added to the centre of the spin membrane and the samples were centrifuged at 6000RPM for 60 secs. DNA purity and concentration were measured using a Nanodrop (ThermoFisher).

4.12) Bioinformatic analysis of pehX and pectinolytic enzymes to distinguish *K. oxytoca* from *K. michiganensis*

The *K. oxytoca* polygalacturonase gene pehX (AY065648.1) was aligned to genomes of *Klebsiella* bacteria using BLAST (Altschul et al., 1997). The presence of polygalacturonases in genomes of *Klebsiella* bacteria was assessed using the Carbohydrate-Active enZymes database (CAZy) (Lombard et al., 2010).

4.13) PCR analysis of pehX and 16S genes

As in Kovtunovych et al. (2003) the pehX gene was amplified using PEH primers and the 16S gene was amplified using 541F/806R primers (Caporaso et al., 2011) (see supplementary materials 4.1). PCR parameters were the same for both assays: 20µl PCR reactions were set up with 10µl of Ultramix PCR buffer (PCR biosystems), 1µl of forward primer (5µM), 1µl of reverse primer (5µM), 1µl of DNA (10ng/µl) and 5µl of molecular water (ThermoFisher). Cycling parameters were: 1) 95°C for 15 mins, 2) 35 cycles of 95°C for 30 secs, 55°C for 30 secs and 72°C for 30 secs, 3)72°C for 10 mins.

4.14) Polygalacturonase enzyme assay

Polygalacturonase production of Medkleb, *Erwinia carotovora* and *Rhizobium leguminosarum* was measured using a DNS colorimetric method (Miller, 1959) with a protocol adapted from Sohail et al. (2016) and Sigma Aldrich protocol EC 3.2.1.1. Cultures were diluted with LB broth (5g/L NaCl; 5g/L yeast extract; 1.5g/L glucose; 10g/L tryptone) to an optical density of 1.0 at 600nm. Bacteria were then filtered from culture media with 0.2µm PES syringe filters (ThermoFisher). Treatment reactions (which were run in triplicate) were set up with 1ml of appropriate filtrate and 1ml of polysaccharide solution

(PS) (0.9% polygalacturonic acid (ThermoFisher) in 0.1M sodium acetate (ThermoFisher) (pH 4.5)), a blank reaction was set up with 1ml of PS only. All reactions were incubated at 45°C for 30mins before 1ml of colour reagent solution (20% 5.3M potassium sodium tartrate, tetrahydrate in 2M sodium hydroxide solution; 50% 96 mM 3,5-Dinitrosalicylic acid solution; 30% molecular water (ThermoFisher)) was added. All reactions were incubated at 100°C for 15 mins, then placed on ice to cool to room temperature. Once cooled, 12ml of molecular water (ThermoFisher) was added to each reaction, followed by hand mixing. Absorbance at 530nm (ΔA530) was measured using a spectrophotometer (Biochrom) which had been blanked for air, with the corrected ΔA530 for treatment reactions being calculated as:

$$\Delta A530\ (Treatment\ reaction) = \Delta A530\ (Treatment\ reaction) - \Delta A530\ (Blank\ reaction)$$

Units of polygalacturonase in filtrate were calculated via comparison to a standard curve of galacturonic acid (Sigma). Standards were made with between 50µl and 1 ml of monosaccharide solution (MS) (1.8% galacturonic acid (ThermoFisher) in 0.1M sodium acetate (ThermoFisher) (pH 4.5)) and topped up to 2ml total volume with molecular water (ThermoFisher). A standard blank was set up containing 2ml of molecular water only. 1ml of colour reagent solution was then added before incubation at 100°C for 15 mins. Standards were placed on ice to cool to room temperature before 12ml of molecular water was added and ΔA530 was measured using a spectrophotometer (Biochrom) which had been blanked for air. The corrected ΔA530 for standards was calculated as:

$$\Delta A530\ (Standard) = A530\ (Standard) - A530\ (Blank)$$

The standard curve was used to estimate mg of galactose released in treatment reactions with linear regression, and units of polygalacturonase per ml of filtrate were then calculated using the formula:

$$Units/ml\ enzyme = (mg\ of\ galactose\ released)/ml\ of\ filtrate$$

4.4) Results

4.1) Classification of Medkleb sequencing contigs

Total Medkleb DNA was sequenced on a PacBio RSII module and assembled using the HGAP.3 algorithm and Quiver (Chin et al., 2013). This process detected one large contig that sequestered 94% of total gene space (Figure 4.2) and four small contigs (mkp1-4; Figure 4.3). Plasmid genomes are generally much smaller than bacterial chromosomes (Shintani et al., 2015), which supports the hypothesis that the total Medkleb DNA complement is formed of one chromosome and four plasmids. Consistent with this, the mlplasmids software (Arredondo-Alonso et al., 2018) classified the large Medkleb contig as chromosomal and the four smaller contigs as plasmids (Table 4.1). In addition, mkp2, mkp3 and mkp4 were sequenced with relatively high coverage depth (Figure 4.1) which indicated high copy number, a common plasmid trait (Providenti et al., 2006). Finally, mkp3 and mkp5 were demonstrated to have low GC content relative to the putative chromosome (Table 4.1), which is characteristic of plasmid DNA (Nishida, 2012). Although mkp2 and mkp4 both exhibited GC content roughly equivalent to that of the chromosome this does not automatically disqualify them from classification as plasmids, as they are likely to be recently acquired, and hence may not yet exhibit the characteristic lower GC content (Rocha et al., 2002).

Table 4.1 Contig derivation and GC content. mlplasmids (Arredondo-Alonso et al., 2018) predicted that the largest of the five Medkleb contigs was chromosomal, with a posterior probability (p(θ\X)) of 0.991. mkp2 and mkp5 were predicted to be plasmid-derived, with posterior probabilities of 0.962 and 0.977 respectively. The posterior probabilities of mkp3 and mkp4 being plasmid-derived were less powerful but still robust, at 0.864 and 0.882 respectively. mkp2 and mkp4 both had GC content comparable to that of the putative chromosomal contig, suggesting they had arisen as a result of a recent horizontal transfer.

contig	**length**	**p(θ\X)**	**Prediction**	**GC**(%)
1	5825435	0.009	Chromosome	56.03
2	136402	0.962	Plasmid (mkp1)	55.18
3	122124	0.864	Plasmid (mkp2)	50.4
4	78046	0.882	Plasmid (mkp3)	56.68
5	63257	0.977	Plasmid (mkp4)	50.65

4.2.1) Quality control – resolution of Medkleb sequencing contigs

PacBio coverage depth >100X is considered sufficient for resolving nucleotide sequences (Rhoads et al., 2015), a threshold met by all contigs other than mkp4 (Figure 4.1). Sequencing resolution >99.999% which equates to a QV of 50 (Chin et al., 2013) is the gold standard for finished bacterial genomes (Bowers et al., 2017; Chain et al., 2009). With a QV of 48.9 (an average error rate of 1 base in every 80100), the resolution of the Medkleb chromosome can be considered very high quality (Figure 4.1). The plasmid QV's were: mkp1) 48, mkp2) 47.3, mkp3) 45.3 and mkp4) 44.8 (Figure 4.1). Although the plasmid sequences had lower resolution than the chromosome, they were robust, with accuracy >99.994% in all cases.

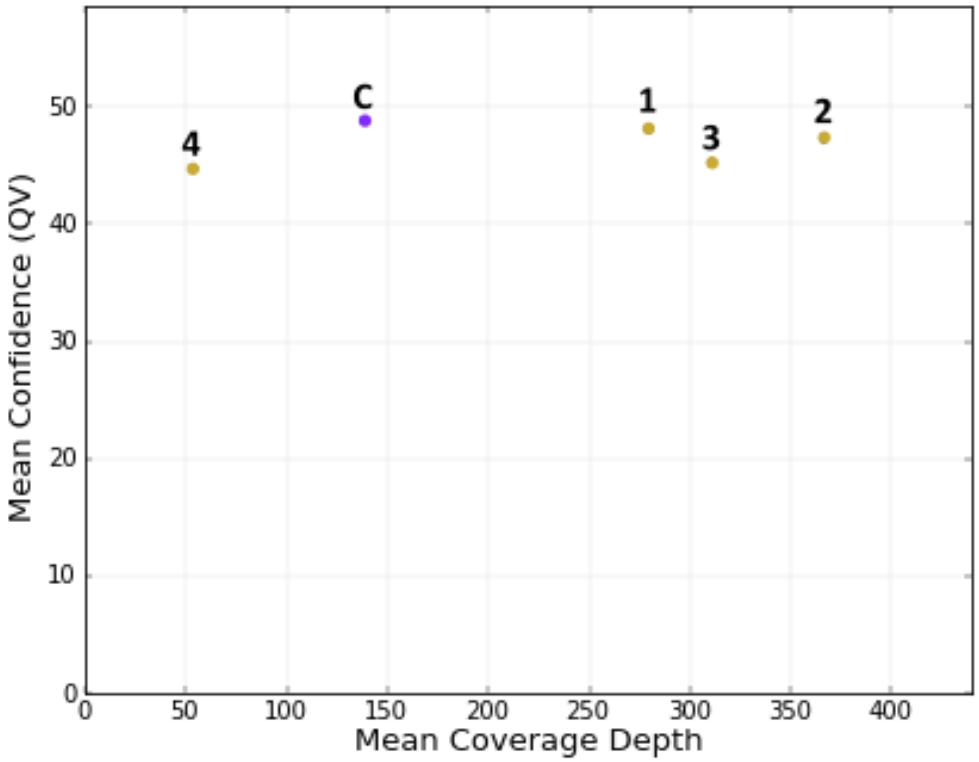

Figure 4.1 Contig coverage Vs quality value for Medkleb sequencing contigs. The mean coverage depth for the Medkleb chromosome (purple dot) was 138.7 reads per base, and mean QV was estimated at 48.9. The four plasmids (yellow dots) had mean coverage depths and QV's of: mkp1) 279.6 and 48; mkp2) 366.4 and 47.3; mkp3) 310.8 and 45.3; mkp4) 53.6 and 44.8. Coverage was relatively high in the cases of mkp1-3, which was as predicted, as plasmids are often retained in high copy number.

4.2.2) Quality control – Medkleb chromosome completeness and contamination

Bowers et al. (2017) state that high quality bacterial genomes have been achieved if assemblies are >90% complete and <5% contaminated. The completeness of the Medkleb genome was measured with BUSCO (Simao et al., 2015), which was used to search the assembly for 781 marker genes associated with bacteria of the *Enterobacteriales* order. BUSCO estimated that the Medkleb chromosomal sequence was 99.5% complete (777 of 781 genes complete and single copy), far surpassing the quality threshold set by Bowers et al. (2017). Furthermore, 3 genes that were predicted by BUSCO to be fragmented were likely heterozygous alleles that failed to collapse during the annotation, and a single missing gene is an acceptable level of gene loss (Wyder et al., 2007). In addition to BUSCO (Simao et al., 2015), CheckM (Parks et al., 2015) was used to assess the Medkleb chromosome for completeness and contamination. Via reference to 1162 marker genes associated with the family *Enterobacteriaceae*, CheckM estimated that the Medkleb genome was 99.7% complete and 0.212% contaminated, which again far exceeded the Bowers et al. (2017) quality threshold.

4.3.1) Medkleb genome synopsis – chromosomal DNA

Gross features of the Medkleb sequence were consistent with genomes published as *Klebsiella oxytoca* (Bao et al., 2013; Shin et al., 2012). The Medkleb genome was found to be 5867451nt in length, with 5388 coding sequences and a GC content of 56.03% (Figure 4.2; Table 4.2). At 87.8%, overall coding sequence was within the expected range (Kuo et al., 2009), and, as predicted by Reva et al. (2004), genes were distributed symmetrically between the two DNA strands. There were 2542 coding sequences on the positive strand, which were predicted to code for 2474 proteins, 50 tRNAs and 18 rRNAs. On the negative strand there were 2847 coding sequences which coded for 2805 proteins, 35 tRNAs and 7 rRNAs. 16 (64%) ribosomal RNAs (rRNA) clustered between nts 62435-700701 near the origin of replication (oriC), a feature which has been suggested to support fast growth through multi-fork replication (Rocha, 2004b; Vieira-Silva et al., 2010). However, Medkleb traits were not all consistent with fast growth. Medkleb was predicted to code for a wide variety of tRNAs in low copy number (see supplementary materials – Table 4.1), but Rocha (2004a) suggests that fast growth is associated with a

low diversity of tRNAs retained in high copy number. Medkleb's GC content was 57.28% for protein coding genes, 53.83% for rRNA and 58.93% for tRNA. Consistent with Lobry (1996) GC skew was asymmetric, with an overrepresentation of G's on the leading strand and C's on the lagging strand. GC skew was inverted (>50% C's) between bases 654265-677695 (GI1), which is indicative of a recent introgression that has resulted in the acquisition of a new gene island (Lawrence et al., 1997; Wixon, 2001).

Table 4.2 Synopsis of the Medkleb genome. The Medkleb genome was 5867451 nt in length and coded for a total of 5388 genes, at a coding density of 0.941 genes per kb.

Length (nt)	5867451
GC content (%)	56.03
CDS (+ve strand)	2542
CDS (-ve strand)	2847
Overall coding sequence (%)	87.8
Gene density (per kb)	0.941

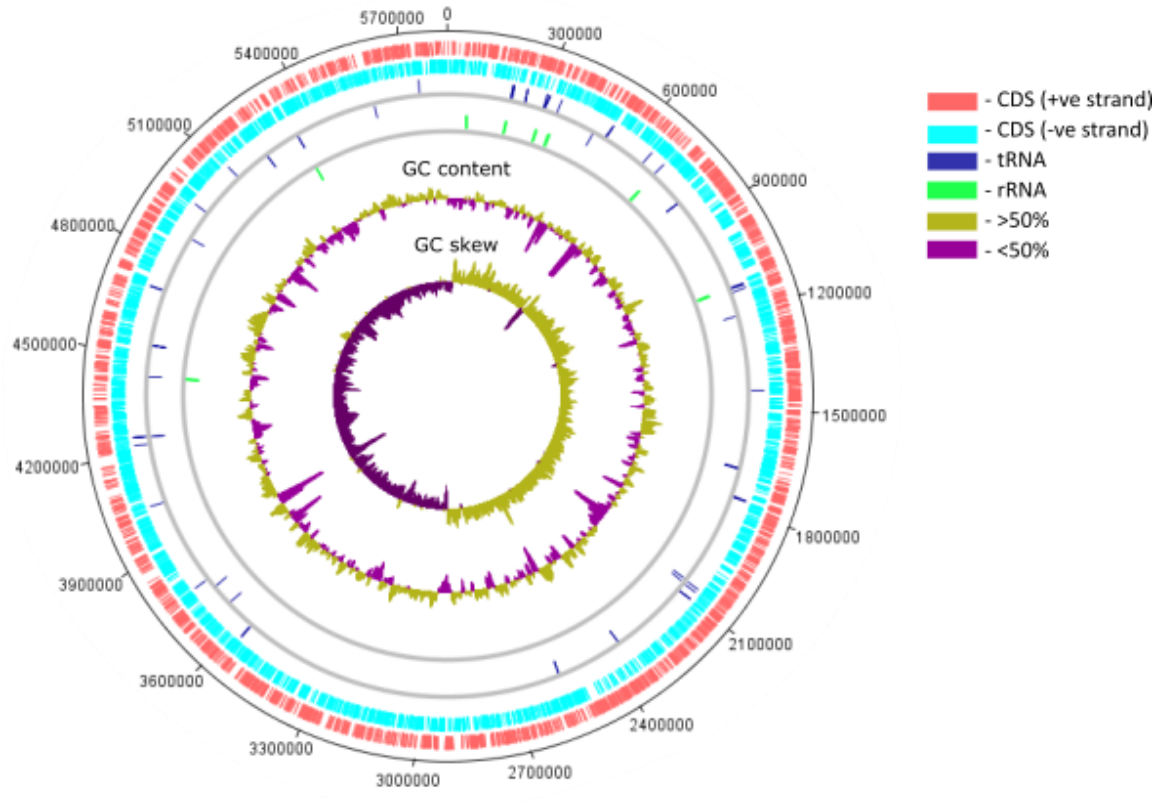

Figure 4.2 Circular summary map of the Medkleb chromosome. The Medkleb chromosome is represented with the stop codon (TGA) of the dnaA gene at position 0. Track one; 2474 red ticks represent gene coding sequences on the positive strand. Track two; 2847 light blue ticks represent gene coding sequences on the negative strand. Track three; 50 dark blue ticks above the grey line represent tRNAs on the positive strand, and 35 ticks below the line represent tRNAs on the negative strand. Track four; 18 green ticks above the grey line represent rRNAs on the positive strand, and 7 ticks below the line represent rRNAs on the negative strand. 64% of rRNAs are found close to oriC. Track five – GC content; regions containing >50% GC content are mustard and regions containing <50% GC content are purple. Track six – GC skew; regions containing >50% Gs are mustard and regions containing <50% Gs are purple. GC skew was asymmetric between leading and lagging strands, with the purple spike between bases 654265-677695 representing recent horizontal gene transfer (Lawrence et al., 1997).

4.3.2) Medkleb genome synopsis – plasmids

The lengths and GC contents of mkps 1-4 (Table 4.3) were all within expected range for plasmids associated with *Klebsiella oxytoca* (see supplementary materials – Table 4.2). mkps 1-4 devote 14-22% of gene space to plasmid associated genes and mobile element coding sequences, which is substantial in comparison to the chromosome which only allocated 0.02% to such features. mkps 1, 3 and 4 exhibited asymmetric gene distribution between DNA strands (coding bias), which is common for plasmid genomes (Reva et al., 2004). The coding bias of mkp3 was particularly apparent, with ~90% of the total gene complement located on the positive strand. mkp4 was the only plasmid predicted by antiSMASH (Weber et al., 2015) to code for secondary metabolites, which included cloacin (de Graaf et al., 1969) and colicin bacteriocins (Cascales et al., 2007).

Table 4.3 Comparison of plasmid genomes. mkps 1-4 varied in length by over 100%, and GC content varied by 6.3%. Plasmid 4 was relatively small but had the greatest gene density, utilising approximately 4% more nucleotide sequence for protein coding than other plasmids.

	mkp1	**mkp2**	**mkp3**	**mkp4**
Length (nt)	136402	122124	78046	63257
GC content (%)	55.2	50.4	56.7	50.7
CDS (+ve strand)	105	95	76	62
CDS (-ve strand)	70	65	8	42
Overall coding sequence (%)	80.3	81.1	81.2	85.3
Gene density (per kb)	1.28	1.31	1.06	1.64
Annotated plasmid CDS (n)	12	3	18	12
Mobile element protein CDS (n)	21	21	1	5

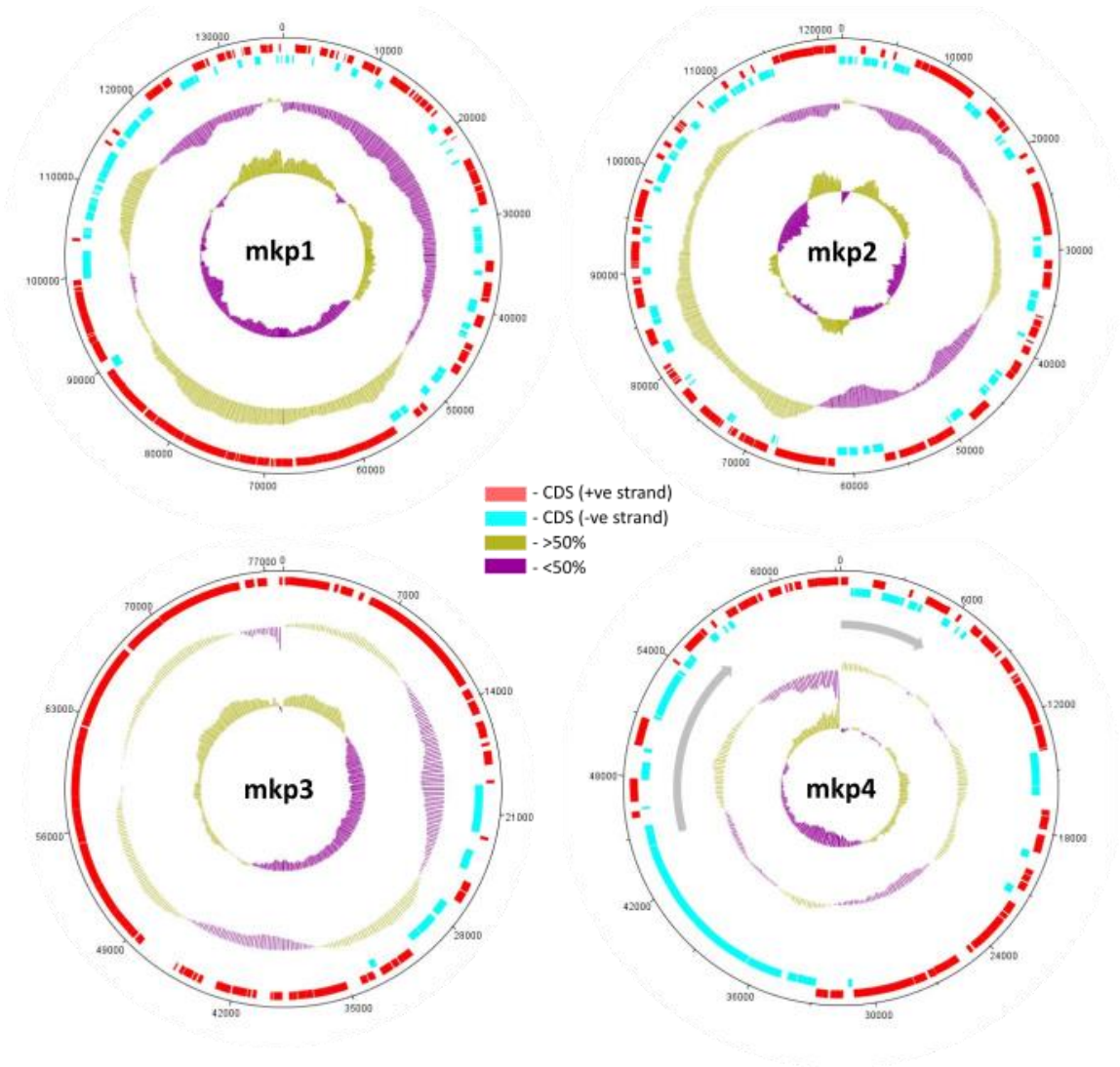

Figure 4.3 Circular maps of mkps 1-4. For all maps: 1) red sectors on track one represent gene coding sequences on the positive strand, 2) light blue sectors on track two represent gene coding sequences on the negative strand, 3) track three represents GC content; regions with >50% GC content are mustard and regions with <50% GC content are purple, 4) track four represents GC skew; regions with a >50% G's are mustard and regions with <50% G's are purple. Interesting individual plasmid features include: 1) mkp4 contains two secondary metabolite clusters indicated by grey arrows, 2) mkps 1,3 and 4 exhibit clear coding bias.

4.4) Isolation of Medkleb's 16S sequence

Medkleb was predicted to be a strain of *K. oxytoca,* which has previously been identified as a major component of the medfly microbiome (Behar, Jurkevitch, et al., 2008). The 16S sequence of *K. oxytoca* strain ATCC 13182 (NR_118853.1) was therefore used to locate homologous sequences in the Medkleb genome using the BLASTn algorithm (Altschul et al., 1997). This analysis revealed that the Medkleb genome contains eight sequences >99% related to NR_118853.1, which were all predicted to code for 16S by RNAmmer 1.2 (Lagesen et al., 2007). Nucleotides 341370-342821 (mk16S) which had the greatest homology with NR_118853.1 were therefore used to represent Medkleb in subsequent taxonomic analyses.

4.5.1) Taxonomic identification of Medkleb - 16S pairwise analysis

As a first approximation to confirm Medkleb to species, the EZBioCloud (Yoon et al., 2017) was searched for sequences homologous to nucleotides 341370-342821 (mk16S). The 16S sequence with greatest homology to mk16S belonged to the W14 strain, which had been classified as *K. michiganensis*. Medkleb's predicted species classification was *K. oxytoca,* hence near identical 16S homology with *K. michiganensis* was unexpected*,* although it is recognised that 16S sequence similarity alone does not confirm species identification (Tindall et al., 2010). As recommended by Tindall et al. (2010), homology between mk16S and 16S sequences that had been classified as either *K. oxytoca* or *K. michiganensis* was calculated using EZBioCloud (Yoon et al., 2017) but this also failed to distinguish Medkleb as either species. mk16S was compared with forty 16S sequences classified as either *K. oxytoca* or *K. michiganensis* and was between 98.5 and 99.93% related to all strains analysed. Stackebrandt (2006) suggest that >98.7% similarity should be the threshold at which 16S sequences are considered to be conspecific but some authors have suggested thresholds need to be as high as 99.5% (Janda et al., 2007). In conclusion, Medkleb could not be specifically characterised as either *K. oxytoca* or *K. michiganensis* via pairwise analysis of its 16S sequence, as according to current taxonomic standards (Janda et al., 2007; Stackebrandt, 2006), the *K. oxytoca* and *K. michiganensis* strains analysed here were themselves conspecific.

4.5.2) Taxonomic identification of Medkleb - 16S phylogenetic analysis

To classify Medkleb's species with improved resolution, a comprehensive 16S phylogenetic analysis (Figure 4.4) was carried out. RefSeq 16S sequences used in the analysis had been classified as either *K. oxytoca, K. michiganensis* or *K. pneumoniae* (Quast et al., 2013), and a single strain of *Pseudomonas aeruginosa* was used as an ancestral root for the phylogeny. *K. pneumoniae* sequences were included as a control, as this species is closely related to *K. oxytoca* (Kovtunovych et al., 2003) but distant enough to be distinguished phylogenetically. Medkleb, *K. oxytoca* and *K. michiganensis* sequences formed one homogenous group and *K. pneumoniae* formed an outgroup, suggesting that Medkleb is both *K. oxytoca* and *K. michiganensis* and hence that the species dichotomy is artificial, as suggested above. However, even though 16S phylogenetic and pairwise analyses failed to delineate *K. oxytoca* from *K. michiganensis*, these species should be considered distinct, as DNA-DNA hybridisation between them is <70% (Saha et al., 2013).

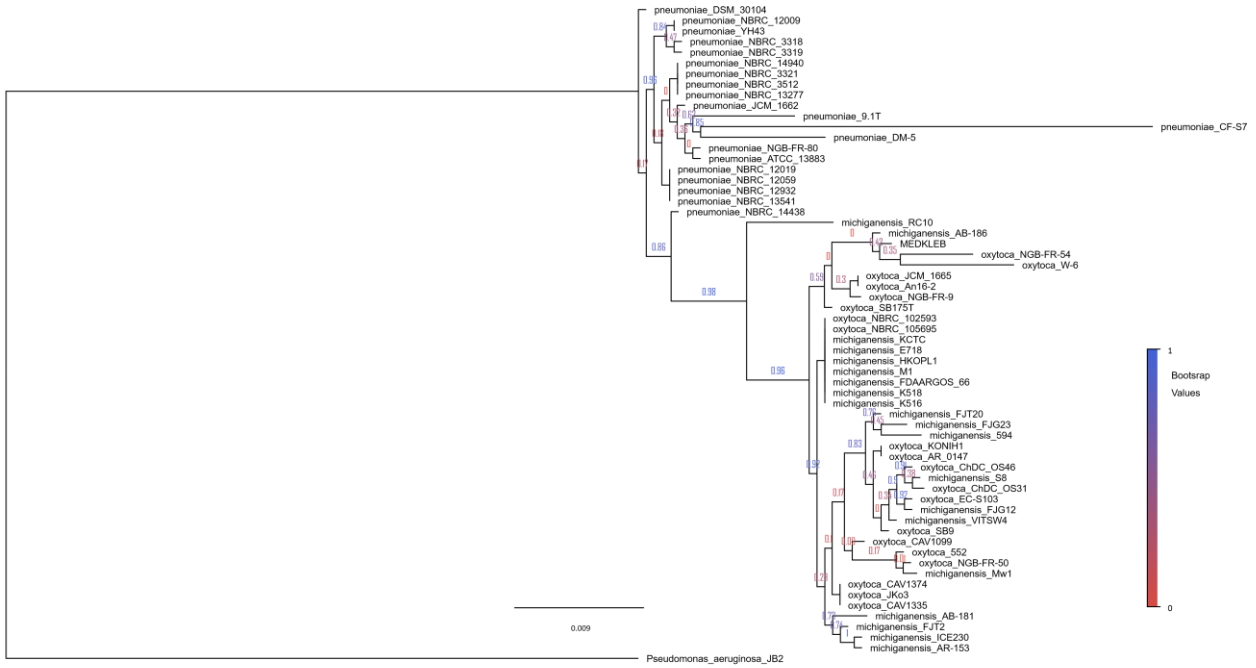

Figure 4.4 Phylogeny showing the evolutionary relationship of 16S genes belonging to Medkleb and three species of the Klebsiella genus. The phylogeny was created with the Silva ACT service (Pruesse et al., 2012), using the FastTree2 program (Price et al., 2010) to compute alignments with bootstrap values. Pseudomonas aeruginosa strain JB2 was used as an ancestral root. The scale bar represents substitutions per site. Bootstrap values are represented at all nodes and are colour coded according to confidence level (blue = 1 and red = 0). 16S sequences of strains that had been identified as K. pneumoniae demonstrated clear evolutionary divergence and segregated definitively from K. oxytoca and K. michiganensis strains. 16S sequences identified as K. oxytoca and K. michiganensis formed one homogenous group, with some species level clustering but no reliable pattern of distribution. Species identification of Medkleb was not possible based on this phylogeny, as it segregated within the K. oxytoca, K. michiganensis group.

4.6) Taxonomic identification of Medkleb – ANI analysis

Medkleb's 16S sequence was too similar to both *K. oxytoca* and *K. michiganensis* for it to be characterised as either species on this basis alone. A more stringent comparative genetic analysis such as Average Nucleotide Identity (ANI) (Konstantinidis et al., 2005) was therefore required. ANI is more powerful than single locus techniques such as 16S-based approaches, as the evolutionary relationship of entire genomes is calculated via pairwise comparisons of all coding sequences, using conservation thresholds of similarity and alignability (Konstantinidis et al., 2005). To determine if Medkleb's species could be resolved by ANI, its genome was positioned in an ANI phylogeny with 35 RefSeq *Klebsiella* genomes that were extracted from the NCBI database (Marchler-Bauer et al., 2009) (Figure 4.5). This analysis placed Medkleb in a lineage with 13 strains of bacteria that had been classified as both *K. oxytoca* and *K. michiganensis*. ANI scores >95% are required to classify bacteria as the same species (Kim et al., 2014; Richter et al., 2009). This 95% similarity threshold was met by all thirteen members of Medkleb's ANI clade (henceforth referred to as the Medkleb group) (Figure 4.6). Therefore, as for the 16S analysis, ANI failed to differentiate between the current named *K. oxytoca* and *K. michiganensis* bacterial species.

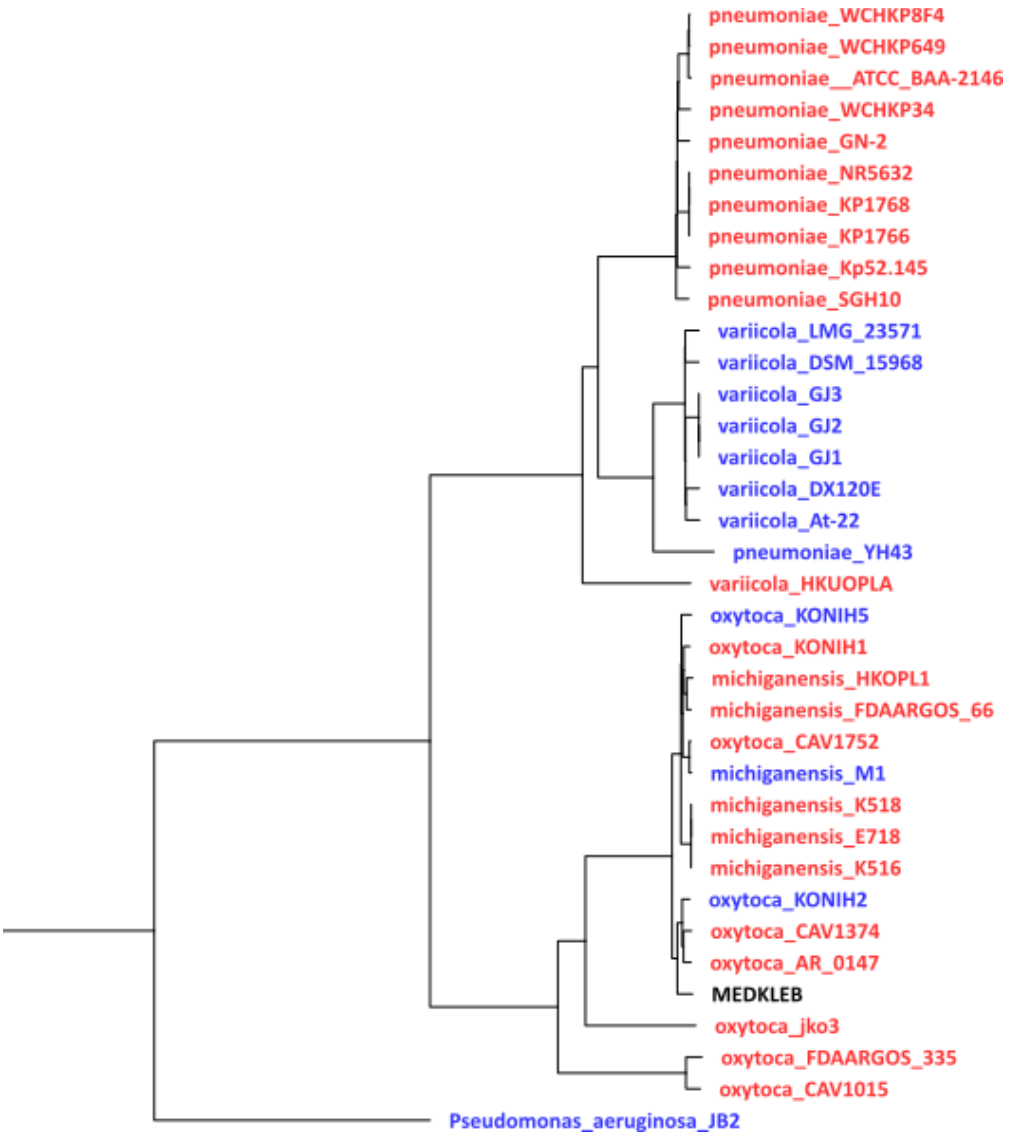

Figure 4.5 ANI phylogeny showing the evolutionary relationship of environmentally derived and host derived Klebsiella bacteria. The phylogeny was created using the ANI calculator (Figueras et al., 2014), with *Pseudomonas aeruginosa* strain JB2 selected as the outgroup. Bacteria derived from animal hosts (red and black), and environmentally derived bacteria (blue), generally fell into three clades: 1) *K. pneumoniae*, 2) *K. variicola* and 3) *K. oxytoca/michiganensis*. With one exception in each group (YH43 and HKUOPLA), all *K. pneumoniae* are host derived and all *K. variicola* are environmentally derived. *K. oxytoca/K. michiganensis* have been isolated from both environmental and animal sources, but their sequences did not cluster according to source status. According to the ANI species threshold set by Kim et al. (2014), Medkleb is conspecific with twelve strains which have been classified as both *K. oxytoca* and *K. michiganensis*.

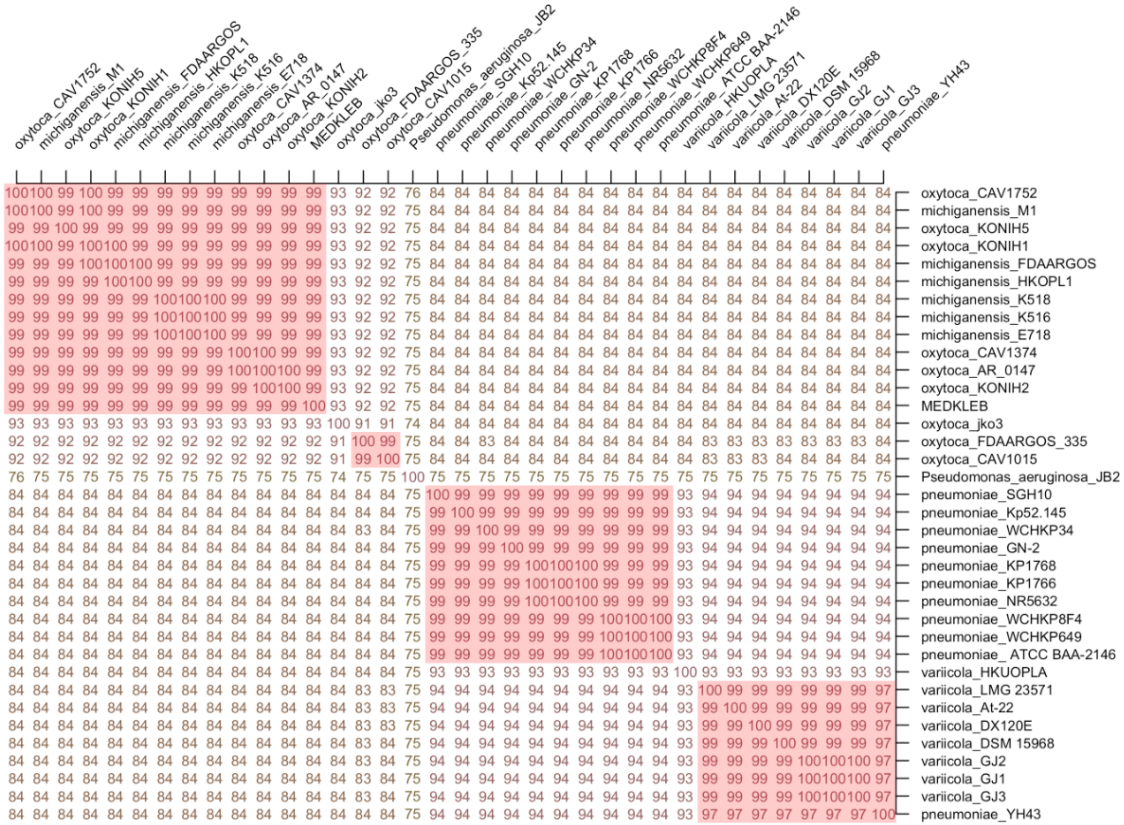

Figure 4.6 Average nucleotide identity matrix for 35 strains of Klebsiella bacteria. The matrix was created using the ANI calculator (Figueras et al., 2014), with Pseudomonas aeruginosa strain JB2 selected as the outgroup. Not including the Pseudomonas outgroup, six distinct *Klebsiella* species were identified by the ANI analysis. Isolates with >95% ANI identity are considered conspecific and are highlighted pink. The "Medkleb group" is located at the top left of the matrix.

4.7.1) Bioinformatic analyses of pectic lyases for classification of Medkleb

The polygalacturonase gene *pehX* of *K. oxytoca* (AY065648.1) is thought to confer the ability to degrade pectate, which is considered a defining phenotype of *K. oxytoca* (Kovtunovych et al., 2003). Furthermore, positive PCR amplification of pehX has previously been used to distinguish *K. oxytoca* from *K. michiganensis* and *K. pneumoniae,* with the latter 2 species both being *pehX* negative (Kovtunovych et al., 2003; Saha et al., 2013). As *pehX* can be PCR amplified from *K. oxytoca* but not *K. michiganensis*, this led to the hypothesis that *K. oxytoca* and *K. michiganensis* within the Medkleb group might be distinguished via their homology with the known *K. oxytoca pehX* sequence (AY065648.1). This analysis did demonstrate significant differences in the respective homology of *pehX* AY065648.1 between the Medkleb group *K. oxytoca* and *K. michiganensis* strains (analysed with BLAST (Altschul et al., 1997)) (t-test, $t = 2.65$, $df = 6.61$, $p<0.05$). However, unexpectedly, *K. michiganensis* shared greater homology with AY065648.1 than did *K. oxytoca* (Table 4.4), despite the fact that *K. michiganensis* does not degrade pectate (Saha et al., 2013). Technically, bioinformatic analysis of *pehX* did facilitate discrimination between *K. oxytoca* and *K. michiganensis* in the Medkleb group, but the result contradicted the hypothesised pattern. The CAZy database (Lombard et al., 2010) did not aid in discrimination of *K. oxytoca* and *K. michiganensis* in the Medkleb group, as it identified two pectate lyases (one from family 2 and one from family 9) in all Medkleb group genomes available on the database (Table 4.3).

Table 4.4 Genomic features of the 'Medkleb group' of bacteria. *pehX* sequence identity (calculated with BLAST (Altschul et al., 1997)) was significantly different between species (t-test, t = 2.65, df = 6.61, p<0.05). *K. michiganensis* genomes contained sequences more closely related to AY065648.1 than was found for *K. oxytoca* genomes (mean relatedness = 88.4% vs 87.9%). All genomes available on the CAZy database (Lombard et al., 2010) are predicted to code for 2 pectate lyases. Medkleb had the second smallest genome in the group, and the second highest GC content.

Species	Strain	Genome size (nt)	GC (%)	pehX identity (%)	CAZy lyases
michiganensis	M1	5865090	56.13	88.47	2
oxytoca	Medkleb	5867541	56.03	87.43	NA
michiganensis	HKOPL1	5914407	55.92	88.56	2
oxytoca	CAV1752	5992008	55.16	88.47	2
michiganensis	FDAARGOS_66	6071464	55.94	88.39	NA
michiganensis	E718	6097032	56.02	88.31	2
michiganensis	K518	6138996	55.95	88.39	2
michiganensis	K516	6139574	55.96	88.31	2
oxytoca	KONIH1	6152190	55.91	88.61	2
oxytoca	KONIH5	6179177	55.81	87.82	2
oxytoca	KONIH2	6190364	55.77	87.43	2
oxytoca	CAV1374	6257473	55.75	87.87	2
oxytoca	AR_0147	6350620	55.57	87.89	2

4.7.2) PCR amplification of *pehX* as a means of characterising Medkleb

Medkleb was *pehX* positive (Figure 4.7), which is reported to be a defining feature of *K. oxytoca* (Kovtunovych et al., 2003) over *K. michiganensis* (Saha et al., 2013).

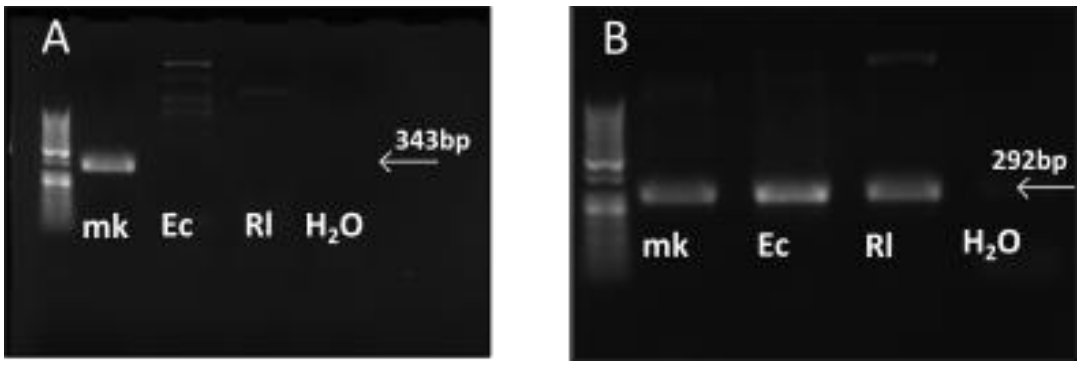

Figure 4.7 PCR amplifications of pehX and 16S genes. Both gels were run with three samples of DNA and a water control. A) PCR amplification of the *pehX* gene produced the expected 343bp product from Medkleb (mk). Neither *Erwinia carotovora* (Ec) nor *Rhizobium leguminosarum* (Rl) amplified this product, but both (Ec in particular) appeared to amplify products larger than 343bp. B) Control reactions: all three species of bacteria produced the expected 292bp 541F-806R amplicon following PCR amplification of 16S, which verified the quality of DNA and reagents used in the PCR assays.

4.8) Polygalacturonase enzyme assay

The capacity of bacteria to produce polygalacturonic acid-degrading polygalacturonases was quantified using a DNS colorimetric method (Miller, 1959). A standard curve of galacturonic acid that had been incubated with DNS (Figure 4.8A), demonstrated a positive relationship between galacturonic acid concentration and colour change, when the OD of the resulting reaction was measured at 530nm (linear model, $F(1,4) = 176.4$, $p<0.001$, $R^2=0.98$). Filtrate of Medkleb and *Erwinia carotovora* culture media both contained pectinolytic enzymes, producing measurable colour change when incubated with polygalacturonic acid and DNS (Figure 4.7B) (Sohail et al., 2016). Medkleb filtrate reduced polygalacturonic acid with around 65% the efficiency of the *Erwinia carotovora* filtrate, but *Rhizobium leguminosarum* filtrate did not demonstrate any measurable pectinolytic activity (Figure 4.8). These phenotypic data suggest that Medkleb should be classified as *K. oxytoca,* as the ability to degrade pectate is a key phenotype associated with this species (Kovtunovych et al., 2003), and is reported to be lacking from *K. michiganensis* (Saha et al., 2013).

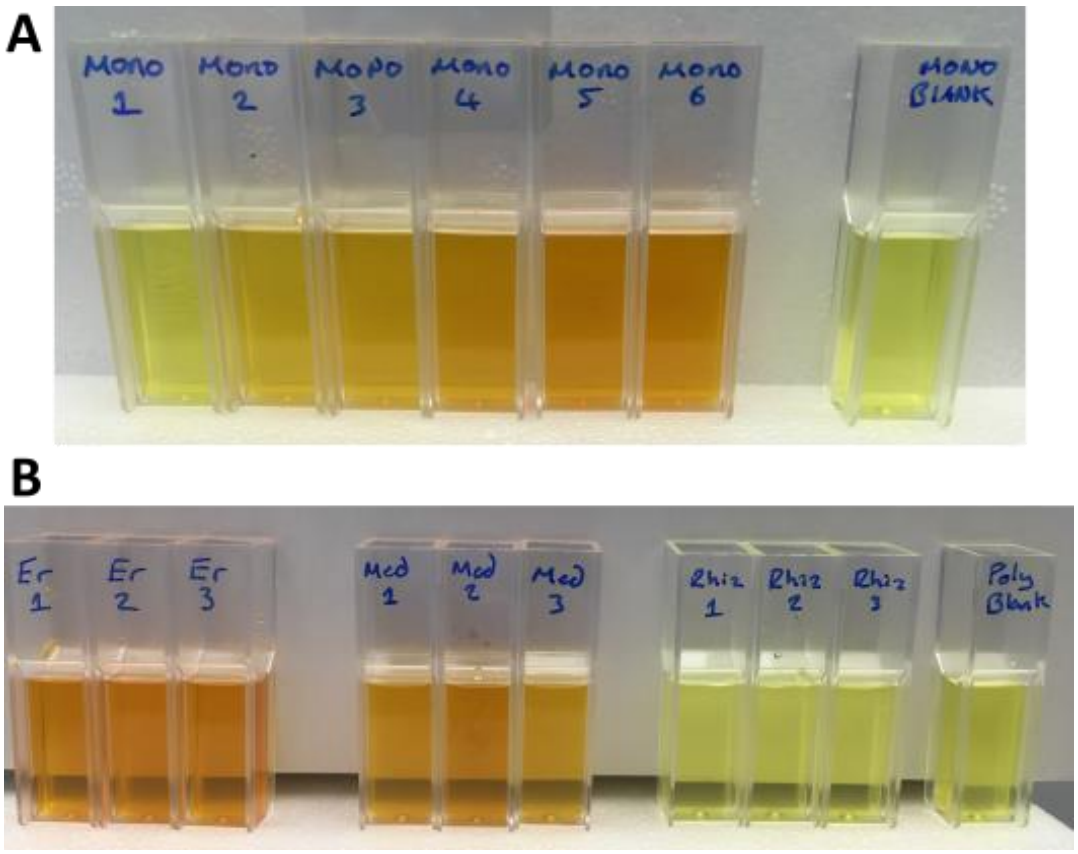

Figure 4.8 Poly/monosaccharide colorimetric reaction mixtures. A) Standard curve – The quantity of sugar in standards ranged from 0.9mg (mono 1) to 18mg (mono 6). Mono blank contained no sugar. When incubated with DNS, colour change (measured at ΔA530) occurred for all reactions relative to the blank. The relationship between sugar quantity and colour change was significant (linear model, $F(1,4) = 176.4$, $p<0.001$, $R^2=0.98$). B) Bacteria reaction mixtures – Polygalacturonase production of three bacteria, *Erwinia carotovora* (Er), Medkleb (Med) and *Rhizobium leguminosarum* (Rhiz) was measured via the quantity of reduced sugar in solution, following incubation of culture filtrate with polygalacturonic acid. Units of polygalacturonase were quantified in terms of colour change via extrapolation from the standard curve. Med and Er filtrate contained 30 and 46 units of polygalacturonase per ml respectively. Rhiz filtrate did not contain any polygalacturonase.

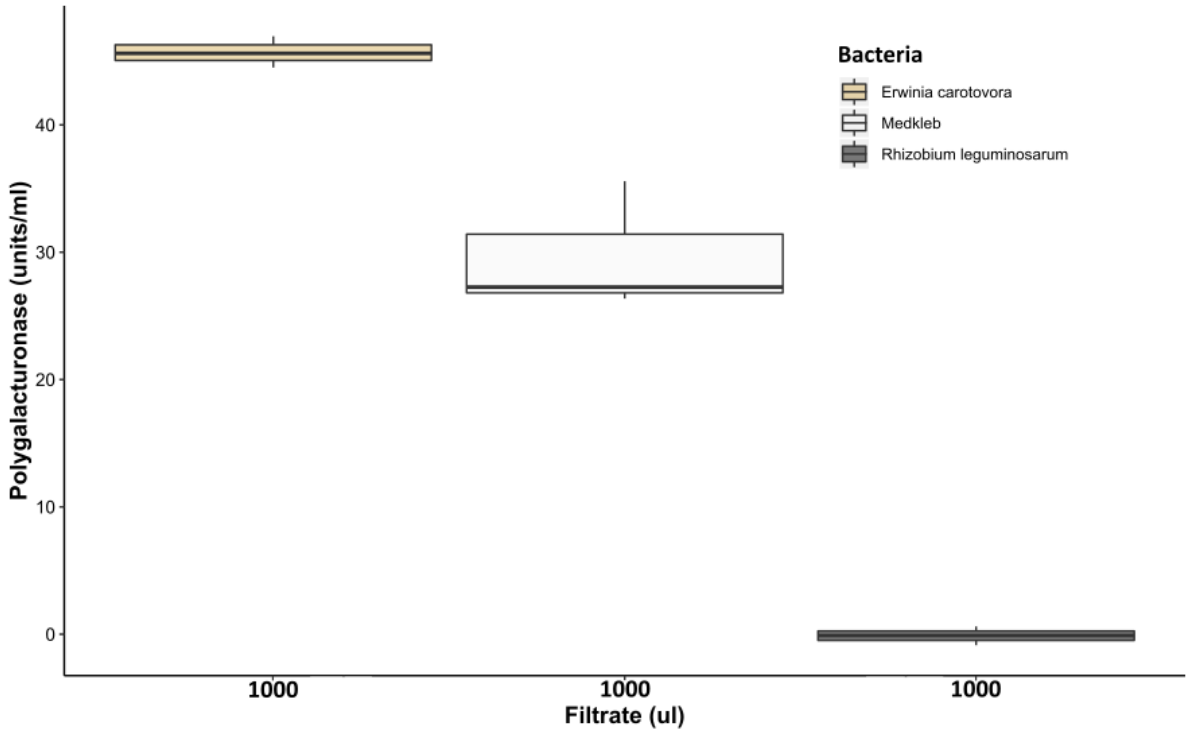

Figure 4.9 Units of polygalacturonase contained in bacterial filtrate. Units of polygalacturonase per ml are represented on the y-axis. 1000ul aliquots of bacterial filtrate processed from three different species of bacteria are represented of the x-axis. Bacteria were assessed for the presence of polygalacturonases using a standard curve of galacturonic acid (linear model, $F(1,4) = 176.4$, $p<0.001$, $R^2=0.98$). The upper and lower hinges of boxplots represent the first and third quartiles of enzyme concentrations in filtrate, calculated from three technical replicates. *Erwinia carotovora* was the highest producer, with an average of 45.7 units/ml and Medkleb produced polygalacturonase with an average of 29.7 units/ml. *Rhizobium leguminosarum* did not produce any polygalacturonase.

4.9) Genomic features associated with symbiotic bacteria

4.9.1) Medkleb's genome size and GC content in comparison to the Medkleb group

Bacterial symbionts undergoing a transition to evolutionary interdependence with their hosts can often be identified by the size and GC content of their genomes, which are reduced relative to purely free-living species (Bennett et al., 2013; Hansen et al., 2011; Husnik et al., 2013; Shigenobu et al., 2000; van Ham et al., 2003; Wu et al., 2006). The Medkleb genome was compared to other members of the Medkleb group in this context (Table 4.4), to evaluate if it were undergoing transition to a host-associated lifestyle. At 5867541bp, the Medkleb genome was 1.5 standard deviations smaller than the mean genome size of bacteria in the 'Medkleb group' (n=13) ($\dot{x}$ = 6093534bp ± 148172 s.d.). However, this is unlikely to be a diagnostic signal of symbiotic transition in this case, as the *K. michiganensis* strain M1 was even smaller than Medkleb (5865090bp) and is a free-living strain isolated from soil on a Korean beach. Medkleb's GC content (56.03%) also conflicted with prediction of a nascent mutualism, being higher than expected, even for bacteria with a free-living life history (Moran et al., 2008). In conclusion, the analysis of the Medkleb genome did not reveal any obvious diagnostic signatures of strongly host-associated bacteria. However, a deeper resolution analysis of the genome might still hold further information required to uncover signatures of a nascent mutualism

4.9.2) Local genomic rearrangement as a signature of nascent mutualism

The Medkleb genome was aligned with progressiveMauve (Darling et al., 2010) to three very closely related strains of *K. oxytoca* (Figure 4.5). This aim of this analysis was to locate any novel loci acquired via lateral gene transfer (Ochman et al., 2000) or loci deleted through genome reduction (Ochman et al., 2006) as these regions are likely to contain signatures of symbiotic transition. Mauve recognised 21 local colinear blocks (LCBs) of conserved DNA in all four genomes (Figure 4.10). These 21 blocks were not uniformly distributed between the genomes. *K. oxytoca* strains AR0147 and CAV1374 both contained inversions between LCBs 11-16 and strain KONIH2 contained several instances of translocation and inversion. However, despite inversion and translocation events, nucleotide sequence in all LBSs other than 18 was very highly conserved. Medkleb LCB18 (≈nts 4973000-5000000) was unique and contained genes associated

with horizontal transfer (an integrase, a mobile element protein and a prophage protein), indicating that this block could have been recently acquired. The annotation of block 18 predicts 25 coding sequences in total, including 13 “hypothetical proteins”, 2 DNA helicases, 2 methyltransferases and an anti-restriction protein.

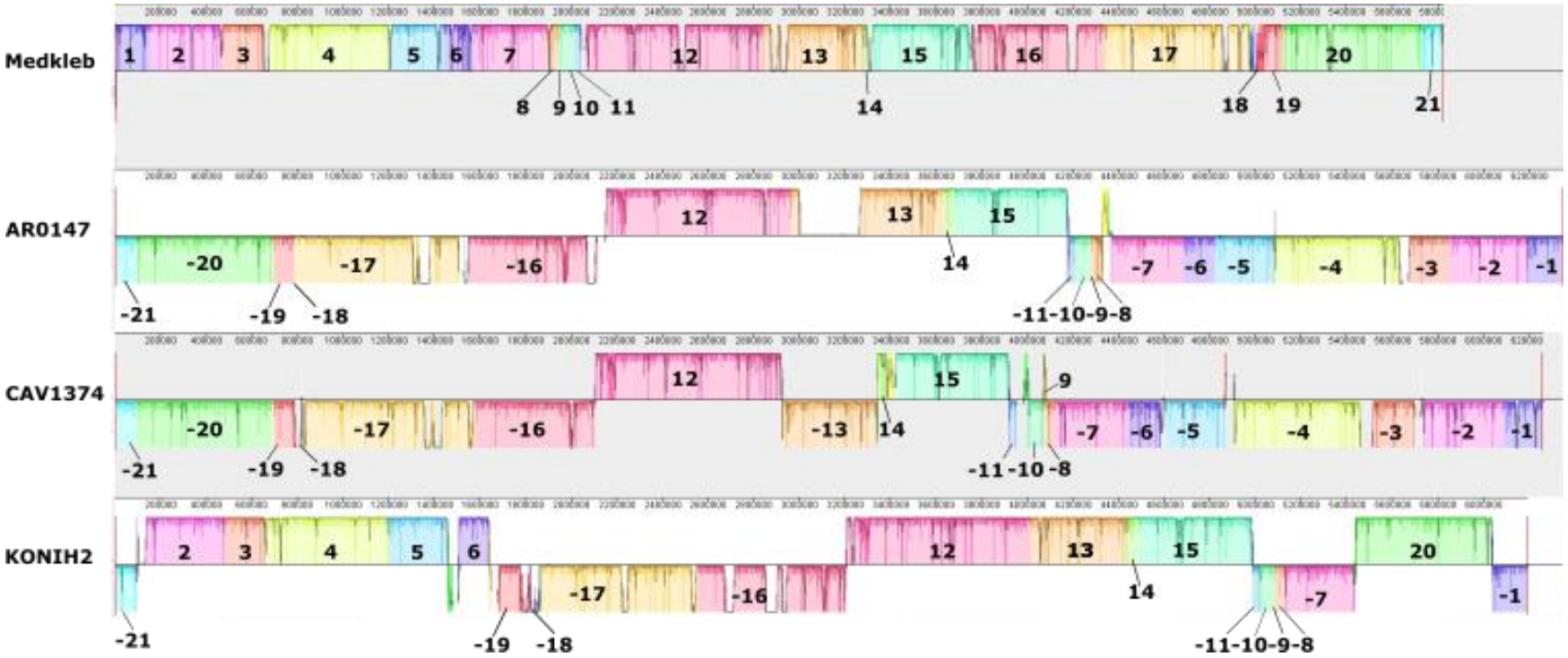

Figure 4.10 Synteny plot of Medkleb and three closely related bacteria. Medkleb was positioned phylogenetically in a clade with three *K. oxytoca* bacteria (Figure 4.5) (strains AR0147, CAV1374 and KONIH2) with ANI analysis (Konstantinidis et al., 2005). progressiveMauve (Darling et al., 2010) was then used to assess synteny between Medkleb, AR0147, CAV1374 and KONIH2. This analysis, which is represented graphically above, uncovered 21 conserved local colinear blocks (LCBs) (Darling et al., 2010) of nucleotide sequence that were shared between all genomes analysed. Block height corresponds the degree of regional sequence conservation. When compared to Medkleb, sequence conservation in block 18 was low for strains AR0147, CAV1374 and KONIH2. There were several instances of LCB inversion between strains but inverted LCBs were not low in height, which indicated that nucleotide sequence was conserved.

4.9.3) Comparative analysis of the higher-level metabolic functions of genes coded by Medkleb and other *K. oxytoca* bacteria

KEGG (Kanehisa et al., 2016) was used to produce a list of gene functions for chromosomes and plasmids associated with all *K. oxytoca* bacteria in the Medkleb group (Table 4.5). The dataset was filtered with R (Team, 2016) to produce two smaller lists: 1) Atypical - gene functions unique to each strain, 2) Absent - gene functions present in all genomes analysed other than the strain in question. The Medkleb genome contained 21 atypical functional orthologues (Figure 4.11). Of Medkleb's 21 atypical genes, 16 were chromosomally derived and 5 located on plasmids. The atypical genes included two putative transposases, 8 enzymes involved in various modes of metabolism and 4 transport proteins. Medkleb has 35 absent gene functions (Figure 4.12). When Medkleb was compared to conspecifics, the largest cluster of absent genes was associated with copper resistance, but several genes associated with the metabolism of amino sugars and genes that sense toxic chemicals in the environment were also missing.

Table 4.5 Comparison of gene functionality for K. oxytoca bacteria in the Medkleb group. The total number of gene functions was fairly consistent in this group, at approximately 2900 per strain. In terms of atypical functions, Medkleb had a relatively large complement, but fewer than CAV1752. The Medkleb genome had more absent gene functions than was found for conspecifics.

	Total gene functions	Atypical functions	Absent functions
Medkleb	2857	21	35
AR0147	2904	12	4
CAV1752	2882	24	5
CAV1374	2944	11	6
KONIH1	2917	18	3
KONIH2	2912	13	10
KONIH5	2882	11	5

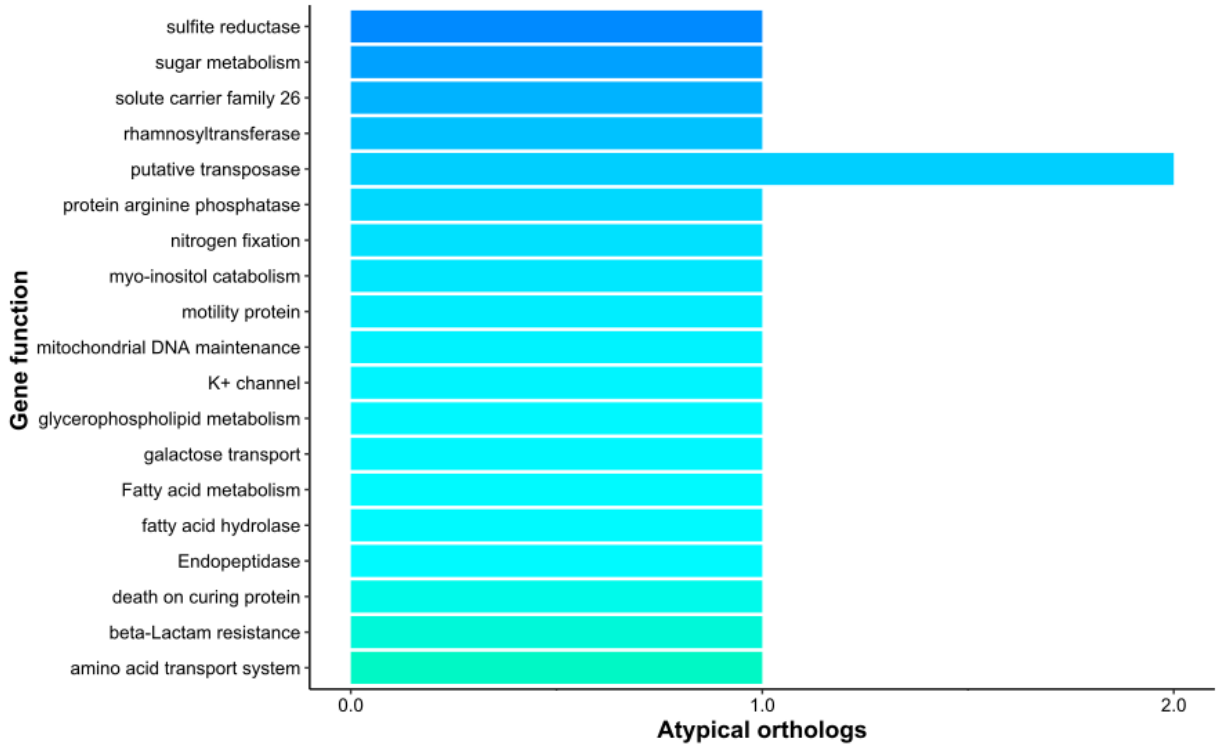

Figure 4.11 The functions of genes present in the Medkleb genome but absent from all other K. oxytoca bacteria in the Medkleb group. The functions of atypical genes that were present in the Medkleb genome but absent from all remaining *K. oxytoca* genomes analysed are represented on the y-axis. Each atypical function is denoted by a discrete colour. The number of atypical genes providing each function is represented on the x-axis. Medkleb codes for 21 atypical genes with 20 different functions. Broadly, these genes were predicted to be involved in metabolism, transport and gene transposition.

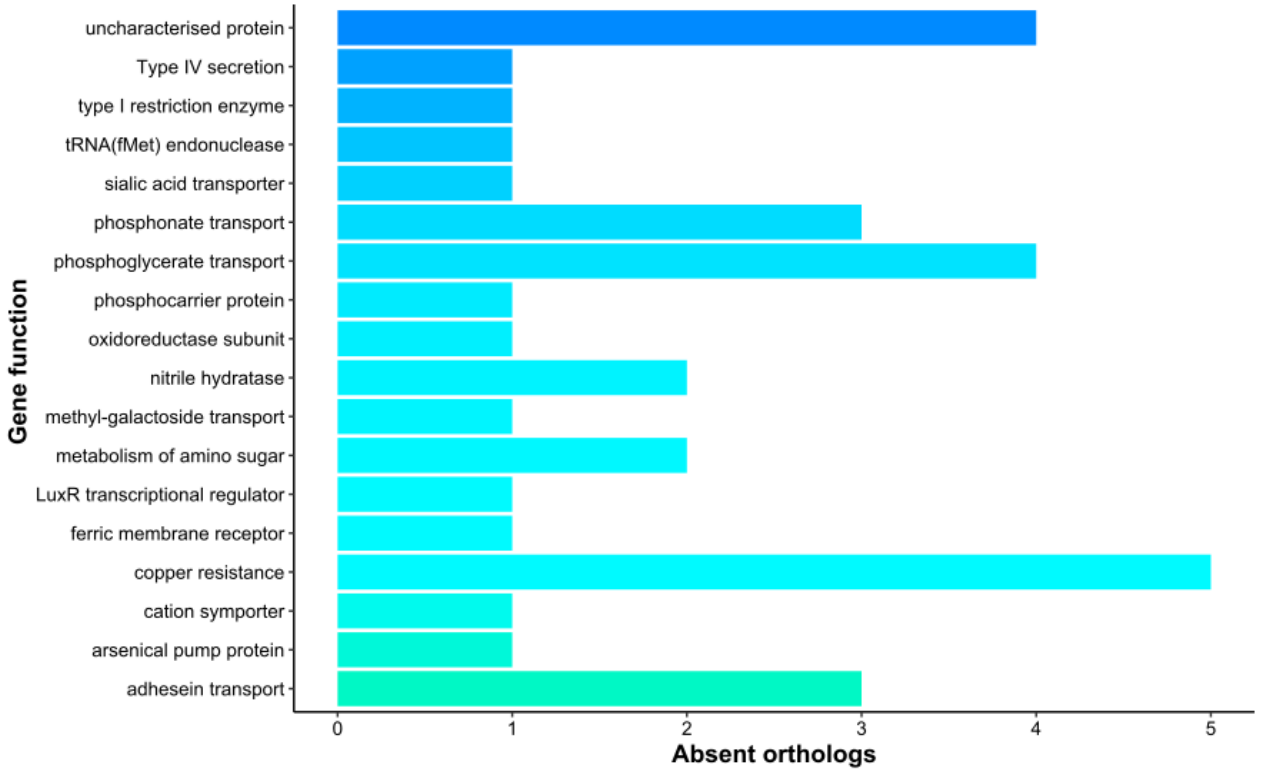

Figure 4.12 The functions of genes that are absent from the Medkleb genome but present in all other K. oxytoca bacteria in the Medkleb group. The functions of genes that were absent from the Medkleb genome but present in all other *K. oxytoca* genomes analysed are represented on the y-axis. Each absent function is denoted by a discrete colour. The number of absent genes associated with each function is represented on the x-axis. Medkleb did not code for 35 genes that were present in every other genome analysed and these were associated with 17 discrete functions. Loss of function occurred in clusters of genes relating to the detoxification of xenobiotic compounds.

4.9.4) Secondary metabolites encoded by *K. oxytoca* bacteria

Secondary metabolites may provide no obvious function for bacteria that produce them (Malik, 1980), but can benefit their host (Barke et al., 2011; Holmes et al., 2016). A gene set recently acquired by a microbe that produces secondary metabolites that benefit an insect host, could therefore be the basis of nascent mutualism. Following this premise, the Medkleb chromosome and plasmids were assessed with antiSMASH 3.0 (Weber et al., 2015) for the presence of novel secondary metabolites. Chromosomes of all *K. oxytoca* members of the Medkleb group (other than KONIH2) coded for four common secondary metabolite clusters: 1) non-ribosomal polypeptide synthetase, 2) thiopeptide antibiotic, 3) polyketide-synthase, non-ribosomal polypeptide synthetase, 4) bacteriocin. The Medkleb chromosome did contain two unique secondary metabolite clusters predicted to code for γ-butyrolactone and N-acyl amides. γ-butyrolactone is utilised by *Streptomyces* bacteria as a signalling molecule to regulate antibiotic production and cell cycle processes (Kitani et al., 2011; Takano, 2006) but the function of N-acyl amides is unclear (Craig et al., 2011). mkp4 was also predicted to code for cloacin and colicin bacteriocins that were not coded by any other *K. oxytoca* strain in the Medkleb group. Interestingly, the cloacin bacteriocin encoded on mkp4 is generally considered to be toxic for *Klebsiella* bacteria (de Graaf et al., 1969).

4.5) Discussion

When a bacterium evolves to become consistently associated with a host species, its genome typically exhibits signatures of this transition. For example, long term association between bacteria and insect hosts often results in reduced genome size and GC content for the bacterium (Bennett et al., 2013; McCutcheon et al., 2011; Moran et al., 2014; Moran et al., 2008). I hypotheised that the Medkleb bacterium that had been isolated from the gut of Toliman medfly, was a strain of *K. oxytoca* and that its genome would exhibit features consistent with transition to a host-associated lifestyle. These hypotheses were based on several studies that have suggested that *K. oxytoca* bacteria are facultative mutualists of the medfly (Behar, Jurkevitch, et al., 2008; Ben Ami et al., 2010). To resolve Medkleb's natural history I first taxonomically characterised its genome, then evaluated it for features of symbiotic transition.

I obtained a fully sequenced Medkleb genome composed of five sequencing contigs that I used for subsequent analyses. Initially, the quality of the Medkleb genome sequence was assessed based on criteria set by Bowers et al. (2017). The Quiver algorithm (Chin et al., 2013) estimated that the sequencing resolution of the five Medkleb contigs was very good and BUSCO (Simao et al., 2015) and CheckM (Parks et al., 2015) software packages both determined that the genome was complete. The Medkleb genome sequence therefore met the Bowers et al. (2017) quality thresholds which allowed me to use it for taxonomic analyses.

For taxonomic characterisation of Medkleb I positioned it in 16S and ANI phylogenies with bacteria of the *Klebsiella* genus. I found that, according to current 16S and ANI standards (Kim et al., 2014; Stackebrandt, 2006), Medkleb could be characterized as either *K. oxytoca* or *K. michiganensis*. Although genomes currently published as *K. oxytoca* and *K. michiganensis* were not distinguished phylogenetically with 16S or ANI these species should be considered distinct, as they are less than 70% similar when analysed with DNA-DNA hybridization (Saha et al., 2013). The final taxonomic analyses of Medkleb were carried out by PCR and an enzyme assay. Medkleb was pehX positive when analysed with PCR, a trait associated with *K. oxytoca* and not *K. michiganensis* (Kovtunovych et al., 2003;

Saha et al., 2013). In addition, Medkleb was demonstrated to be a producer of pectinolytic enzymes which is diagnostic of *K. oxytoca* (Kovtunovych et al., 2003; Saha et al., 2013). Therefore, the Medkleb genome cannot be definitively characterised as *K. oxytoca* as available reference genomes are do no support this claim but it does exhibit a phenotype that is characteristic of the species.

I then used differences in GC content and sequence homology with the *K. oxytoca pehX* gene, to distinguish the genomes of *K. oxytoca* and *K. michiganensis* bacteria in the Medkleb group. Of the 15 RefSeq *K. oxytoca/K. michiganensis* genomes that were available at the time of analysis, three were demonstrated by ANI to be mischaracterised. This reduced the study to a population of thirteen *K. oxytoca/K. michiganensis* genomes (including Medkleb). GC content, and pehX sequence identity were both significantly lower in *K. oxytoca* than *K. michiganensis* genomes. Therefore, future attempts to distinguish these species might be guided by comparing the GC content and *pehX* homology of representative genomes, which will become progressively more reliable as more genomes become available.

Characterisation of Medkleb represents a positive first step towards selecting it as a platform for paratransgenic control, as *Klebsiella* bacteria are known to co-occur with the medfly at a frequency that suggests an incipient facultative mutualism may be developing (Behar et al., 2008a; Ben Ami et al., 2010; Leftwich et al., unpublished). However, facultative mutualism is not defined simply by co-occurrence, so I then further investigated the nature of the Medkleb/medfly relationship by analysing Medkleb's genome.

Both size and GC content are generally reduced when a bacterium adopts a facultative lifestyle (McCutcheon et al., 2011). The Medkleb genome was the second smallest of all *Klebsiella* bacteria in the Medkleb group (n=13). However, this did not represent a signal of facultative transition, as Medkleb was larger than the free-living *K. michiganensis* strain M1. If Medkleb was compared only with strains of *K. oxytoca*, then its genome was the smallest in the group (n=7) by 124Kb. However, a size differential of 124Kb is not substantial, as the smallest reported symbiont genome is only 112Kb in total (Bennett et al., 2013). Medkleb's GC content was also the second highest for any bacteria in the

Medkleb group, counter to the prediction of facultative mutualism. In addition to genome reduction and reduced GC content, a strongly symbiotic transition is generally associated with an increased mutation rate, which causes facultative symbionts to occupy extended branches of phylogenetic trees (McCutcheon et al., 2011). Phylogenetically, Medkleb was slightly distinct from the three bacteria most closely related to it, but not markedly so. In conclusion, the Medkleb genome did not exhibit three of the key features associated with the genomes of facultative mutualists.

High resolution comparative genetic techniques were also used to test for signals of a putative mutualistic transition. These analyses were designed to test two key hypotheses. 1) That mutualism would be evident if Medkleb possessed genetic loci/functions that were absent from closely related *Klebsiella* bacteria, if these loci/functions had an obvious mutualistic capacity for the fly. 2) That a facultative mutualistic transition might be evident if Medkleb was lacking genetic loci/functions found in all closely related *Klebsiella* bacteria , if these loci/functions were necessary for life in varied environments. Set against this is that gene loss may be unpredictable in the early stages of facultative mutualistic transitions (Moran et al., 2014).

According to synteny predicted with progressiveMauve (Darling et al., 2010), a single region of the Medkleb genome (LCB18 ; ≈nts 4973000-5000000) was unique in comparison to conspecifics (Figure 4.10). LCB18 contained several genes that facilitate horizontal gene transfer and may have been recently acquired. However, it is not yet evident whether LCB18 contains genes that might confer fitness to the medfly. LCB18 contains 25 predicted coding sequences. None of the coding sequences are obviously 'mutualistic', though this conclusion is hampered as thirteen sequences are annotated as "hypothetical proteins".

When a circular map of the Medkleb genome was created an inversion of GC skew highlighted a region of putative horizontal gene transfer (nts 654265-677695; GI1). GI1 was therefore assessed for genes with putative mutualistic signatures. GI1 is thought to have transferred from a plasmid, as it codes for *TraY* which facilitates plasmid conjugal transfer (Nelson et al., 1995). Although plasmid integration into host chromosomes is not uncommon (Bire et al., 2012; Dobrindt et al., 2004), GI1 did not appear to have

integrated from mkps 1-4, as it did not contain homologous nucleotide sequence with those elements. GI1 is predicted to code for 25 proteins including Bll0873. Bll0873 was first sequenced in *Bradyrhizobium diazoefficiens,* a bacterium that is a known nitrogen fixing symbiont of legumes (Kaneko et al., 2002). However, the specific function of Bll0873 is unknown, so there is as yet no evidence that it provides a mutualistic benefit the medfly. Interestingly, both GI1 and LCB18 code for genes that facilitate molybdopterin biosynthesis, a group of co-factors that are known to aid nitrogen reduction (Moreno-Vivian et al., 1999). As for LCB18, GI1 is predicted to code for thirteen hypothetical proteins.

In summary, the Medkleb genome contained two loci (LCB18 and GI1) that were unique when compared to other closely related *Klebsiella* bacteria in the Medkleb group. Both LCB18 and GI1 appear to have been horizontally acquired, but there is no evidence yet that either confer a mutualistic benefit to the medfly. Further analyses of LCB18 and GI1 would be extremely useful to elucidate the functions of the hypothetical proteins encoded.

According to KEGG analysis (Kanehisa et al., 2016), Medkleb contains coding sequences for 21 genes that were not coded by any other closely related *Klebsiella* bacteria in the Medkleb group. Some of these genes encode potentially mutualistic functions such as sugar metabolism and nitrogen fixation and it is plausible that the medfly is benefitting from these traits. Medkleb is also missing clusters of genes that are present in all other bacteria analysed. It is possible that these missing genes may have been deleted due to incipient mutualism with the medfly. Medkleb was the only bacteria analysed that does not code for specific enzymes related to copper resistance and phosphonate transport. At high concentrations copper can become toxic to bacteria (Grey et al., 2001), and phosphonate transport proteins provide feedback to bacteria regarding toxicity in their local environment (Rizk et al., 2006). Medkleb could have lost these abilities as they are not required in the medfly gut. To test this hypothesis, one could compare the concentrations of copper and phosphonate in the medfly gut with the concentrations of those materials in locations where *Klebsiella* bacteria are commonly found. However, as *Klebsiella* are ubiquitous in nature (Brisse et al., 2006) a full test of this hypothesis would

require a comprehensive survey of environmental concentrations of copper and phosphonate.

Finally, Medkleb's ability to synthesise secondary metabolites was compared to conspecifics with antiSMASH 3.0 (Weber et al., 2015), as secondary metabolites can provide insect hosts with a fitness benefit (Barke et al., 2011; Holmes et al., 2016). Medkleb was found to synthesise two clusters of secondary metabolites not produced by conspecifics. 1) Medkleb synthesises a butyrolactone, a signalling molecule utilised by *Streptomyces* bacteria to elicit the transcription of antibiotics (Kitani et al., 2011; Takano, 2006). 2) Medkleb synthesises an N-acyl amino acid cluster. N-acyl amino acids are common in soil dwelling bacteria but their function is unclear (Craig et al., 2011). It is not yet evident how either of these compounds might confer a mutualistic benefit to the medfly.

Table 4.5 Unique features of the Medkleb genome in comparison to conspecifics

Unique feature	Identified via	Possible mutualistic function
LCB18	progressiveMauve (Darling et al., 2010) analysis of Medkleb and three conspecifics	Codes for genes related to molybdopterin biosynthesis and several proteins with unknown function. These genes may confer fitness to the medfly.
GI1	Inversion of GC skew	Codes for genes related to molybdopterin biosynthesis and several proteins with unknown function. These genes may confer fitness to the medfly.

Atypical metabolic genes	Comparison of KEGG (Kanehisa et al., 2016) gene functions between Medkleb group members.	Expands the range of nutrients available to the medfly.
Absent sensory genes	Comparison of KEGG (Kanehisa et al., 2016) gene functions between Medkleb group members.	The medfly gut may be relatively non-toxic allowing Medkleb to survive without genes that detect and metabolise certain chemical threats in its environment.
Butyrolactone biosynthesis	antiSMASH 3.0 (Weber et al., 2015)	Unknown
N-acyl amino acid biosynthesis	antiSMASH 3.0 (Weber et al., 2015)	Unknown

In conclusion, if *K. oxytoca* is a facultative mutualist of the medfly as suggested (Behar et al., 2008a; Behar et al., 2008b; Ben Ami et al., 2010), then this relationship could be exploited for paratransgenic control. The Medkleb bacterium that was isolated from medfly guts was not definitively characterised as a strain of *K. oxytoca* although this may be rectified as more reliable reference genomes become available. Medkleb was demonstrated to be pectinolytic and comparative genomics highlighted that the genome contained two discrete loci (GI1 and LCB18) with mutualistic potential. Global analyses of Medkleb's gene functions in comparison to conspecifics highlighted that several genes were encoded exclusively by Medkleb. Some exclusive genes were metabolic enzymes

that could allow the medfly to utilise otherwise unattainable nutrients. However, as potentially mutualistic traits are not evidence of mutualism itself and many signals that are commonly associated with facultative mutualism are not captured by the Medkleb genome, there is insufficient evidence, so far, to definitively classify Medkleb as a facultative mutualist of the medfly. What has been established is that *Klebsiella* bacteria and the medfly appear to co-occur almost universally (Behar et al., 2008a; Leftwich et al., unpublished). Therefore, Medkleb might provide a platform for paratransgenic control of the medfly even if *Klebsiella* are not technically facultative or mutualistic. To establish whether Medkleb is a mutualist, medflies that are Medkleb deficient (*aMed*) would be extremely useful. In this scenario fitness parameters such as longevity and mating success could be compared between *aMed* and wild type flies to definitively evaluate if the medfly benefits from its association with *Klebsiella*.

4.6 Supplementary material

Table 4.1 Composition of tRNAs encoded by the Medkleb genome. An abundance of tRNA genes >61 is predictive of fast growth when anticodon diversity is <34 (Rocha, 2004a). The Medkleb tRNA complement is not predictive of fast or slow growth as the genome exhibits a total of 39 anticodons encoded by 85 tRNA genes.

Amino	Codon	Copy number	Amino	Codon	Copy number
Isoleucine	GAT	3	Glycine	TTG	2
Alanine	TGC	3	Leucine	TAG	1
Aspartatic acid	GTC	3	Lysine	TTT	5
Tryptophan	CCA	3	Valine	TAC	4
Arginine	CCG	3	Asparagine	GTT	4
Histidine	GTG	1	Serine	CGA	1
Leucine	GAG	1	Cystine	GCA	1
Proline	TGG	1	Leucine	TAA	1
Threonine	TGT	1	Valine	GAC	3
Tyrosine	GTA	4	Serine	GGA	2
Glycine	TCC	1	Serine	TGA	1
Threonine	GGT	2	Proline	GGG	1
Glutamic acid	TTC	4	Argenine	CCT	1
Phenylalanine	GAA	2	Alanine	GGC	2
Glycine	GCC	4	Argenine	AGC	4
Leucine	CAA	1	Serine	GCT	1
Threonine	CGT	1	Glycine	CCC	1
Argenine	TCT	1	Leucine	GAG	1
Glycine	CTG	2	Proline	CGG	1
Methionine	CAT	7			

Table 4.2 The length and GC content of 30 plasmids associated with K. oxytoca bacteria. The lengths of the four Medkleb associated plasmids (63257-136402 nt) fall within the expected range of plasmids associated with *K. oxytoca* (Mean = 124973nt ± s.d. 88432). Medkleb associated plasmids fall within the range of expected GC content for *K. oxytoca* associated plasmids (Mean = 52.95% ± s.d. 3.21).

Host bacteria	Length (nt)	GC content (%)	NCBI Accession
AR-0147	110705	54.09	CP020359.1
CAV1015	76186	52.68	CP017932
CAV1015	113105	54.17	CP009466.1
CAV1015	114139	51.49	CP017930.1
CAV1015	111395	48.86	CP017931.1
CAV1015	11638	58.08	CP017933.1
CAV1374	332956	51.46	CP011635.1
CAV1374	227680	53.83	CP011634.1
CAV1374	150318	53.67	CP011633.1
CAV1374	83652	53.17	CP011632.1
CAV1374	16069	59.34	CP011628.1
KONIH1	205586	53.9	CP008791.1
KONIH1	133397	52.12	CP008789.1
KONIH5	282476	51.34	CP026276.1
KONIH5	56561	53.39	CP026277.1
TJ11	275300	46.42	MG845200.1
CAV1752	277616	53.16	CP018361.1
ATCC51983	89977	54.41	KJ541681.1
pKOX3	61680	52.23	KY913900.1
KONIH4	235655	52.4	CP026274.1
CAV1335	113105	54.18	CP011615.1
7121	42461	51.69	KX784502.1
AR_0028	91175	50.64	CP026718.1
AR_0028	189585	51.14	CP026716.1
pKOX3	55246	49.08	KY913899.1
pKOI-34	87343	53.25	AB715422.2
pKLox-45574cz	46161	46.66	MG833406.1

97_38	68763	53.13	KY128483.1
AR_0028	80970	56.88	CP026717.1
H146340707	8300	61.6	KX946994.1
Medkleb (Mkp1)	136402	55.2	
Medkleb (Mkp2)	122124	50.4	
Medkleb (Mkp3)	78046	56.7	
Medkleb (Mkp4)	63257	50.7	

4.1) Primers used for PCR analysis of *pehX* and 16S

As in Kovtunovych et al. (2003) the pehX gene was amplified using PEH-C forward primer (5'GATACGGAGTATGCCTTTACGGTG-3') and PEH-D reverse primer (5'TAGCCTTTATCAAGCGG ATACTGG-3'). The 16S gene was amplified using 541F forward primer (5'GTGCCAGCMGCCGCGGTAA-3') and 806R reverse primer (5'GGACTACHVGGGTWTCTAAT-3') as in Caporaso et al. (2011).

4.7) References

Abraham, N. M., Liu, L., Jutras, B. L., Yadav, A. K., Narasimhan, S., Gopalakrishnan, V., . . . Fikrig, E. (2017). Pathogen-mediated manipulation of arthropod microbiota to promote infection. *Proceedings of the National Academy of Sciences, 114*(5), E781. doi:10.1073/pnas.1613422114

Aharon, Y., Pasternak, Z., Ben Yosef, M., Behar, A., Lauzon, C., Yuval, B., & Jurkevitch, E. (2013). Phylogenetic, metabolic, and taxonomic diversities shape Mediterranean fruit fly microbiotas during ontogeny. *Appl Environ Microbiol, 79*(1), 303-313. doi:10.1128/aem.02761-12

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped blast and psi-blast: A new generation of protein database search programs. *Nucleic Acids Res, 25*(17), 3389-3402.

Anbutsu, H., Moriyama, M., Nikoh, N., Hosokawa, T., Futahashi, R., Tanahashi, M., . . . Fukatsu, T. (2017). Small genome symbiont underlies cuticle hardness in beetles. *Proceedings of the National Academy of Sciences, 114*(40), E8382. doi:10.1073/pnas.1712857114

Arredondo-Alonso, S., Rogers, M. R. C., Braat, J. C., Verschuuren, T. D., Top, J., Corander, J., . . . Schurch, A. C. (2018). Mlplasmids: A user-friendly tool to predict plasmid- and chromosome-derived sequences for single species. *Microb Genom*. doi:10.1099/mgen.0.000224

Ballinger, M. J., & Perlman, S. J. (2017). Generality of toxins in defensive symbiosis: Ribosome-inactivating proteins and defense against parasitic wasps in *Drosophila*. *PLoS Pathog, 13*(7), e1006431. doi:10.1371/journal.ppat.1006431

Bao, G., Zhang, Y., Du, C., Chen, Z., Li, Y., Cao, Z., & Ma, Y. (2013). Genome sequence of *Klebsiella oxytoca* m5al, a promising strain for nitrogen fixation and chemical production. *Genome Announc, 1*(1). doi:10.1128/genomeA.00074-12

Barke, J., Seipke, R. F., Yu, D. W., & Hutchings, M. I. (2011). A mutualistic microbiome: How do fungus-growing ants select their antibiotic-producing bacteria? *Commun Integr Biol, 4*(1), 41-43. doi:10.4161/cib.4.1.13552

Behar, A., Jurkevitch, E., & Yuval, B. (2008a). Bringing back the fruit into fruit fly–bacteria interactions. *Molecular Ecology, 17*(5), 1375-1386. doi:10.1111/j.1365-294X.2008.03674.x

Behar, A., Yuval, B., & Jurkevitch, E. (2008b). Gut bacterial communities in the Mediterranean fruit fly (*Ceratitis capitata*) and their impact on host longevity. *J Insect Physiol, 54*(9), 1377-1383. doi:10.1016/j.jinsphys.2008.07.011

Ben-Yosef, M., Pasternak, Z., Jurkevitch, E., & Yuval, B. (2014). Symbiotic bacteria enable olive flies (bactrocera oleae) to exploit intractable sources of nitrogen. *J Evol Biol, 27*(12), 2695-2705. doi:10.1111/jeb.12527

Ben Ami, E., Yuval, B., & Jurkevitch, E. (2010). Manipulation of the microbiota of mass-reared mediterranean fruit flies *Ceratitis capitata (Diptera: Tephritidae)* improves sterile male sexual performance. *Isme j, 4*(1), 28-37. doi:10.1038/ismej.2009.82

Bosco-Drayon, V., Poidevin, M., Boneca, I. G., Narbonne-Reveau, K., Royet, J., & Charroux, B. (2012). Peptidoglycan sensing by the receptor pgrp-le in the drosophila gut induces immune responses to infectious bacteria and tolerance to microbiota. *Cell Host Microbe, 12*(2), 153-165. doi:10.1016/j.chom.2012.06.002

Bennett, G. M., & Moran, N. A. (2013). Small, smaller, smallest: The origins and evolution of ancient dual symbioses in a phloem-feeding insect. *Genome Biol Evol, 5*(9), 1675-1688. doi:10.1093/gbe/evt118

Bire, S., & Rouleux-Bonnin, F. (2012). Transposable elements as tools for reshaping the genome: It is a huge world after all! *Methods Mol Biol, 859*, 1-28. doi:10.1007/978-1-61779-603-6_1

Bowers, R. M., Kyrpides, N. C., Stepanauskas, R., Harmon-Smith, M., Doud, D., Reddy, T. B. K., . . . Woyke, T. (2017). Minimum information about a single amplified genome (misag) and a metagenome-assembled genome (mimag) of bacteria and archaea. *Nature Biotechnology, 35*, 725. doi:10.1038/nbt.3893

Brisse, S., Grimont, F., & Grimont, P. A. D. (2006). The genus *Klebsiella*. In M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, & E. Stackebrandt (Eds.), *The prokaryotes: Volume 6: Proteobacteria: Gamma subclass* (pp. 159-196). New York, NY: Springer New York.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., . . . Knight, R. (2011). Global patterns of 16s rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci U S A, 108 Suppl 1*, 4516-4522. doi:10.1073/pnas.1000080107

Carver, T., Thomson, N., Bleasby, A., Berriman, M., & Parkhill, J. (2009). DNAplotter: Circular and linear interactive genome visualization. *Bioinformatics, 25*(1), 119-120.
doi:10.1093/bioinformatics/btn578

Cascales, E., Buchanan, S. K., Duché, D., Kleanthous, C., Lloubès, R., Postle, K., . . . Cavard, D. (2007). Colicin biology. *Microbiology and molecular biology reviews : MMBR, 71*(1), 158-229.
doi:10.1128/MMBR.00036-06

Chain, P. S. G., Grafham, D. V., Fulton, R. S., Fitzgerald, M. G., Hostetler, J., Muzny, D., . . . Detter, J. C. (2009). Genomics. Genome project standards in a new era of sequencing. *Science (New York, N.Y.), 326*(5950), 236-237.
doi:10.1126/science.1180614

Chaisson, M. J., & Tesler, G. (2012). Mapping single molecule sequencing reads using basic local alignment with successive refinement (blasr): Application and theory. *BMC Bioinformatics, 13*, 238.
doi:10.1186/1471-2105-13-238

Chandler, J. A., Morgan Lang, J., Bhatnagar, S., Eisen, J. A., & Kopp, A. (2011). Bacterial communities of diverse drosophila species: Ecological context of a host–microbe model system. *Plos Genetics, 7*(9), e1002272.
doi:10.1371/journal.pgen.1002272

Chin, C. S., Alexander, D. H., Marks, P., Klammer, A. A., Drake, J., Heiner, C., . . . Korlach, J. (2013). Nonhybrid, finished microbial genome assemblies from long-read smrt sequencing data. *Nature Methods, 10*(6), 563-569.
doi:10.1038/nmeth.2474

Craig, J. W., Cherry, M. A., & Brady, S. F. (2011). Long-chain n-acyl amino acid synthases are linked to the putative pep-cterm/exosortase protein-sorting system in gram-negative bacteria. *Journal of bacteriology, 193*(20), 5707-5715.
doi:10.1128/JB.05426-11

Darling, A. E., Mau, B., & Perna, N. T. (2010). Progressivemauve: Multiple genome alignment with gene gain, loss and rearrangement. *Plos One, 5*(6), e11147.
doi:10.1371/journal.pone.0011147

Deguenon, J. M., Travanty, N., Zhu, J., Carr, A., Denning, S., Reiskind, M. H., . . . Ponnusamy, L. (2019). Exogenous and endogenous microbiomes of wild-caught phormia regina (diptera: Calliphoridae) flies from a suburban farm by 16s rrna gene sequencing. *Scientific Reports, 9*(1), 20365.
doi:10.1038/s41598-019-56733-z

de Graaf, F. K., Spanjaerdt Speckman, E. A., & Stouthamer, A. H. (1969). Mode of action of a bacteriocin produced by enterobacter cloacae df13. *Antonie Van Leeuwenhoek, 35*(3), 287-306.

de Souza, D. J., Bézier, A., Depoix, D., Drezen, J.-M., & Lenoir, A. (2009). *Blochmannia* endosymbionts improve colony growth and immune defence in the ant *Camponotus fellah*. *BMC Microbiology, 9*(1), 29. doi:10.1186/1471-2180-9-29

Dobrindt, U., Hochhut, B., Hentschel, U., & Hacker, J. (2004). Genomic islands in pathogenic and environmental microorganisms. *Nature Reviews Microbiology, 2*, 414. doi:10.1038/nrmicro884

Durvasula, R. V., Gumbs, A., Panackal, A., Kruglov, O., Aksoy, S., Merrifield, R. B., . . . Beard, C. B. (1997). Prevention of insect-borne disease: An approach using transgenic symbiotic bacteria. *Proceedings of the National Academy of Sciences of the United States of America, 94*(7), 3274-3278. doi:10.1073/pnas.94.7.3274

Figueras, M. J., Beaz-Hidalgo, R., Hossain, M. J., & Liles, M. R. (2014). Taxonomic affiliation of new genomes should be verified using average nucleotide identity and multilocus phylogenetic analysis. *Genome Announc, 2*(6). doi:10.1128/genomeA.00927-14

Gascuel, O. (1997). Bionj: An improved version of the nj algorithm based on a simple model of sequence data. *Mol Biol Evol, 14*(7), 685-695. doi:10.1093/oxfordjournals.molbev.a025808

Gavriel, S., Jurkevitch, E., Gazit, Y., & Yuval, B. (2011). Bacterially enriched diet improves sexual performance of sterile male Mediterranean fruit flies. *Journal of Applied Entomology, 135*(7), 564-573. doi:10.1111/j.1439-0418.2010.01605.x

Gould, A. L., Zhang, V., Lamberti, L., Jones, E. W., Obadia, B., Korasidis, N., . . . Ludington, W. B. (2018). Microbiome interactions shape host fitness. *Proceedings of the National Academy of Sciences, 115*(51), E11951. doi:10.1073/pnas.1809349115

Grey, B., & Steck, T. R. (2001). Concentrations of copper thought to be toxic to *Escherichia coli* can induce the viable but nonculturable condition. *Appl Environ Microbiol, 67*(11), 5325-5327. doi:10.1128/AEM.67.11.5325-5327.2001

Guerfali, M. M., Parker, A., Fadhl, S., Hemdane, H., Raies, A., & Chevrier, C. (2011). Fitness and reproductive potential of irradiated mass-reared Mediterranean fruit fly males *Ceratitis capitata (Diptera: Tephritidae):* Lowering radiation doses. *Florida Entomologist, 94*(4), 1042-1050.

Hansen, A. K., & Moran, N. A. (2011). Aphid genome expression reveals host–symbiont cooperation in the production of amino acids. *Proceedings of the National Academy of Sciences, 108*(7), 2849. doi:10.1073/pnas.1013465108

Heine, D., Holmes, N. A., Worsley, S. F., Santos, A. C. A., Innocent, T. M., Scherlach, K., . . . Wilkinson, B. (2018). Chemical warfare between leafcutter ant symbionts and a co-evolved pathogen. *Nature Communications, 9*(1), 2208. doi:10.1038/s41467-018-04520-1

Holmes, N. A., Innocent, T. M., Heine, D., Bassam, M. A., Worsley, S. F., Trottmann, F., . . . Hutchings, M. I. (2016). Genome analysis of two *Pseudonocardia* phylotypes associated with *Acromyrmex* leafcutter ants reveals their biosynthetic potential. *Front Microbiol, 7*, 2073. doi:10.3389/fmicb.2016.02073

Hurst, G. D. D., & Frost, C. L. (2015). Reproductive parasitism: Maternally inherited symbionts in a biparental world. *Cold Spring Harbor perspectives in biology, 7*(5), a017699. doi:10.1101/cshperspect.a017699

Husnik, F., Nikoh, N., Koga, R., Ross, L., Duncan, Rebecca P., Fujie, M., . . . McCutcheon, John P. (2013). Horizontal gene transfer from diverse bacteria to an insect genome enables a tripartite nested mealybug symbiosis. *Cell, 153*(7), 1567-1578. doi:10.1016/j.cell.2013.05.040

Hyatt, D., Chen, G. L., Locascio, P. F., Land, M. L., Larimer, F. W., & Hauser, L. J. (2010). Prodigal: Prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics, 11*, 119. doi:10.1186/1471-2105-11-119

Janda, J. M., & Abbott, S. L. (2007). 16s rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. *J Clin Microbiol, 45*(9), 2761-2764. doi:10.1128/jcm.01228-07

Jurkevitch, E. (2011). Riding the trojan horse: Combating pest insects with their own symbionts. *Microb Biotechnol, 4*(5), 620-627. doi:10.1111/j.1751-7915.2011.00249.x

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., & Tanabe, M. (2016). Kegg as a reference resource for gene and protein annotation. *Nucleic Acids Res, 44*. doi:10.1093/nar/gkv1070

Kaneko, T., Nakamura, Y., Sato, S., Minamisawa, K., Uchiumi, T., Sasamoto, S., . . . Tabata, S. (2002). Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* usda110. *DNA Res, 9*(6), 225-256. doi:10.1093/dnares/9.6.225

Kim, M., Oh, H. S., Park, S. C., & Chun, J. (2014). Towards a taxonomic coherence between average nucleotide identity and 16s rRNA gene sequence similarity for species demarcation of prokaryotes. *Int J Syst Evol Microbiol, 64*(Pt 2), 346-351. doi:10.1099/ijs.0.059774-0

Kitani, S., Miyamoto, K. T., Takamatsu, S., Herawati, E., Iguchi, H., Nishitomi, K., . . . Nihira, T. (2011). Avenolide, a hormone controlling antibiotic production in *Streptomyces avermitilis*. *Proceedings of the National Academy of Sciences, 108*(39), 16410. doi:10.1073/pnas.1113908108

Knipling, E. F. (1955). Possibilities of insect control or eradication through the use of sexually sterile males. *Journal of Economic Entomology, 48*(4), 459.

Konstantinidis, K. T., & Tiedje, J. M. (2005). Genomic insights that advance the species definition for prokaryotes. *Proceedings of the National Academy of Sciences of the United States of America, 102*(7), 2567-2572. doi:10.1073/pnas.0409727102

Kovtunovych, G., Lytvynenko, T., Negrutska, V., Lar, O., Brisse, S., & Kozyrovska, N. (2003). Identification of *Klebsiella oxytoca* using a specific PCR assay targeting the polygalacturonase pehX gene. *Res Microbiol, 154*(8), 587-592. doi:10.1016/s0923-2508(03)00148-7

Kuo, C. H., Moran, N. A., & Ochman, H. (2009). The consequences of genetic drift for bacterial genome complexity. *Genome Res, 19*(8), 1450-1454. doi:10.1101/gr.091785.109

Lagesen, K., Hallin, P., Rødland, E. A., Staerfeldt, H.-H., Rognes, T., & Ussery, D. W. (2007). Rnammer: Consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Research, 35*(9), 3100-3108. doi:10.1093/nar/gkm160

Lawrence, J. G., & Ochman, H. (1997). Amelioration of bacterial genomes: Rates of change and exchange. *J Mol Evol, 44*(4), 383-397.

Lee, C., Grasso, C., & Sharlow, M. F. (2002). Multiple sequence alignment using partial order graphs. *Bioinformatics, 18*(3), 452-464. doi:10.1093/bioinformatics/18.3.452

Leftwich, P. T., & Chapman, T. *unpublished*.

Lhocine, N., Ribeiro, P., Buchon, N., Wepf, A., Wilson, R., Tenev, T., . . . Leulier, F. (2008). Pims modulates immune tolerance by negatively regulating drosophila innate immune signaling. *Cell host & microbe, 4*, 147-158. doi:10.1016/j.chom.2008.07.004

Lindberg, B. G., Tang, X., Dantoft, W., Gohel, P., Seyedoleslami Esfahani, S., Lindvall, J. M., & Engstrom, Y. (2018). Nubbin isoform antagonism governs drosophila intestinal immune homeostasis. *PLoS Pathog, 14*(3), e1006936. doi:10.1371/journal.ppat.1006936

Liu, L. J., Martinez-Sañudo, I., Mazzon, L., Prabhakar, C. S., Girolami, V., Deng, Y. L., . . . Li, Z. H. (2016). Bacterial communities associated with invasive populations of bactrocera dorsalis (diptera: Tephritidae) in china. *Bulletin of Entomological Research, 106*(6), 718-728. doi:10.1017/S0007485316000390

Lobry, J. R. (1996). Asymmetric substitution patterns in the two DNA strands of bacteria. *Mol Biol Evol, 13*(5), 660-665. doi:10.1093/oxfordjournals.molbev.a025626

Lombard, V., Bernard, T., Rancurel, C., Brumer, H., Coutinho, P. M., & Henrissat, B. (2010). A hierarchical classification of polysaccharide lyases for glycogenomics. *Biochem J, 432*(3), 437-444. doi:10.1042/bj20101185

Malik, V. S. (1980). Microbial secondary metabolism. *Trends in Biochemical Sciences, 5*(3), 68-72. doi:10.1016/0968-0004(80)90071-7

Marchler-Bauer, A., Liu, C., He, J., Geer, L. Y., Han, L., Geer, R. C., . . . Shi, W. (2009). The NCBI biosystems database. *Nucleic Acids Research, 38*. doi:10.1093/nar/gkp858

McCutcheon, J. P., & Moran, N. A. (2011). Extreme genome reduction in symbiotic bacteria. *Nat Rev Microbiol, 10*(1), 13-26. doi:10.1038/nrmicro2670

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Domazet-Lošo, T., Douglas, A. E., . . . Wernegreen, J. J. (2013). Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences of the United States of America, 110*(9), 3229-3236. doi:10.1073/pnas.1218525110

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry, 31*(3), 426-428. doi:10.1021/ac60147a030

Moran, N. A., & Bennett, G. M. (2014). The tiniest tiny genomes. *Annu Rev Microbiol, 68*, 195-215. doi:10.1146/annurev-micro-091213-112901

Moran, N. A., McCutcheon, J. P., & Nakabachi, A. (2008). Genomics and evolution of heritable bacterial symbionts. *Annu Rev Genet, 42*, 165-190. doi:10.1146/annurev.genet.41.110306.130119

Moran, N. A., Tran, P., & Gerardo, N. M. (2005). Symbiosis and insect diversification: An ancient symbiont of sap-feeding insects from the bacterial phylum . *Appl Environ Microbiol, 71*(12), 8802-8810. doi:10.1128/aem.71.12.8802-8810.2005

Moreno-Vivian, C., Cabello, P., Martinez-Luque, M., Blasco, R., & Castillo, F. (1999). Prokaryotic nitrate reduction: Molecular properties and functional distinction among bacterial nitrate reductases. *J Bacteriol, 181*(21), 6573-6584.

Negri, I., Franchini, A., Gonella, E., Daffonchio, D., Mazzoglio, P. J., Mandrioli, M., & Alma, A. (2009). Unravelling the *Wolbachia* evolutionary role: The reprogramming of the host genomic imprinting. *Proc Biol Sci, 276*(1666), 2485-2491. doi:10.1098/rspb.2009.0324

Nelson, W. C., Howard, M. T., Sherman, J. A., & Matson, S. W. (1995). The traY gene product and integration host factor stimulate *Escherichia coli* DNA helicase i-catalyzed nicking at the f-plasmid oriT. *J Biol Chem, 270*(47), 28374-28380.

Nishida, H. (2012). Comparative analyses of base compositions, DNA sizes, and dinucleotide frequency profiles in archaeal and bacterial chromosomes and plasmids. *International Journal of Evolutionary Biology, 2012*. doi:10.1155/2012/342482

Ochman, H., & Davalos, L. M. (2006). The nature and dynamics of bacterial genomes. *Science, 311*(5768), 1730-1733. doi:10.1126/science.1119966

Ochman, H., Lawrence, J. G., & Groisman, E. A. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature, 405*(6784), 299-304. doi:10.1038/35012500

Oliver, K. M., Russell, J. A., Moran, N. A., & Hunter, M. S. (2003). Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proc Natl Acad Sci U S A, 100*(4), 1803-1807. doi:10.1073/pnas.0335320100

Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J., Disz, T., . . . Stevens, R. (2014). The seed and the rapid annotation of microbial genomes using subsystems technology (rast). *Nucleic Acids Research, 42*. doi:10.1093/nar/gkt1226

Parks, D. H., Imelfort, M., Skennerton, C. T., Hugenholtz, P., & Tyson, G. W. (2015). Checkm: Assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res, 25*(7), 1043-1055. doi:10.1101/gr.186072.114

Peplies, J., Kottmann, R., Ludwig, W., & Glöckner, F. O. (2008). A standard operating procedure for phylogenetic inference (soppi) using (rRNA) marker genes. *Systematic and Applied Microbiology, 31*(4), 251-257. doi:10.1016/j.syapm.2008.08.003

Price, M. N., Dehal, P. S., & Arkin, A. P. (2010). Fasttree 2-approximately maximum-likelihood trees for large alignments. *Plos One, 5*(3), e9490. doi:10.1371/journal.pone.0009490

Providenti, M. A., O'Brien, J. M., Ewing, R. J., Paterson, E. S., & Smith, M. L. (2006). The copy-number of plasmids and other genetic elements can be determined by SYBR-green-based quantitative real-time PCR. *Journal of Microbiological Methods, 65*(3), 476-487. doi:10.1016/j.mimet.2005.09.007

Pruesse, E., Peplies, J., & Glöckner, F. O. (2012). Sina: Accurate high-throughput multiple sequence alignment of ribosomal RNA genes. *Bioinformatics, 28*(14), 1823-1829. doi:10.1093/bioinformatics/bts252

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., . . . Glöckner, F. O. (2013). The silva ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research, 41*. doi:10.1093/nar/gks1219

Rambaut, A., & Drummond, A. (2009). Figtree version 1.4.3. Retrieved from http://tree.bio.ed.ac.uk/

Reva, O. N., & Tummler, B. (2004). Global features of sequences of bacterial chromosomes, plasmids and phages revealed by analysis of oligonucleotide usage patterns. *BMC Bioinformatics, 5*, 90. doi:10.1186/1471-2105-5-90

Rhoads, A., & Au, K. F. (2015). Pacbio sequencing and its applications. *Genomics, Proteomics & Bioinformatics, 13*(5), 278-289. doi:10.1016/j.gpb.2015.08.002

Richter, M., & Rossello-Mora, R. (2009). Shifting the genomic gold standard for the prokaryotic species definition. *Proc Natl Acad Sci U S A, 106*(45), 19126-19131. doi:10.1073/pnas.0906412106

Rizk, S. S., Cuneo, M. J., & Hellinga, H. W. (2006). Identification of cognate ligands for the *Escherichia coli* phnd protein product and engineering of a reagentless fluorescent biosensor for phosphonates. *Protein Sci, 15*(7), 1745-1751. doi:10.1110/ps.062135206

Rocha, E. P. (2004a). Codon usage bias from tRNA's point of view: Redundancy, specialization, and efficient decoding for translation optimization. *Genome Res, 14*(11), 2279-2286. doi:10.1101/gr.2896904

Rocha, E. P. (2004b). The replication-related organization of bacterial genomes. *Microbiology, 150*(Pt 6), 1609-1627. doi:10.1099/mic.0.26974-0

Rocha, E. P. C., & Danchin, A. (2002). Base composition bias might result from competition for metabolic resources. *Trends in Genetics, 18*(6), 291-294. doi:10.1016/S0168-9525(02)02690-2

Sabree, Z. L., Huang, C. Y., Okusu, A., Moran, N. A., & Normark, B. B. (2013). The nutrient supplying capabilities of *uzinura*, an endosymbiont of armoured scale insects. *Environ Microbiol, 15*(7), 1988-1999. doi:10.1111/1462-2920.12058

Saha, R., Farrance, C. E., Verghese, B., Hong, S., & Donofrio, R. S. (2013). *Klebsiella michiganensis* sp. Nov., a new bacterium isolated from a tooth brush holder. *Curr Microbiol, 66*(1), 72-78. doi:10.1007/s00284-012-0245-x

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y., & Ishikawa, H. (2000). Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. Aps. *Nature, 407*(6800), 81-86. doi:10.1038/35024074

Shin, S. H., Kim, S., Kim, J. Y., Lee, S., Um, Y., Oh, M. K., . . . Yang, K. S. (2012). Complete genome sequence of *Klebsiella oxytoca* kctc 1686, used in production of 2,3-butanediol. *J Bacteriol, 194*(9), 2371-2372. doi:10.1128/jb.00026-12

Shintani, M., Sanchez, Z. K., & Kimbara, K. (2015). Genomics of microbial plasmids: Classification and identification based on replication and transfer systems and host taxonomy. *Frontiers in microbiology, 6*, 242-242. doi:10.3389/fmicb.2015.00242

Simao, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V., & Zdobnov, E. M. (2015). Busco: Assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics, 31*(19), 3210-3212. doi:10.1093/bioinformatics/btv351

Sinotte, V. M., Freedman, S. N., Ugelvig, L. V., & Seid, M. A. (2018). *Camponotus floridanus* ants incur a trade-off between phenotypic development and pathogen susceptibility from their mutualistic endosymbiont *Blochmannia*. *Insects, 9*(2). doi:10.3390/insects9020058

Sloan, D. B., & Moran, N. A. (2012). Endosymbiotic bacteria as a source of carotenoids in whiteflies. *Biol Lett, 8*(6), 986-989. doi:10.1098/rsbl.2012.0664

Sohail, M., & Latif, Z. (2016). Phylogenetic analysis of polygalacturonase-producing *Bacillus* and *Pseudomonas* isolated from plant waste material. *Jundishapur J Microbiol, 9*(1), e28594. doi:10.5812/jjm.28594

Stackebrandt, E. (2006). Taxonomic parameters revisited : Tarnished gold standards. *Microbiol. Today, 33*, 152-155.

Storelli, G., Strigini, M., Grenier, T., Bozonnet, L., Schwarzer, M., Daniel, C., . . . Leulier, F. (2018). *Drosophila* perpetuates nutritional mutualism by promoting the fitness of its intestinal symbiont *Lactobacillus plantarum*. *Cell Metabolism, 27*(2), 362-377.e368. doi:10.1016/j.cmet.2017.11.011

Takano, E. (2006). Ɣ-butyrolactones: *Streptomyces* signalling molecules regulating antibiotic production and differentiation. *Current Opinion in Microbiology, 9*(3), 287-294. doi:10.1016/j.mib.2006.04.003

Tamas, I., Klasson, L., Canback, B., Naslund, A. K., Eriksson, A. S., Wernegreen, J. J., . . . Andersson, S. G. (2002). 50 million years of genomic stasis in endosymbiotic bacteria. *Science, 296*(5577), 2376-2379. doi:10.1126/science.1071278

Team, R. C. (2016). R: A language and environment for statistical computing: R Foundation for Statistical Computing, Vienna, Austria. Retrieved from www.R-project.org

Tindall, B. J., Rossello-Mora, R., Busse, H. J., Ludwig, W., & Kampfer, P. (2010). Notes on the characterization of prokaryote strains for taxonomic purposes. *Int J Syst Evol Microbiol, 60*(Pt 1), 249-266. doi:10.1099/ijs.0.016949-0

Toledo, J., Rull, J., Oropeza, A., Hernandez, E., & Liedo, P. (2004). Irradiation of *Anastrepha obliqua (Diptera: Tephritidae)* revisited: Optimizing sterility induction. *J Econ Entomol, 97*(2), 383-389.

van Ham, R. C. H. J., Kamerbeek, J., Palacios, C., Rausell, C., Abascal, F., Bastolla, U., . . . Moya, A. (2003). Reductive genome evolution in *Buchnera aphidicola*. *Proceedings of the National Academy of Sciences, 100*(2), 581. doi:10.1073/pnas.0235981100

Vieira-Silva, S., & Rocha, E. P. C. (2010). The systemic imprint of growth and its uses in ecological (meta)genomics. *Plos Genetics, 6*(1), e1000808. doi:10.1371/journal.pgen.1000808

Weber, T., Blin, K., Duddela, S., Krug, D., Kim, H. U., Bruccoleri, R., . . . Medema, M. H. (2015). Antismash 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic Acids Res, 43*(W1), W237-243. doi:10.1093/nar/gkv437

Weiss, B., & Kaltenpoth, M. (2016). Bacteriome-localized intracellular symbionts in pollen-feeding beetles of the *genus Dasytes (Coleoptera, Dasytidae)*. *Frontiers in microbiology, 7*(1486). doi:10.3389/fmicb.2016.01486

Whitten, M. M. A., Facey, P. D., del Sol, R., Fernández-Martínez, L. T., Evans, M. C., Mitchell, J. J., . . . Dyson, P. J. (2015). Symbiont-mediated RNA interference in insects. *Proceedings of the Royal Society B: Biological Sciences, 283*(1825). doi:10.1098/rspb.2016.0042

Wixon, J. (2001). Featured organism: Reductive evolution in bacteria*: Buchnera* sp., *rickettsia prowazekii* and *Mycobacterium leprae*. *Comparative and functional genomics, 2*(1), 44-48. doi:10.1002/cfg.70

Woruba, D. N., Morrow, J. L., Reynolds, O. L., Chapman, T. A., Collins, D. P., & Riegler, M. (2019). Diet and irradiation effects on the bacterial community composition and structure in the gut of domesticated teneral and mature queensland fruit fly, bactrocera tryoni (diptera: Tephritidae). *BMC Microbiology, 19*(1), 281. doi:10.1186/s12866-019-1649-6

Wu, D., Daugherty, S. C., Van Aken, S. E., Pai, G. H., Watkins, K. L., Khouri, H., . . . Eisen, J. A. (2006). Metabolic complementarity and genomics of the dual bacterial symbiosis of sharpshooters. *PLoS Biol, 4*(6), e188. doi:10.1371/journal.pbio.0040188

Wyder, S., Kriventseva, E. V., Schröder, R., Kadowaki, T., & Zdobnov, E. M. (2007). Quantification of ortholog losses in insects and vertebrates. *Genome Biology, 8*(11), R242-R242. doi:10.1186/gb-2007-8-11-r242

Yang, Z. (1994). Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: Approximate methods. *J Mol Evol, 39*(3), 306-314.

Yoon, S.-H., Ha, S.-M., Kwon, S., Lim, J., Kim, Y., Seo, H., & Chun, J. (2017). Introducing ezbiocloud: A taxonomically united database of 16s rRNA gene sequences and whole-genome assemblies. *Int J Syst Evol Microbiol, 67*(5), 1613-1617. doi:doi:10.1099/ijsem.0.001755

Chapter 5 - Assessing egg smearing as a mechanism for vertical transmission of *Klebsiella oxytoca* bacteria by the Mediterranean Fruit Fly

5.1) Abstract

The Mediterranean fruit fly (*Ceratitis capitata*; medfly) is a highly invasive, economically important agricultural pest. *Klebsiella oxytoca* bacteria have frequently been detected in the guts of medfly adults and larvae and it has been suggested that they may be transmitted vertically between medfly generations. The relevance of this association in the context of pest control is that heritable bacteria can be manipulated for the control of insect hosts through paratransgenesis. I hypothesised that *K. oxytoca* were being passed vertically by medflies on the egg surface and tested this idea by dechorionating eggs to disrupt the process. I analysed the gut microbiomes of flies with 16S metagenomics and found no evidence that vertical transmission of *K. oxytoca* had been obstructed by egg dechorionation. However, there was good evidence that *K. oxytoca* can be introduced to medfly guts via the diet, reinforcing the premise that *K. oxytoca* offer a potential platform for paratransgenic control of the medfly.

5.2) Introduction

The Mediterranean fruit fly (*Ceratitis capitata;* medfly) is a pest of over 300 plants (Liquido et al., 1991). It is native to West Africa (Gasperi et al., 1991) but is highly invasive and has spread to over 100 countries on 7 continents (CABI, 2019). The medfly gut microbiome has consistently been reported to be numerically dominated by *Klebsiella oxytoca* bacteria (Aharon et al., 2013; Behar et al., 2008; Ben Ami et al., 2010; Leftwich et al., unpublished). Leftwich et al. (unpublished) detected large quantities of *K. oxytoca* in the guts of medflies that were reared both in distinct environments and on distinct diets, which suggests that medflies may acquire *K. oxytoca* by a conserved mechanism and not via ecological drift.

Behar et al. (2008) suggest that *K. oxytoca* is transmitted transovarially (on the egg surface) (Salem et al., 2015) by female medflies to their offspring, based on the observation that fruit rots at an accelerated rate when exposed to medfly eggs. They infer that fruit rots when it comes into contact with medfly eggs, due to secondary infection with transovarial *K. oxytoca.* Consistent with this, Aharon et al. (2013) identified bacteria on the exterior of medfly eggs with Fluorescence In Situ Hybridisation (FISH). However, Behar et al. (2008) did not include a negative control (such as surface sterilised eggs (Heys et al., 2018; Kaltenpoth et al., 2009; Koyle et al., 2016) to confirm that fruit rot is caused by transovarial *K. oxytoca*.

If *K. oxytoca* is transovarially transmitted by the medfly, then the *K. oxytoca/*medfly relationship could be defined as facultative (Moran et al., 2008). Facultative bacteria are vertically transmitted by hosts to their offspring but are able to survive when separated from the host (Moran et al., 2008). As such, *K. oxytoca* could offer a potential platform for control of the medfly, as facultative bacteria can be manipulated for control purposes through paratransgenesis.

Paratransgenesis is the genetic manipulation of heritable bacteria for transfer of desired traits to host organisms (Durvasula et al., 1997). It can be applied for control purposes, either of the host (Whitten et al., 2015), or for control of organisms that are host-associated (Arora et al., 2018). For example, Arora et al. (2018) modified *Pantoea*

agglomerans bacteria, a symbiont of the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) to synthesise (AMP)-melittin and scorpine-like molecule (SLM), antimicrobial peptides. Modified *P. agglomerans* were unaffected by melittin and SLM proteins but the agricultural pathogen *Xylella fastidiosa* (which is vectored by GWSS) was unable to grow in their presence (Arora et al., 2018). Arora et al. (2018) suggest that wild GWSS populations infected with modified *P. agglomerans* could be used to control *X. fastidiosa,* as GWSS infected with modified *P. agglomerans* exhibited a reduced *X. fastidiosa* burden of at least 96%.

Despite the consistent association between *K. oxytoca* and the medfly (Aharon et al., 2013; Behar 2008; Ben Ami et al., 2010; Leftwich et al., unpublished), the findings to date are not sufficient to definitively categorise the relationship as facultative. Paratransgenic control of the medfly with *K. oxytoca* will not be a tenable proposition unless the precise mechanism by which the medfly is acquiring *K. oxytoca* can be uncovered, as until this mechanism has been described in detail, it is possible that it could spontaneously fail. Hence, the mechanism that facilitates the *K. oxytoca/*medfly relationship requires further scrutiny, if *K. oxytoca* is to be developed as a platform for paratransgenic control.

If Behar et al. (2008) are correct and *K. oxytoca* is transovarially transmitted by the medfly, then the medfly egg chorion (Figure 5.1) must be infected with *K. oxytoca*. Heys et al. (2018) and Koyle et al. (2016) describe bleaching protocols for dechorionation of *Drosophila* eggs. Furthermore, Koyle et al. (2016) report that dechorionation facilitates the generation of axenic *Drosophila* (flies that are free of all microorganisms) and that gnotobiotic flies (flies that have a defined microbiome) are generated if specific bacteria are added to the diet of axenic flies. Therefore, I hypotheised that, if *K. oxytoca* is transovarially transmitted by the medfly, then dechorionation of medfly eggs should produce *K.oxytoca* deficient/axenic flies. Furthermore, I hypothesised that *K. oxytoca* could be reintroduced to the gut microbiome of axenic medflies, if administered in the diet. This would offer the potential to infect adults with engineered *K. oxytoca* to effect control.

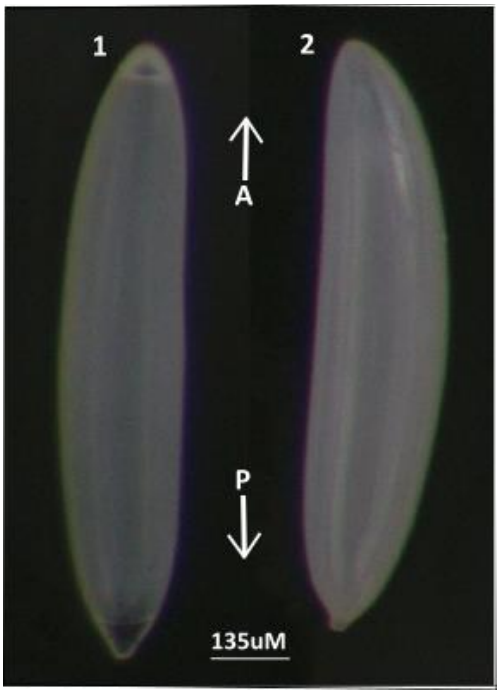

Figure 5.1 Chorionated and dechorionated medfly eggs: Two medfly eggs are positioned with anterior poles at the top of the image. 1) dechorionated egg - visibly clear tips on anterior and posterior poles of membrane, 2) chorionated egg – egg is covered with opaque chorion obscuring the clear membrane at the anterior and posterior poles.

To test my hypotheses, I reared both dechorionated and chorionated medfly eggs in a sterile environment. Flies that developed from dechorionated eggs were either supplied with sterile food (dechorionated treatment) or diet that had been supplemented with *K. oxytoca* (*+Kleb* treatment). Flies that developed from chorionated eggs were supplied with sterile diet as a negative control. I predicted that the gut microbiome of dechorionated flies would be small and *K. oxytoca* deficient, in comparison to the gut microbiome of both *+Kleb* flies and chorionated controls. Gut DNA was analysed with qPCR targeting the 16S gene to provide an absolute quantification of the microbiome (Glassing et al., 2016; Jimeno et al., 2018). To provide a relative estimation of the different species present in the gut, DNA was analysed with 16S metagenomics (Di Salvo et al., 2019; Suenami et al., 2019). To complement this analysis, I analysed DNA extracted from the guts of wild-caught medflies (isolated from Morocco) and compared them to my treatments and controls. I predicted that the microbiomes of Morocco flies would be similar to the chorionated controls, both in terms of size and community structure but exhibit a smaller proportion of *K. oxytoca* and be reduced in comparison to *+Kleb* flies.

However, the 16S metagenomic data appeared to contain a highly abundant contaminating bacterial species of unknown origin. Therefore, I developed a proprietary qPCR protocol to quantify a *K. oxytoca*-specific DNA sequence within medfly gut DNA, which would allow me to answer my original research questions. In a study by Kovtunovych et al. (2003), *K. oxytoca* bacteria were specifically identified via PCR amplification of the *pehX* gene (Kovtunovych et al., 2003). I hypothesised that the primers used by Kovtunovych et al. (2003) to identify *K. oxytoca* DNA, could be utilised to quantify *K. oxytoca* DNA in medfly guts via qPCR. In this way, despite the lack of clean 16S metagenomic data, I could nevertheless test whether the guts of dechorionated flies contained fewer *K. oxytoca* bacteria than the guts of *+Kleb*, wild type Morocco or chorionated control flies.

5.3) Methods

5.1) Medfly strains used for microbiome investigations

Gut bacterial DNA was obtained from both lab-reared Toliman medflies and a wild medfly strain collected from the field in Morocco. The Toliman strain was originally captured in Guatemala in 1990 and has been reared in the lab by Oxitec (Oxitec, 2019) since 2004. The wild Morocco strain was collected from Argan fruit in March 2014, by Elani Rachid on behalf of Oxitec ltd.

5.2.1) Toliman husbandry – lab conditions

Medflies were reared in a controlled environment: 25^{o}C temperature, approximately 50% relative humidity and 12L: 12D photoperiod. Day 1: approximately 500 eggs (<24 hrs old) were collected by pipette from adult cages and transferred to 100ml of larval diet (Agar 12.5g/L; Sucrose 73.5g/L; Maize 67g/L; Brewer's yeast 47.5g/L; 10% Nipagin 25ml/L; Propionic acid 2ml/L) in a plastic bottle. Day 7: the bottle was laid down in 2cm of childrens' play-sand in a plastic pupation box (170mm x 130mm x 50mm) that had a mesh breathing hole (50mm Ø) cut in the lid. From Day 8 to Day 15: L3 stage larvae naturally dispersed from the bottles to pupate in the sand. Day 16: 100 pupae were collected from the sand and transferred to a plastic rearing enclosure (120mm x 120mm x 135mm) which had a 90mm (Ø) opening cut into the side enclosed with fine nylon mesh. In the cage, flies had *ad libitum* access to ddH_2O and artificial diet (3:1 sucrose: hydrolysed yeast). Day 24: caged females laid eggs through the nylon mesh which dropped into a reservoir of ddH_2O to be collected for infestation onto larval diet for the next generation.

5.2) Dechorionation protocol

Prior to use, embryo collection cages, cover slips and brushes were sterilised with ethanol and 10 mins exposure to UV radiation in a class 2 microbial fume hood (Herasafe). Eggs were dechorionated in a collection cage by vigorous washing in 1% sodium hypochlorite for 1.5 mins. The dechorionated eggs were held under a continuous flow of autoclaved ddH_2O for 30 secs to remove residual sodium hypochlorite then placed in a reservoir of clean, autoclaved ddH_2O in a 90mm petri dish. A group of dechorionated eggs was

inspected with a Leica M80 stereo microscope to assess if the procedure had been successful (Figure 5.1).

5.3.1) Culture of *Klebsiella oxytoca* bacteria

K. oxytoca bacteria were cultured in 10ml LB broth (5g/L NaCl; 5g/L yeast extract; 1.5g/L glucose; 10g/L tryptone) which had been stored in 50ml glass bottles and autoclaved prior to use. Each bottle was inoculated with a "loop" of bacteria then incubated in an orbital incubator (New Brunswick Scientific Innova 44) at 200RPM and 37^{o}C.

5.3.2) Preparation of *Klebsiella oxytoca* bacteria for *+Kleb* treatment

6ml of culture broth (OD600 ≈ 0.7) was centrifuged at 5K RPM for 10 mins in 2ml Eppendorf tubes. The supernatants were removed from the tubes and 2ml of autoclaved sugar water (20% w/v) was added under a Bunsen burner. The bacterial pellet was re-suspended by vortex and the outside of tubes were wiped with ethanol. The tubes were then transferred to a sterile, class 2 microbial fume hood (Herasafe).

5.4.1) Larval husbandry – microbial fume hood

Prior to use, embryo collection cages, cover slips and brushes were sterilised with ethanol and 10 mins exposure to UV radiation in a class 2 microbial fume hood (Herasafe). Day 0 – 7 days after the first fly in an enclosure had eclosed, Toliman eggs were collected and moved to a class 2 microbial fume hood (Herasafe). Once in the hood, eggs were separated into three treatment groups: 1) Dechorionated, 2) Chorionated +oil, 3) Chorionated -oil. The Dechorionated group were treated with protocol 5.2 and the remaining two groups were placed in a reservoir of autoclaved ddH_2O. Eggs were moved with a brush onto 22mm cover slips, then placed on 250ml of autoclaved larval diet (agar 12.5g/L; sucrose 73.5g/L; maize 67g/L; brewer's yeast 47.5g/L; 10% nipagin 25ml/L; propionic acid 2ml/L) in 1275cm^3 glass butter dishes. Each treatment group occupied a discrete butter dish that contained 8 cover slips of eggs. Eggs from the Dechorionated and Chorionated +oil treatment groups were covered with a drop of blended oil (7:1, halocarbon oil 700 (Sigma): paraffin oil (Sigma)) but oil was not added to the Chorionated -oil group. To maintain humidity in the dish, three 10x10cm sterile gauze swabs (LCH

medical) were moistened with molecular H_2O (ThermoFisher) and fastened on the underside of the dish lid with electrical tape, that had been wiped with ethanol. The lid was placed on the dish and the gap was sealed with electrical tape. Gauzes were re-moistened with molecular H_2O (ThermoFisher) every day, for two days. Day 3 - cover slips were placed in the larval diet at a 90^o angle with sterile tweezers to guide larvae onto the food.

5.4.2) Pupal husbandry – microbial fume hood

Prior to use, pupation boxes and butter knives were wiped with ethanol and exposed to 10 mins UV radiation in a class 2 microbial fume hood (Herasafe). Day 7 - larvae from the Chorionated +oil and Chorionated -oil groups were moved to 2cm of autoclaved childrens' play-sand in a plastic pupation box (170mm x 130mm x 50mm), that had a mesh breathing hole (50mm Ø) cut in the lid. Larval food was cut around the edge of the butter dishes with a butter knife and the dish was turned, to allow the food and larvae to drop into the pupation box. Day 10 - larvae from the Dechorionated group were moved to a pupation box as described above.

5.4.3 Adult rearing enclosure – microbial fume hood

Prior to use, rearing enclosures, dental rolls and fine tweezers were sterilised with ethanol and 10 mins exposure to UV radiation in a class 2 microbial fume hood (Herasafe). Day 16 - 50 pupae from the Chorionated +oil and Chorionated -oil treatment groups were picked from the pupation boxes with fine tweezers and placed in discrete plastic rearing enclosures (100mm x 100mm x 100mm). Each rearing enclosure was supplied with 6ml of autoclaved sugar water (20% w/v), which had been soaked into two 10mm dental rolls. The rearing enclosure was then placed inside a larger rearing enclosure (120mm x 120mm x 135mm) to prevent cross-contamination between treatment groups. Day 18 - pupae from the Dechorionated group were picked from pupation boxes and split into two subgroups: 1) Dechorionated, 2) *+Kleb*. Dechorionated and *+Kleb* pupae were placed in two discrete rearing enclosures (100mm x 100mm x 100mm). The Dechorionated enclosure was set up with sterile sugar water as described above but the *+Kleb* group was supplied with 6ml of sugar water that had been infected with *K. oxytoca* bacteria

according to protocol 5.3.2. Rearing enclosures were moved to -80°C for storage, 7 days after the first fly in the enclosure had eclosed.

5.5) DNA extractions from medfly guts for qPCR and 16S metagenomic analyses

DNA was extracted from medfly guts with a DNeasy blood and tissue kit (Qiagen) and microbe lysis buffer (MLB) (20 mg/ml of lysozyme (Sigma) and 5mg/ml of achromopeptidase (Sigma) in 20 mM Tris-HCl, 2 mM EDTA, 1.2% Triton X (pH 8.0)). For each DNA sample, 5 guts were dissected and pooled in 2ml of 0.2 µm filtered PBS (ThermoFisher). Samples were centrifuged at 13K RPM for 5 mins, before the supernatants were removed. Gut pellets were homogenised with a clean pestle in liquid nitrogen. 180µl of Buffer ATL and 20µl of proteinase K were added to each sample before they were vortexed and incubated at 56°C. Every thirty minutes for three hours, the samples were vortexed then incubated at 56°C. The samples were then incubated for 12 hours overnight at 56°C. In the morning, 180µl of MLB was added to each sample before they were vortexed and incubated at 37°C for two hours. Samples were vortexed every 30 mins during the two-hour incubation. Buffer AL and ethanol were mixed 1:1 (Buffer ALE) and warmed to 55°C. Each sample had 400µl of warm Buffer ALE added before being immediately vortexed for 10-15 secs. Samples were transferred to a spin column and centrifuged at 8000RPM for 60 secs. 500µl of Buffer AW1 was added and the sample was centrifuged again at 8000RPM for 60 secs. 500µl of Buffer AW2 was added and the samples were centrifuged at 13 000 RPM for 4 mins. 35µl of warm Buffer AE (60°C) was added to the centre of the spin membrane and the samples were centrifuged at 6000RPM for 60 secs. DNA purity and concentration were measured using a Nanodrop (ThermoFisher).

5.6.) DNA extraction from *Klebsiella oxytoca* bacteria in broth culture to estimate the absolute quantity of bacteria in medfly guts

DNA was extracted from *K. oxytoca* culture media produced according to protocol 5.3.1. 1ml of *K. oxytoca* culture was centrifuged at 13K RPM for 5 mins, before the supernatant was removed. The pellet was homogenised with a clean pestle in liquid nitrogen. Bacterial DNA was extracted using a DNeasy blood and tissue kit (Qiagen) and microbe

lysis buffer (MLB) (20 mg/ml of lysozyme (Sigma) and 5mg/ml of achromopeptidase (Sigma) in 20 mM Tris-HCl, 2 mM EDTA, 1.2% Triton X (pH 8.0)). 180µl of MLB was added before the sample was vortexed and incubated at 37°C for two hours. The samples were vortexed every 30 mins during the two-hour incubation. Buffer AL and ethanol were mixed 1:1 (Buffer ALE) and warmed to 55°C. The sample had 400µl of warm Buffer ALE added before being immediately vortexed for 10-15 secs. The sample was transferred to a spin column and centrifuged at 8000RPM for 60 secs. 500µl of Buffer AW1 was added and the sample was centrifuged again at 8000RPM for 60 secs. 500µl of Buffer AW2 was added and the sample was centrifuged at 13 000 RPM for 4 mins. 35µl of warm Buffer AE (60°C) was added to the centre of the spin membrane and the sample were centrifuged at 6000RPM for 60 secs. DNA purity and concentration were measured using a Nanodrop (ThermoFisher).

5.7) qPCR of 16S and *pehX* genes in medfly gut DNA and *Klebsiella oxytoca* DNA

Copies of 16S and *pehX* were quantified in medfly gut DNA via extrapolation from a standard curve of *K. oxytoca* DNA. DNA extracted from medfly guts and *K. oxytoca* bacteria was analysed for on a StepOnePlus thermocycler (Applied Biosystems) with iTaq Universal SYBR Green Supermix (BioRad). Primers for 16S and *pehX* were purchased from Eurofins Genomics (see supplementary materials 5.1). Each qPCR well contained either 7.5ng of medfly gut DNA or a specified quantity of *K. oxytoca* DNA, 2µl of each primer at a concentration of 5µM, 10µl of iTaq and 5µl of molecular H_2O (ThermoFisher). The PCR cycling parameters were: 95°C for ten minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 min.

5.8) Amplification, gel extraction and 16S metagenomic sequencing of medfly gut DNA

5.8.1) 16S PCR amplification and gel extraction of medfly gut DNA

The 16S gene was amplified from medfly gut DNA with PCRBIO Ultra Mix (PCRbiosystems). Two PCR reactions were run for each DNA sample and these were pooled for gel extraction. Each PCR reaction mixture contained 12.5µl of mastermix, 100ng of DNA and 1µl of each 541F/806R primer (5µM) (see supplementary materials 5.1). PCR reactions were topped up to total volume of 25µl with molecular H_2O (ThermoFisher). The PCR cycling parameters were: 95°C for two minutes, then 40 cycles

of 95°C for 15 seconds, 55°C for 15 seconds, then 72°C for 10 minutes. Gel extractions were carried out with a QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions but DNA was extracted in 30µl molecular H_2O rather than buffer EB (ThermoFisher).

5.8.2) 16S metagenomic sequencing of gel extracted DNA

16S metagenomic sequencing of medfly gut DNA was carried out by a secondary provider (Mr DNA, 2019). Samples were PCR amplified with barcoded 515F/806R 16S PCR primers (Caporaso et al., 2011) using HotStarTaq Plus Master Mix Kit (Qiagen, USA). PCR cycling parameters were: 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, then 72°C for 5 minutes. The PCR products were then used to prepare an Illumina DNA library and sequencing was performed on a MiSeq platform (Illumina).

5.8.3) Processing of 16S sequence data

Sequence data were processed with Mr DNA's analysis pipeline (Mr DNA, 2019). Sequences were joined and barcodes were removed, then sequences with ambiguous bases and sequences <150bp were deleted. Operational taxonomic units (OTUs) were generated and defined by clustering at 97% similarity. BLASTn (Altschul et al., 1997) was used to assign OTUs to specific taxa via analysis against the RDPII (RDP, 2019) and NCBI (Marchler-Bauer et al., 2009) databases. Data supplied by Mr DNA was analysed in R Studio (Team, 2016).

5.4) Results

5.1) DNA samples used for qPCR and 16S metagenomic analyses

DNA was extracted from the guts of medflies that had been reared according to the five distinct sterile / non sterile, chorionated / dechorionated experimental treatments listed in Table 5.1. A total of 5 biological replicates of DNA were produced for the Dechorionated, *+Kleb* and Chorionated +oil conditions and three biological replicates of DNA were produced for the Morocco, Chorionated -oil and Toliman conditions. High quality DNA (measured by Nanodrop), suitable for analysis with both qPCR and 16S metagenomic sequencing was collected for all samples. The reservoir of H_2O (hereafter 'Wash') in which dechorionated eggs had been bathed immediately prior to being placed on larval diet, was also analysed with 16S metagenomic sequencing, to evaluate whether it contained bacteria that could have contaminated the dechorionated eggs. As the concentration of Wash DNA was low, Wash PCR reactions contained less than 100ng of DNA as had been prescribed but amplification did occur, and Wash DNA was gel extracted then sequenced.

Table 5.1 Medfly rearing treatment conditions. Medfly gut DNA was extracted from flies that had been reared according to five distinct conditions. Flies reared in the Dechorionated and *+Kleb* treatments developed from dechorionated eggs that were covered in oil then reared in a microbial fume hood. Dechorionated flies were reared in a sterile environment but *+Kleb* flies had *K. oxytoca* administered to them in their diet. The Chorionated +oil and Chorionated -oil control flies developed from chorionated eggs and were reared under sterile conditions in a microbial fume hood. Chorionated +oil eggs were covered in oil but Chorionated -oil eggs were not. Morocco flies were captured in the field in Morocco and were subject to naturally occurring conditions.

Treatment	Dechorionated	Oil	Location	Microbe exposure
Dechorionated	Yes	Yes	Fume hood	Sterile environment
+Kleb	Yes	Yes	Fume hood	Sterile environment + *K. oxytoca*
Chorionated +oil	No	Yes	Fume hood	Sterile environment
Chorionated -oil	No	No	Fume hood	Sterile environment
Morocco	No	No	Morocco	Natural conditions

5.2) Estimating microbe abundance in medfly guts via quantification of the 16S gene

5.2.1) Calculating a standard curve for copies of 16S with serial dilutions of DNA extracted from *Klebsiella oxytoca* bacteria

Serial dilutions of *K. oxytoca* DNA were analysed with qPCR targeting the 16S gene and the data were utilised to calculate a standard curve. At the highest concentration on the standard curve, *K. oxytoca* DNA was diluted 1:10 with molecular water. Each successive datapoint on the standard curve represented a 1:1 dilution of *K. oxytoca* DNA in molecular water. 16S copy number was therefore estimated to drop by 50% at each dilution on the standard curve. The relationship between DNA dilution factor and qPCR cycling threshold was highly significant (linear model, $F(1,4) = 1768$, $p<0.001$, $R^2=0.99$).

5.2.2) Quantifying 16S copy number in medfly gut DNA via extrapolation from a standard curve of *K. oxytoca* DNA

DNA was extracted from the guts of medlfies that had been reared according to five distinct conditions. Copies of the microbial 16S gene in 7.5ng of DNA were quantified based on a standard curve of *K. oxytoca* DNA. Thus, the relative load of bacteria in medfly guts was calculated based on the quantity of 16S genes in 7.5ng of gut DNA. It was predicted that egg dechorionation would block maternal transmission of *K. oxytoca* to larvae and that DNA extracted from Dechorionated fly guts would contain fewer copies of 16S than DNA extracted from flies reared according to the remaining four conditions. DNA extracted from the guts of Morocco and *+Kleb* flies (HighCOP group) contained approximately 25-fold more copies of 16S (and thus approximately 25 times more bacteria) than DNA extracted from the guts of Dechorionated, Chorionated +oil and Chorionated -oil flies (LowCOP group) (Figure 5.2). Contrary to the prediction, there was no significant difference in 16S copy numbers in gut DNA extracted from Dechorionated flies, relative to Chorionated +oil or Chorionated -oil flies (ANOVA, $F(2,6) =0.17$, $p>0.05$). Therefore, it appears that Dechorionated flies exhibited a diminished microbiome but that this was a function of having been reared in a sterile environment, rather than due to the egg dechorionation treatment. 16S copy numbers were not significantly different in DNA extracted from *+Kleb* and Morocco fly guts (t-test, $t = 1.76$, $df = 2$, $p<0.05$).

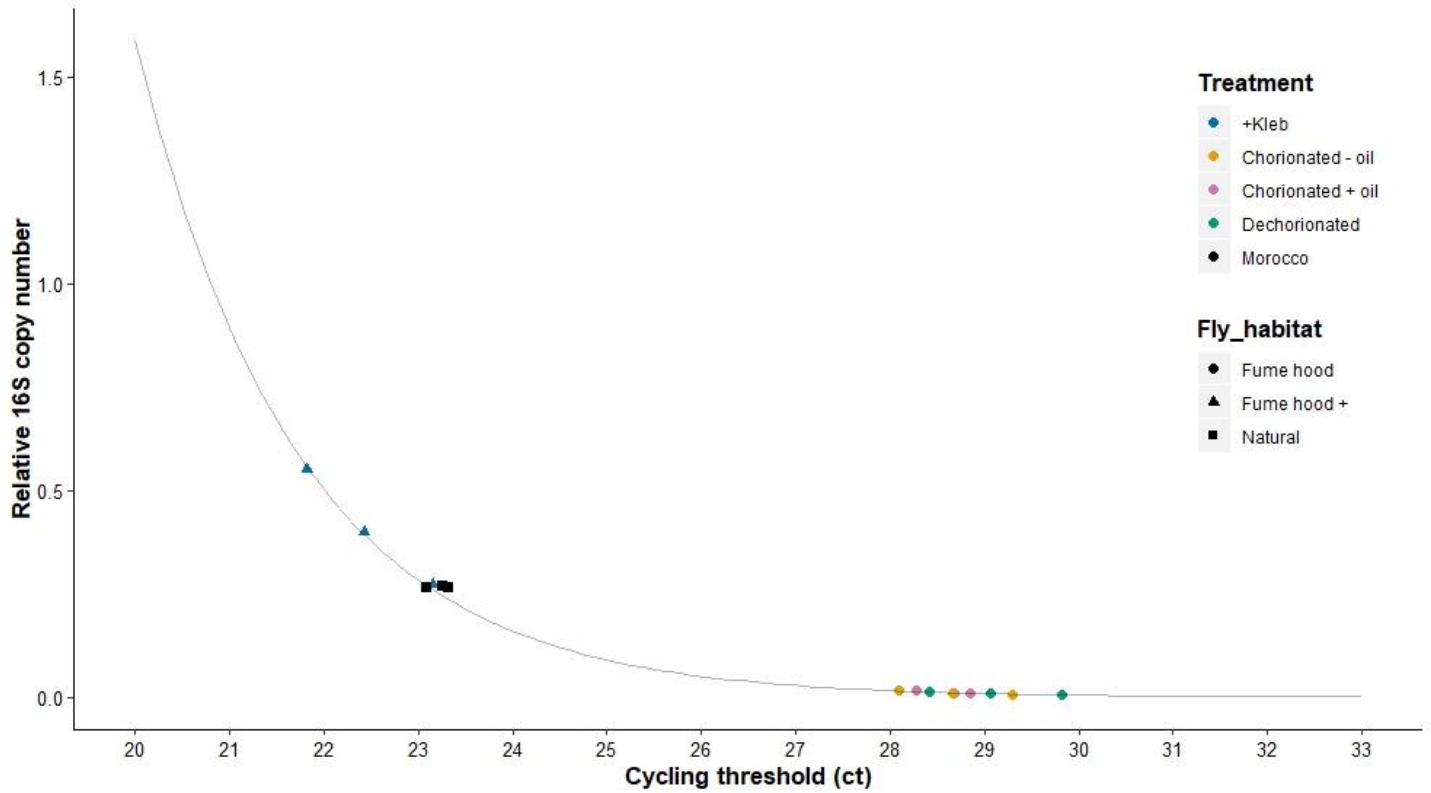

Figure 5.2 The relative quantity of 16S genes in 7.5ng of DNA extracted from medfly guts. The x axis represents qPCR cycling threshold. The y axis represents the relative quantity of 16S genes in 7.5ng of medfly gut DNA, based on a standard curve of *K. oxytoca* DNA. The standard curve of *K. oxytoca* DNA is represented by a grey line. Datapoint colours represent the different rearing treatment conditions. Flies were reared in either a sterile fume hood (circles), a sterile fume hood + *K. oxytoca* (triangles) or in a natural environment (squares). Based on the standard curve there was a significant relationship between 16S copy number and qPCR cycling threshold (linear model, $F(1,4) = 1768$, $p<0.001$, $R^2=0.99$). DNA extracted from the guts of flies reared in a fume hood contained approximately 25 times fewer copies of 16S than flies reared in a fume hood *+Kleb* or flies reared under natural conditions.

5.3) 16S metagenomic sequencing of medfly gut DNA and a Wash control

The gut microbiomes of the different treatments of medflies were characterised with 16S metagenomic sequencing (Di Salvo et al., 2019; Suenami et al., 2019). Wash DNA was sequenced to evaluate whether it contained bacteria that could have contaminated eggs during the dechorionation process. It was predicted that dechorionation would prevent maternal transmission of *K. oxytoca* and that Dechorionated flies would be *K. oxytoca* deficient. *K. oxytoca* and *Lysobacter enzymogenes* were by far the most abundant bacteria in all gut samples and Wash DNA (Table 5.2; Figure 5.3). At 11.5%, 8.8%, and 12.2% respectively, the mean relative abundance of *K. oxytoca* in the microbiomes of Dechorionated, Chorionated -oil, and Chorionated +oil flies was fairly consistent. There was no evidence that dechorionation prevented maternal transmission of *K. oxytoca.* However, as Wash contained *K. oxytoca*, it is possible that dechorionation did prevent maternal transmission of *K. oxytoca* but dechorionated eggs had been subsequently contaminated with bacteria when bathed in Wash. The mean percentage of *K.oxytoca* in the gut microbiomes of Morrocan flies and *+Kleb* flies was 31.4% and 54.4% respectively. *+Kleb* flies had *K. oxytoca* administered to them and Morocco flies were caught in the field, so it was unsurprising that they harboured a large amount of *K. oxytoca* in their gut microbiomes. Surprisingly, *L. enzymogenes* represented a substantial proportion of bacteria in all samples, a species that has not been described in any previous investigation of the medfly gut microbiome (Ben Ami et al., 2010; Leftwich et al., unpublished). Furthermore, *L. enzymogenes* does not appear to have been described in the gut microbiome of any insect to date. It appears that *L. enzymogenes* was detected in these DNA samples due to a contamination that occurred during dissection, extraction or sequencing.

Table 5.2 The mean relative abundance of *K. oxytoca* and *L. enzymogenes* in medfly gut DNA samples and Wash. The mean relative abundance of *K. oxytoca and L. enzymogenes* for all biological replicates of DNA are represented below. *K. oxytoca* and *L. enzymogenes* represented approximately 10% and 40% respectively of bacteria in the Wash control and DNA extracted from the guts of LowCOP flies. *K. oxytoca* formed 31.4% of the microbiome of Morocco flies and *L. enzymogenes* formed 15.6%. *K. oxytoca* formed a greater proportion of the gut microbiome of *+Kleb* flies (54.4%) than in flies from any other treatment.

Treatment	**Sterile/non-sterile**	***K. oxytoca (%)***	***L. enzymogenes (%)***
Chorionated -oil	Sterile	8.8	40.3
Dechorionated	Sterile	11.5	43.6
Chorionated +oil	Sterile	12.2	45.3
Morocco	Non-sterile	31.4	15.6
+Kleb	Sterile	54.4	12.6
Wash	N/A	10.9	44.3

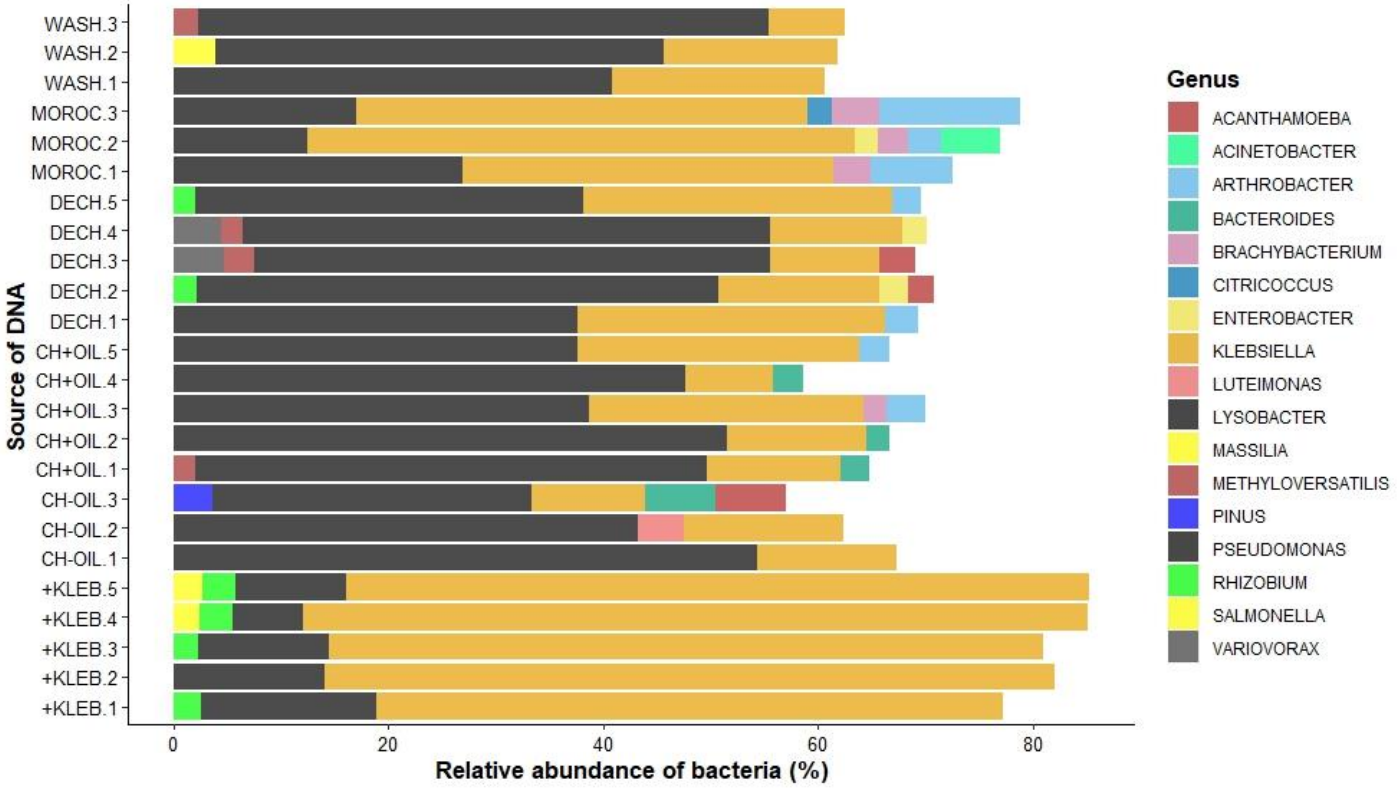

Figure 5.3 The relative abundance of bacterial genera in medfly gut samples and a Wash control. Relative abundance as a percentage of the overall microbiome is represented on the x-axis. Specific rearing treatment conditions and a Wash control are represented on the y axis. Bacteria that formed less than 2% of the total microbiome were deleted from the analysis. *Klebsiella* and *Lysobacter* formed the vast majority of bacteria in the gut microbiomes of all flies and the Wash control. The microbiomes of Dechorionated, Chorionated +oil and Chorionated -oil flies contained a smaller proportion of *Klebsiella* than *Lysobacter*. The microbiomes of Morocco and +*Kleb* flies contained a greater proportion of *Klebsiella* than *Lysobacter*. The wash control contained a greater proportion of *Lysobacter* than *Klebsiella*.

5.4) 16S metagenomic sequencing of Chorionated +oil DNA and pure DNA extraction reagents to assess if previously sequenced DNA samples had been contaminated during the extraction process

The gut microbiomes of medflies analysed in a preliminary round of 16S metagenomic sequencing (section 5.3) all contained a large proportion of *L. enzymogenes* bacteria. *L. enzymogenes* detected in those samples appeared to arise due to a contamination that occurred during either gut dissection, DNA extraction or sequencing. Therefore, a second round of 16S metagenomic sequencing was performed using pure DNA extraction reagents to identify potential sources of *L. enzymogenes* contamination. Three Chorionated +oil DNA samples analysed during round 1 were re-analysed to act as a control between sequencing runs. Bacteria of the *Klebsiella* genus represented 8% of the total bacterial load in DNA extracted from reagents and *K. oxytoca* represented 5.7% of that total. *L. enzymogenes* represented a large proportion of bacteria (mean = 28.9%) in DNA extracted exclusively from DNA extraction reagents. The microbial community of Chorionated +oil guts was reasonably consistent between sequencing runs 1 and 2. The mean relative abundance of *Klebsiella* bacteria and *L. enzymogenes* in Chorionated +oil DNA had been 14.6% and 45.3% during run 1, which dropped to 9% and 33% respectively in round 2 (Figure 5.4). It is possible therefore that *L. enzymogenes* bacteria detected in medfly guts during round 1 of sequencing, arose due contamination from the reagents used during extraction or sequencing. However, as the reagents also contained *K. oxytoca* it is also possible that the *K. oxytoca* signal detected in medfly gut DNA arose due to a cross-contamination.

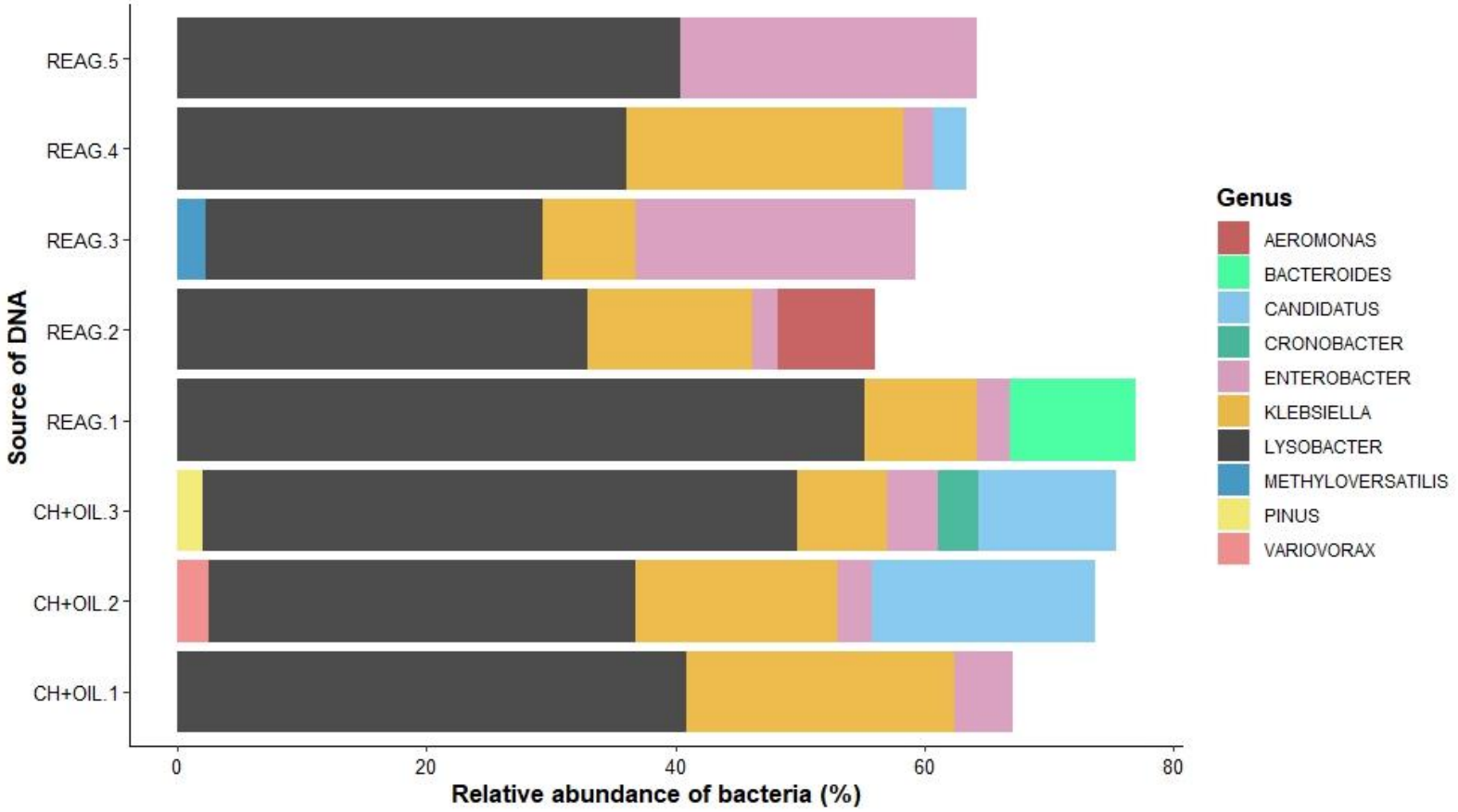

Figure 5.4 The relative abundance of bacterial genera in chemical reagents and Chorionated +oil gut samples. Relative abundance of bacteria as a percentage of the microbiome is represented on the x-axis. Chorionated +oil and reagent DNA samples are represented on the y axis. Bacteria that formed less than 2% of the total microbiome were deleted from the analysis. Bacteria of the *Klebsiella* and *Lysobacter* genera formed the vast majority of bacteria in both Chorionated +oil medfly gut and reagent DNA samples.

5.5) Estimating the relative abundance of Klebsiella oxytoca bacteria in medfly guts via qPCR of the *pehX* gene

5.5.1) Evaluation of *pehX* primers for quantification of *K. oxytoca* bacteria in medfly gut DNA

All RefSeq *Proteobacteria* genomes in the NCBI database (Marchler-Bauer et al., 2009) and a medfly genome (GCA_000347755.4) were analysed with Primer-BLAST (Ye et al., 2012), to identify DNA sequences amenable to amplification with the PEHC/PEHD primers used by Kovtunovych et al. (2003) to amplify *pehX*. The only genomes predicted by this analysis to contain DNA that would be prone to amplification with the Kovtunovych et al. (2003) primers, were characterised as *K. oxytoca* and *K. michiganensis* bacteria. It was plausible therefore that the Kovtunovych et al. (2003) primers could be used to quantify *K. oxytoca* in DNA extracted from medfly guts.

5.5.2) qPCR of *pehX* and 16S to quantify the relative abundance of *Klebsiella oxytoca* bacteria in medfly guts

In my earlier assay, the absolute abundance of bacteria in medfly guts was quantified via qPCR of the 16S gene (section 5.2). However, the relative quantity of *K. oxytoca* in those gut samples was not convincingly described with 16S metagenomic sequencing, due to the presence of a contaminating bacterial species (sections 5.3/5.4). To avoid a potentially lengthy search for the source of the contamination, I developed the following alternative approach to quantify *K. oxytoca* in medfly gut DNA. It was hypothesised that *K. oxytoca* itself could be quantified within gut samples with qPCR targeting a *K. oxytoca* specific DNA sequence (*pehX)*. Morocco and Chorionated -oil DNA had been depleted during the original analyses. Therefore, qPCR of *pehX* was carried out for Chorionated +oil, Dechorionated and *+Kleb* samples, as well novel DNA extracted from Morocco flies and lab-reared Toliman flies. DNA extraction was attempted for pure extraction reagents as a negative control, but this did not produce DNA in high enough concentration to be used for qPCR. As for the previous qPCR analysis, 7.5ng samples of medfly gut DNA were subjected to qPCR targeting the 16S gene and compared to a standard curve of *K. oxytoca* DNA. As for the first analysis, there was no significant difference in bacterial load

between the guts of Chorionated +oil and Dechorionated flies (ANOVA, $F(1,4) = 0.76$, $p > 0.05$) (Figure 5.5). Bacterial load was significantly different between the guts of *+Kleb,* Morocco and Toliman flies (ANOVA, $F(2,6) = 11.99$, $p < 0.05$) (Figure 5.6). Equivalent DNA samples were then analysed with qPCR targeting the *pehX* gene (Figure 5.7). As for 16S, *pehX* was quantified via comparison to a standard curve of *K. oxytoca* DNA. Serial dilutions of *K. oxytoca* DNA were analysed with qPCR targeting the *pehX* and the relationship between DNA dilution factor and qPCR cycling threshold was highly significant (linear model, $F(1,4) = 2210$, $p < 0.001$, $R^2 = 0.99$). qPCR of *pehX* produced a signal for the *+Kleb* DNA samples and a sample of Morocco DNA. This suggests that the guts of *+Kleb* flies contained a large amount of *K. oxytoca* bacteria which was supported by 16S metagenomic analysis (section 5.4). Toliman flies harboured more bacteria overall than *+Kleb* flies but did produce a *pehX* signal, which suggests that lab reared Toliman flies host a large gut microbiome but that the proportion of it formed by *K. oxytoca* was not detectable by this assay.

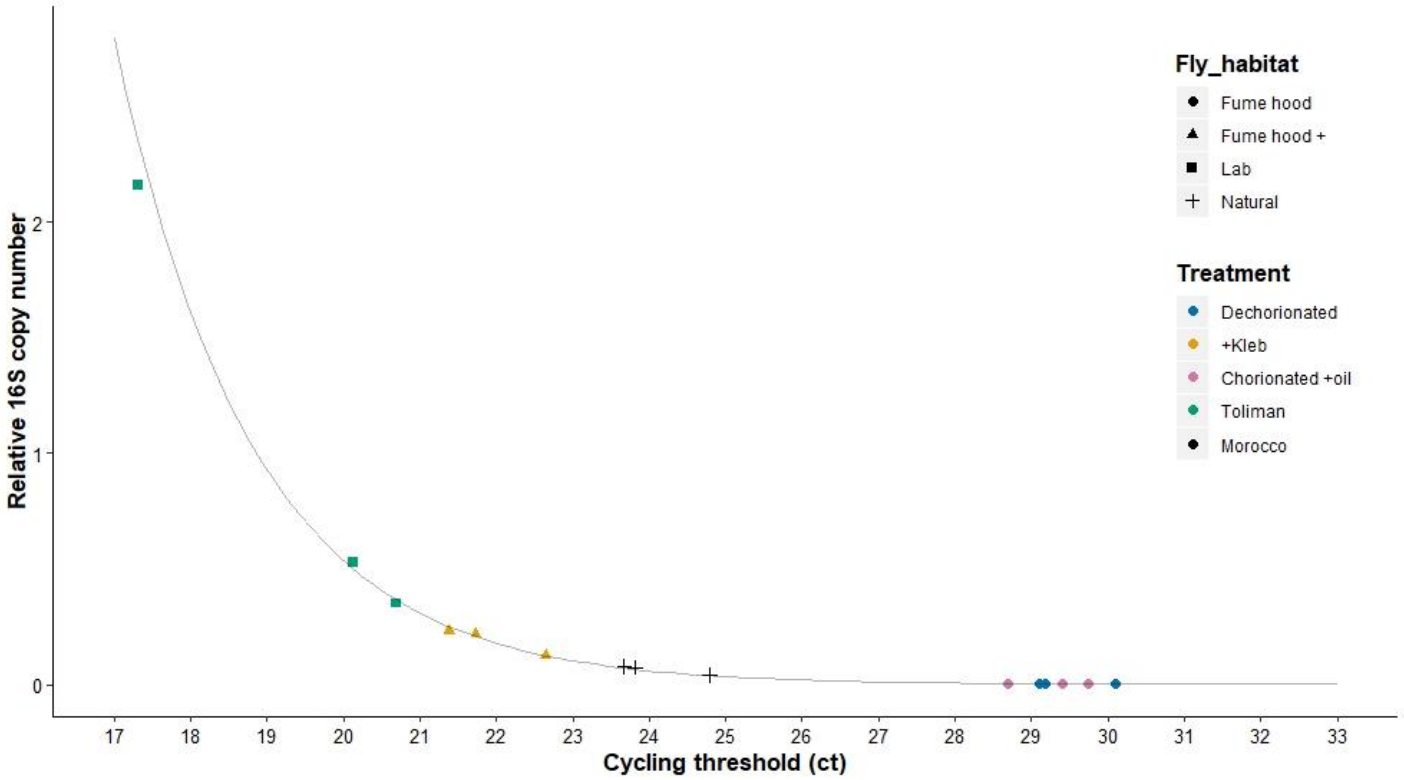

Figure 5.5 The relative quantity of 16S in 7.5ng of DNA extracted from medfly guts. The x axis represents qPCR cycling threshold. The y axis represents the quantity of 16S genes in 7.5ng of medfly gut DNA based on a standard curve of *K. oxytoca* DNA. The standard curve of *K.oxytoca* DNA is represented by a grey line. Datapoint colours represent rearing conditions. Flies were reared in either a sterile fume hood (circles), a sterile fume hood + *K. oxytoca* (triangles), in a lab (squares) or in a natural environment (crosses). Based on the standard curve there was a significant relationship between 16S copy number and qPCR cycling threshold (linear model, $F(1,4) = 418.9$, $p<0.001$, $R^2=0.99$). There was no significant difference in bacterial load between the guts of Chorionated +oil and Dechorionated flies (ANOVA, $F(1,4) =0.76$, $p>0.05$).

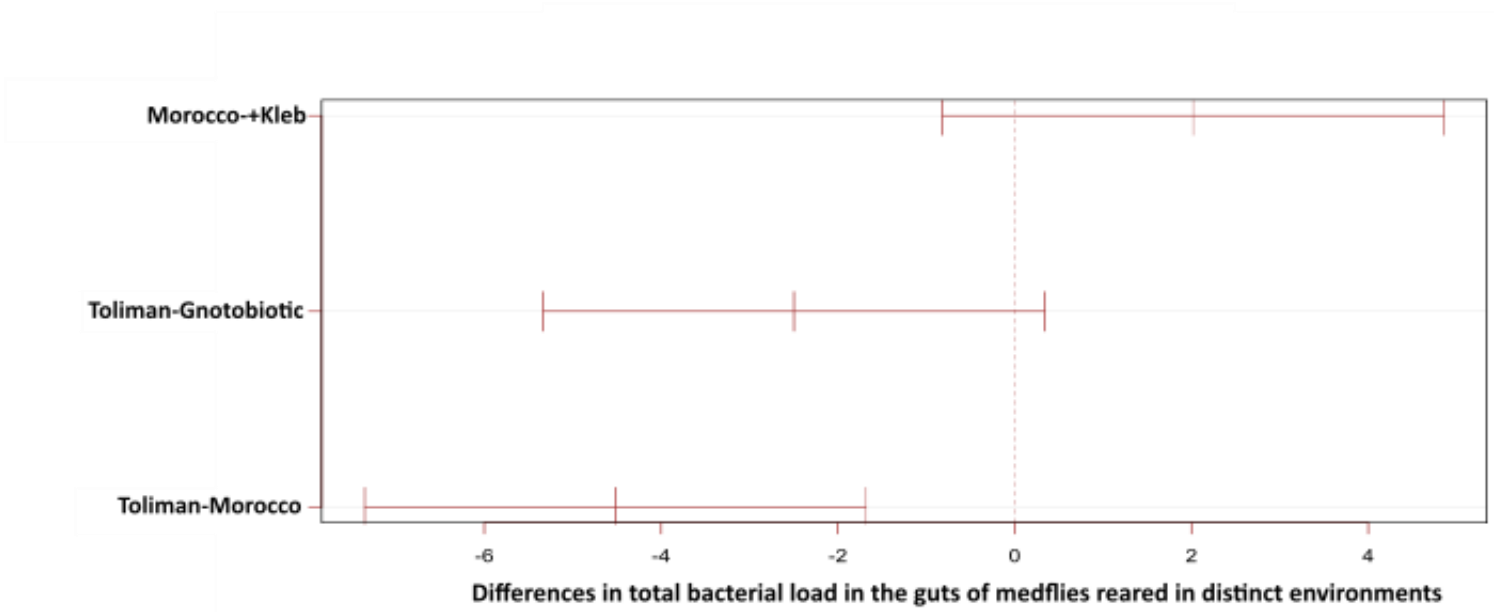

Figure 5.6 Post-hoc Tukey comparison of the bacterial load in the guts of *+Kleb*, Toliman and Morocco flies. Calculated at a 95% confidence interval the guts of Morocco flies contained significantly less bacteria than the guts of Toliman flies.

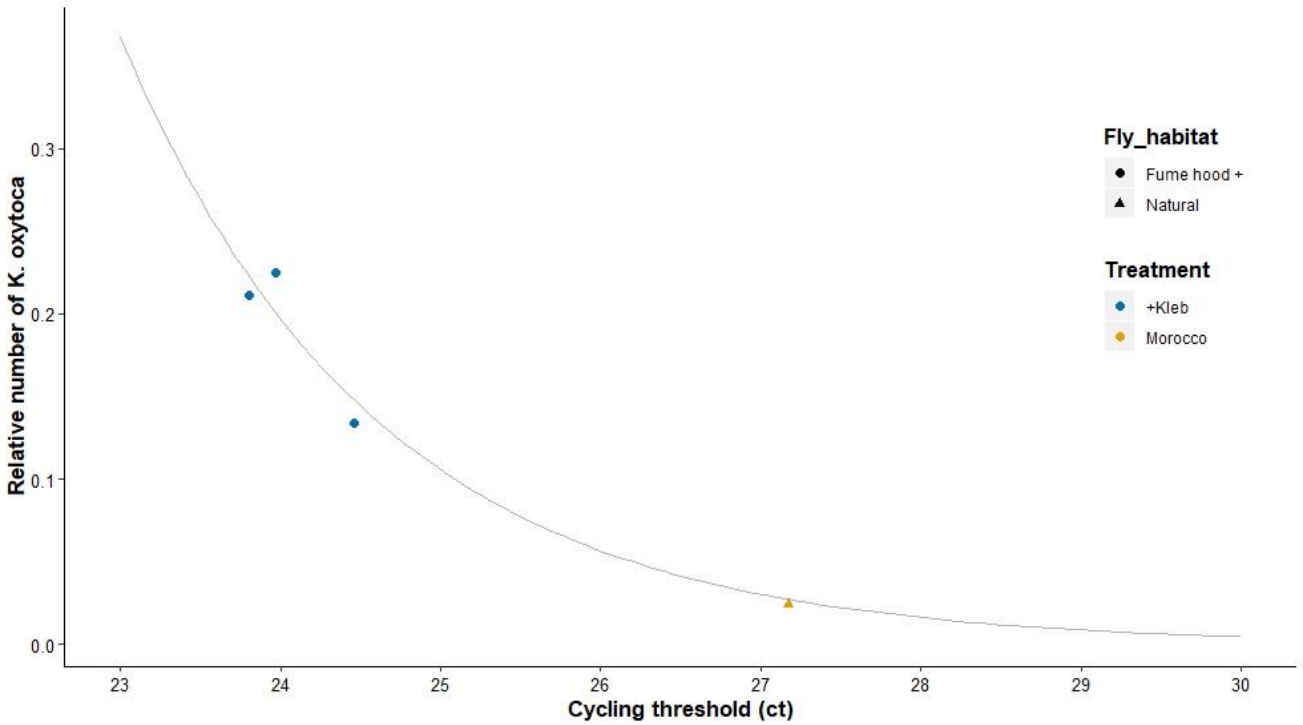

Figure 5.7 The relative quantity of pehX in 7.5ng of DNA extracted from medfly guts. The x axis represents qPCR cycling threshold. The y axis represents the quantity of pehX genes in 7.5ng of medfly gut DNA based on a standard curve of *K. oxytoca* DNA. The standard curve of *K.oxytoca* DNA is represented by a grey line. Datapoint colours represent rearing conditions. Flies were reared in either a sterile fume hood + *K. oxytoca* (circles) or in a natural environment (triangles). Based on the standard curve there was a significant relationship between pehX copy number and qPCR cycling threshold (linear model, $F(1,4) = 2210$, $p<0.001$, $R^2=0.99$). The guts of flies *+Kleb* flies contained significantly more pehX genes and thus more *Klebsiella* bacteria than the guts of Morocco flies (ANOVA, $F(1,2) = 39.11$, $p<0.05$). DNA extracted from the guts of Toliman flies and chorionated controls did not amplify *pehX* via qPCR.

5.5) Discussion

Klebsiella oxytoca have frequently been reported to form a large proportion of bacteria in the medfly gut microbiome (Aharon et al., 2013; Behar et al., 2008; Ben Ami et al., 2010). Furthermore, *K.* oxytoca appear to dominate the medfly gut microbiome, regardless of which environments or diets flies have been exposed to (Leftwich et al., unpublished). This apparently constant association between *K. oxytoca* and the medfly, indicates that the relationship is stable, and it has been suggested that *K. oxytoca* are transmitted by medflies to their offspring on the egg chorion (Behar et al., 2008).

I hypothesised that dechorionation of medfly embryos would disrupt vertical transmission of *K. oxytoca*. Following from this premise, I hypothesised that medflies reared from dechorionated eggs would exhibit a gut microbiome that was *K. oxytoca* deficient and thus, reduced in size. To test my hypotheses, I reared dechorionated eggs in a sterile environment then analysed the gut microbiome of flies that developed from those eggs quantitatively with qPCR (Glassing et al., 2016; Jimeno et al., 2018) and qualitatively with 16S metagenomics (Di Salvo et al., 2019; Suenami et al., 2019).

Contrary to the prediction, when analysed with qPCR, flies reared from dechorionated eggs did not exhibit a reduced microbiome relative to chorionated controls. These data appear to be robust, because as predicted, *+Kleb* flies (that had bacteria administered to them in their diet) and Morocco flies (that were captured in the field) both harboured a large quantity of gut bacteria. Therefore, it seems that the reduced microbiome of dechorionated flies was a consequence of being reared in a sterile environment, rather than egg dechorionation.

I predicted that the gut microbiomes of dechorionated medflies would contain a smaller proportion of *K. oxytoca* bacteria than the microbiomes of chorionated controls, an idea that I tested by using 16S metagenomics. Although the fraction of *K. oxytoca* in gut microbiomes varied between samples, *K. oxytoca* was not significantly reduced in the microbiome of dechorionated flies. This could suggest that *K. oxytoca* are not

transovarially transmitted by the medfly, though these experiments would need to be repeated without the contaminating bacterial species present.

The majority of analysed gut microbiomes were dominated by *Lysobacter enzymogenes* bacteria. *L. enzymogenes* has never previously been detected in the medfly gut (Aharon et al., 2013; Behar et al., 2008; Ben Ami et al., 2010). Furthermore, Leftwich et al. (unpublished) (see supplementary materials Figure 5.1) did not detect *L. enzymogenes* in the guts of Morocco flies, despite the fact that those flies were extracted from the same population as analysed here. It seems likely that *L. enzymogenes* had contaminated: 1) the fly guts during dissection, 2) the extraction reagents at UEA, 3) the sequencing reagents used by the provider (Mr DNA, 2019).

A possible source of contamination were the reagents and associated consumables used for DNA extraction and sequencing (Glassing et al., 2016). However, it was also possible that *L. enzymogenes* contamination occurred during gut dissection. To elucidate potential contamination sources, a second round of 16S metagenomic sequencing was carried out on DNA that was extracted from pure chemical reagents. Chorionated +oil DNA was also re-sequenced to act as a control between sequencing runs. The data produced during round 2 of sequencing were fairly consistent with those produced during round 1, in that all samples analysed (other than reagent sample 5) contained a large proportion of *K. oxytoca* and *L. enzymogenes.* These data did not pinpoint a source of *L. enzymogenes* contamination but excluded the dissection process, as the reagents did not contain guts. As *L. enzymogenes* was detected in Chorionated +oil DNA during in both sequencing rounds, DNA samples could not have been contaminated by an irregular phenomenon during round 1 at the provider (Mr DNA, 2019). Equally, DNA could not have been contaminated by an irregular phenomenon at UEA during round 1, as the reagents were not sequenced during round 1, yet they contained *L. enzymogenes*. Therefore, the contamination source was constant between sequencing rounds and did not occur during rearing or dissection.

L. enzymogenes contamination could have arisen from at least two sources that are yet to be eliminated from this analysis: 1) DNA extraction reagents and buffers used in the UEA labs, 2) sequencing reagents and buffers used in the labs of the sequencing provder

(Mr DNA, 2019). DNA extraction reagents should be filter sterilised in future investigations. It is of note that *L. enzymogenes* is used commercially to produce achromopeptidase (Takami et al., 2017), which was integral to the microbe lysis buffer (MLB) used in protocol 5.5. MLB was passed through a 0.2um filter and hence, should have been microbe free. However, it is possible that the achromopeptidase component of MLB (Sigma - product A3457) was contaminated with *L. enzymogenes* DNA that passed through the 0.2um filters (Mueller et al., 2014) and confounded the subsequent analysis. Therefore, all buffers and reagents should be passed through 0.1um filters in future investigations, to eliminate contaminating DNA with improved efficiency and achromopeptidase should be treated with DNase prior to being added to MLB. Furthermore, 16S metagenomic sequencing should be carried out a different lab, to eliminate Mr DNA's reagents as a source of contamination.

Finally, I quantified the microbe specific 16S gene and the *K. oxytoca* specific *pehX* gene, in equivalent samples of medfly gut DNA with qPCR. I hypotheised that this analysis should provide a general microbe signal and a *K. oxytoca* specific signal for each sample, which could then be compared to reveal which of the microbiomes contained the greatest proportion of *K. oxytoca* relative to their overall size. Three new Morocco DNA samples and three samples of DNA extracted from the Toliman lab strain were used for this analysis, along with the *+Kleb*, Dechorionated and Chorionated +oil DNA from the earlier analysis.

As for the first round, qPCR of 16S revealed that the guts of Dechorionated and Chorionated +oil flies contained significantly less bacteria than *+Kleb*, Morocco or Toliman flies. The guts of Toliman flies contained significantly more bacteria than the guts of Morocco flies. However, only the *+Kleb* samples and a single Morocco sample amplified *pehX.* This analysis suggests that a the *+Kleb* samples contained a large quantity of *K. oxytoca,* which is consistent with the 16S metagenomic data. Overall, this analysis requires further optimisation. Currently, the assay lacks the sensitivity required to quantify small amounts of *K. oxytoca* as seen by the lack of amplification in the Toliman and several of the Morocco samples. Future studies should refine the *pehX* assay by optimising qPCR cycling parameters or using the more sensitive TaqMan mastermix.

Alternatively, qPCR of gut DNA with 16S primers that target different taxa should reveal the relative abundance of different genera in the microbiome (Yang et al., 2015).

5.6) Supplementary material

5.1) Primers used for qPCR of *pehX* and 16S and PCR of 16S

As in Kovtunovych et al. (2003) the pehX gene was amplified using PEH-C forward primer (5'GATACGGAGTATGCCTTTACGGTG-3') and PEH-D reverse primer (5'TAGCCTTTATCAAGCGG ATACTGG-3'). The 16S gene was amplified using 541F forward primer (5'GTGCCAGCMGCCGCGGTAA-3') and 806R reverse primer (5'GGACTACHVGGGTWTCTAAT-3') as in Caporaso et al. (2011).

5.2) Overview of the Leftwich et al. (unpublished) study

Dr Philip Leftwich analysed the guts of adult Toliman medflies, untreated Toliman larvae and Toliman larvae that had been exposed to antibiotics (100 μg/ml kanamycin, 200 μg/ml ampicillin, 200 μg/ml streptomycin, 50 μg/ml chloramphenicol,100μg/ml apramycin, 100 μg/ml hygromycin and 200 μg/ml tetracycline) with 16S metagenomics. To complement this, Dr. Leftwich also analysed the guts of medflies collected from the field in Crete and Morocco. I subsequently analysed the guts of Morrocan medlfies, from the same population as had been analysed by Dr Leftwich. Both Dr Leftwich (Figure 5.8) and I (Figure 5.3) detected a large proportion of *Klebsiella* bacteria in the guts of Morrocan flies but my analysis detected *Lysobacter enzymogenes* bacteria, which had been absent from Dr Leftwich's analysis.

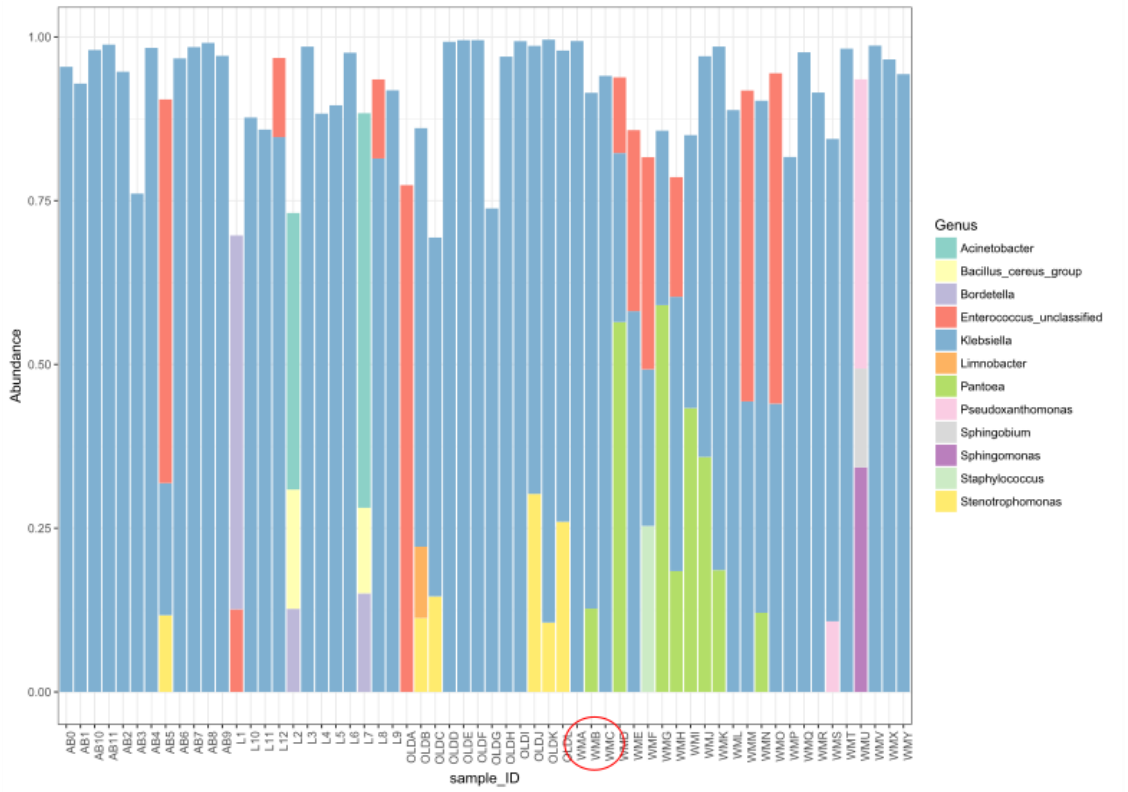

Figure 5.8 The relative abundance of bacterial genera in medfly gut samples analysed by Dr Philip Leftwich. Relative abundance as a fraction of the overall microbiome is represented on the y-axis. Specific rearing treatment conditions represented on the x axis – flies collected from Morocco are circled. Bacteria that formed less than 2% of the total microbiome were deleted from the analysis. The guts of flies collected from Morocco all contained a large proportion of *Klebsiella* bacteria (blue bars), but none contained *Lysobacter* bacteria.

5.7) References

Aharon, Y., Pasternak, Z., Ben Yosef, M., Behar, A., Lauzon, C., Yuval, B., & Jurkevitch, E. (2013). Phylogenetic, metabolic, and taxonomic diversities shape mediterranean fruit fly microbiotas during ontogeny. *Appl Environ Microbiol, 79*(1), 303-313. doi:10.1128/aem.02761-12

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped blast and psi-blast: A new generation of protein database search programs. *Nucleic Acids Res, 25*(17), 3389-3402.

Arora, A. K., Pesko, K. N., Quintero-Hernández, V., Possani, L. D., Miller, T. A., & Durvasula, R. V. (2018). A paratransgenic strategy to block transmission of *Xylella fastidiosa* from the glassy-winged sharpshooter *Homalodisca vitripennis*. *BMC Biotechnol, 18*(1), 50. doi:10.1186/s12896-018-0460-z

Behar, A., Jurkevitch, E., & Yuval, B. (2008a). Bringing back the fruit into fruit fly–bacteria interactions. *Molecular Ecology, 17*(5), 1375-1386. doi:10.1111/j.1365-294X.2008.03674.x

Ben Ami, E., Yuval, B., & Jurkevitch, E. (2010). Manipulation of the microbiota of mass-reared mediterranean fruit flies *Ceratitis capitata* (*Diptera: Tephritidae*) improves sterile male sexual performance. *Isme j, 4*(1), 28-37. doi:10.1038/ismej.2009.82

CABI. (2019). Invasive species compendium. Retrieved from https://www.cabi.org/isc/datasheet/12367

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., . . . Knight, R. (2011). Global patterns of 16s rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci U S A, 108 Suppl 1*, 4516-4522. doi:10.1073/pnas.1000080107

Di Salvo, M., Calcagnile, M., Talà, A., Tredici, S. M., Maffei, M. E., Schönrogge, K., . . . Alifano, P. (2019). The microbiome of the *Maculinea myrmica* host-parasite interaction. *Scientific Reports, 9*(1), 8048. doi:10.1038/s41598-019-44514-7

Gasperi, G., Guglielmino, C. R., Malacrida, A. R., & Milani, R. (1991). Genetic variability and gene flow in geographical populations of *Ceratitis capitata* (wied.) (medfly). *Heredity, 67*(3), 347-356. doi:10.1038/hdy.1991.98

Glassing, A., Dowd, S. E., Galandiuk, S., Davis, B., & Chiodini, R. J. (2016). Inherent bacterial DNA contamination of extraction and sequencing reagents may affect interpretation of microbiota in low bacterial biomass samples. *Gut Pathogens, 8*(1), 24. doi:10.1186/s13099-016-0103-7

Heys, C., Lizé, A., Blow, F., White, L., Darby, A., & Lewis, Z. J. (2018). The effect of gut microbiota elimination in drosophila melanogaster: A how-to guide for host–microbiota studies. *Ecology and evolution, 8*(8), 4150-4161. doi:10.1002/ece3.3991

Jimeno, R., Brailey, P. M., & Barral, P. (2018). Quantitative polymerase chain reaction-based analyses of murine intestinal microbiota after oral antibiotic treatment. *J Vis Exp*(141). doi:10.3791/58481

Kaltenpoth, M., Winter, S. A., & Kleinhammer, A. (2009). Localization and transmission route of *Coriobacterium glomerans*, the endosymbiont of *Pyrrhocorid* bugs. *FEMS Microbiology Ecology, 69*(3), 373-383. doi:10.1111/j.1574-6941.2009.00722.x

Kovtunovych, G., Lytvynenko, T., Negrutska, V., Lar, O., Brisse, S., & Kozyrovska, N. (2003). Identification of *Klebsiella oxytoca* using a specific PCR assay targeting the polygalacturonase pehX gene. *Res Microbiol, 154*(8), 587-592. doi:10.1016/s0923-2508(03)00148-7

Koyle, M. L., Veloz, M., Judd, A. M., Wong, A. C. N., Newell, P. D., Douglas, A. E., & Chaston, J. M. (2016). Rearing the fruit fly *Drosophila melanogaster* under axenic and gnotobiotic conditions. *J Vis Exp*(113), 54219. doi:10.3791/54219

Leftwich, P. T., & Chapman, T. (unpublished). *unpublished*.

Liquido, N. J., Shinoda, L. A., & Cunningham, R. T. (1991). *Host plants of the* Mediterranean fruit fly *(Diptera: Tephritidae): An annotated world review*: Entomological Society of America.

Marchler-Bauer, A., Liu, C., He, J., Geer, L. Y., Han, L., Geer, R. C., . . . Shi, W. (2009). The NCBI biosystems database. *Nucleic Acids Research, 38*(suppl_1), D492-D496. doi:10.1093/nar/gkp858

Moran, N. A., McCutcheon, J. P., & Nakabachi, A. (2008). Genomics and evolution of heritable bacterial symbionts. *Annu Rev Genet, 42*, 165-190. doi:10.1146/annurev.genet.41.110306.130119

Mr DNA (2019). Mr DNA Retrieved from http://www.mrdnalab.com/

Mueller, J. A., Culley, A. I., & Steward, G. F. (2014). Variables influencing extraction of nucleic acids from microbial plankton (viruses, bacteria, and protists) collected on nanoporous aluminum oxide filters. *Appl Environ Microbiol, 80*(13), 3930-3942. doi:10.1128/AEM.00245-14

RDP. (2019). Retrieved from http://rdp.cme.msu.edu/

Salem, H., Florez, L., Gerardo, N., & Kaltenpoth, M. (2015). An out-of-body experience: The extracellular dimension for the transmission of mutualistic bacteria in insects. *Proceedings of the Royal Society B: Biological Sciences, 282*(1804), 20142957. doi:doi:10.1098/rspb.2014.2957

Suenami, S., Konishi Nobu, M., & Miyazaki, R. (2019). Community analysis of gut microbiota in hornets, the largest eusocial wasps, *Vespa mandarinia and V. Simillima*. *Scientific Reports, 9*(1). doi:10.1038/s41598-019-46388-1

Takami, H., Toyoda, A., Uchiyama, I., Itoh, T., Takaki, Y., Arai, W., . . . Ikeda, H. (2017). Complete genome sequence and expression profile of the commercial lytic enzyme producer *Lysobacter enzymogenes* m497-1. *DNA Res, 24*(2), 169-177. doi:10.1093/dnares/dsw055

Team, R. C. (2016). R: A language and environment for statistical computing: R Foundation for Statistical Computing, Vienna, Austria. Retrieved from www.R-project.org

Whitten, M. M. A., Facey, P. D., del Sol, R., Fernández-Martínez, L. T., Evans, M. C., Mitchell, J. J., . . . Dyson, P. J. (2015). Symbiont-mediated RNA interference in insects. *Proceedings of the Royal Society B: Biological Sciences, 283*(1825). doi:10.1098/rspb.2016.0042

Yang, Y.-W., Chen, M.-K., Yang, B.-Y., Huang, X.-J., Zhang, X.-R., He, L.-Q., . . . Hua, Z.-C. (2015). Use of 16s rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in mouse feces. *Appl Environ Microbiol, 81*(19), 6749-6756. doi:10.1128/aem.01906-15

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-blast: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics, 13*, 134. doi:10.1186/1471-2105-13-13

Chapter 6 – Evaluating the Fall Armyworm (*Spodoptera frugiperda)* as a candidate for control with Oxitec's self-limiting technology

6.1) Abstract

The polyphagous, migratory Fall Armyworm (*Spodoptera frugiperda;* FAW) is a significant agricultural pest, which has been endemic to the Western Hemisphere for at least 100 years. Currently FAW is progressively invading new territories in the Eastern Hemisphere, and novel methods of control are urgently required to mitigate its damaging effects. I performed several preliminary investigations to evaluate whether the FAW is an appropriate candidate species for control with Oxitec's self-limiting technology. I hypothesised that optimal self-limiting control would be delivered in the FAW model if: 1) females exhibited polyandry during dispersal 2) females exhibited last-male sperm-precedence. Initially, I developed a system for marking FAW spermatophores with fluorescent dye that facilitated their identification once they had been transferred to female moths. Using the labelled spermatophores I was then able to demonstrate that FAW females practiced polyandrous behaviour both in the lab and in cage trials. Finally, I investigated whether FAW females exhibited last-male sperm precedence by consecutively mating females with a wild-type male and then a transgenic male. By recording the frequency of transgenics in the F1 generation I was able to show that last-male sperm precedence does appear to be typical of FAW. Therefore, the results support the idea that FAW is a suitable candidate for self-limiting control.

6.2) Introduction

The Fall Armyworm (*Spodoptera frugiperda;* FAW) is a polyphagous, migratory noctuid moth (Luginbill, 1928). Adult FAW can live for several weeks (Ashley et al., 1989; Hardke et al., 2015; Luginbill, 1928) and may disperse over hundreds of miles (Nagoshi et al., 2012; Westbrook et al., 2016). Females can lay up to 1000 eggs, which are laid on the underside (Hardke et al., 2015) of the leaves of over 80 plant species (Luginbill, 1928; Pashley, 1988). FAW larvae consume host plants (Day et al., 2017; Luginbill, 1928; Sparks, 1979) and can reduce crop yields by up to 57% (Cruz et al., 1999). The FAW is therefore considered to be a significant agricultural pest (Day et al., 2017; Luginbill, 1928; Sparks, 1986).

The FAW species group comprises two discrete strains (FAW^C and FAW^R) that are morphologically identical and live in sympatry but favour different habitats (Pashley, 1986; Pashley et al., 1985). The C strain (FAW^C) preferentially inhabits corn while the R stain (FAW^R) preferentially inhabits rice and grasses (Pashley, 1988). The FAW^C/FAW^R strains produce distinct pheromone blends (Groot et al., 2008) and preferentially mate at different times of the night (Schofl et al., 2009) but remain conspecifics and can naturally hybridise in the field (Machado et al., 2008; Nagoshi et al., 2012). However, the FAW^C/FAW^R strain dichotomy may represent incipient speciation, as FAW^C male/FAW^R female interstrain matings result in reduced fertility (Kost et al., 2016) and Gouin et al. (2017) report interstrain variation in genes associated with chemosensation, digestion and detoxification.

Historically, the FAW has been a pest of the Western Hemisphere, where it has been endemic throughout North and South America from Southern Canada to Argentina (Ashley et al., 1989; Johnson, 1987). However, the FAW cannot survive the extreme winters in North America and is restricted to two annual overwintering sites in Texas and Florida (Ashley et al., 1989; Luginbill, 1928). Analysis of the FAW's Cytochrome Oxidase I (*COI*) gene reveals four discrete, non-randomly distributed haplotypes (h1-h4) that are associated with the FAW^C populations in Texas and Florida (Nagoshi et al., 2012; Nagoshi et al., 2007). The majority of Texan FAW^C express the h2 haplotype and the majority of

Floridian FAW^{C} express the h4 haplotype, which may indicate that these groups show some genetic differentiation that could be a precursor to incipient reproductive isolation (Nagoshi et al., 2012). Each year at the beginning of summer, FAW^{C} moths that express the h2 haplotype disperse north from Texas to Canada and moths that express the h4 haplotype disperse north-east from Florida along the eastern seaboard (Nagoshi et al., 2012).

In the Western Hemisphere, FAW is usually controlled with insecticides and GM crops, that express insecticidal toxins extracted from *Bacillus thuringiensis* (Bt) microbes (Burtet et al., 2017; Huang et al., 2014). However, resistance has evolved rapidly, as these modes of control have frequently been intensively applied as a monotherapy (Burtet et al., 2017). Hence, the FAW has developed field resistance to at least four chemical insecticides (Burtet et al., 2017; Carvalho et al., 2013; Nascimento et al., 2016) and resistance to Bt corn has been documented in Brazil (Farias et al., 2014; Omoto et al., 2016), Puerto Rico (Storer et al., 2010) and the USA (Huang et al., 2014).

The first reports of FAW^{C} in the Eastern Hemisphere occurred in São Tomé and Príncipe in 2016 (Goergen et al., 2016) and unrestricted by climate, FAW^{C} quickly spread across sub-Saharan Africa (Day et al., 2017) and has since invaded India (Deshmukh et al., 2018), South-East Asia (CABI, 2019), and China (Liu et al., 2019). It appears that the invasive FAW^{C} in the Eastern Hemisphere are descendants of the Floridian (h4) FAW^{C} strain (Nagoshi et al., 2018) and that the FAW^{R} lineage remains isolated in the Western hemisphere at this time (Nagoshi, 2019), although there is some evidence of FAW^{R} in China (Liu et al., 2019).

FAW^{C}'s invasion of the Eastern Hemisphere will inevitably have consequences for agriculture and food security (Day et al., 2017; Health et al., 2018). In Africa alone, FAW^{C} is predicted to reduce corn yields by up to 20.6M tonnes per annum (Day et al., 2017). The European Food Safety Authority advise that FAW^{C} could invade Europe from North Africa and establish year-round populations in Spain, Italy and Greece (Health et al., 2018). Health et al. (2018) recommend Integrated Pest Management (IPM) and broad spectrum insecticides as a means of FAW^{C} control in Europe. However, it is acknowledged that current EU legislation regarding GM organisms may need to be relaxed, to allow for

control of FAW^{C} with crops that express insecticidal Bt toxins (Health et al., 2018). If Health et al.'s (2018) prediction is correct and EU legislation is relaxed to allow control of FAW^{C} with genetically modified organisms, then a useful approach for control could include Oxitec's self-limiting system (Thomas et al., 2000).

Oxitec's self-limiting technology (Thomas et al., 2000) is a method of GM pest control that adheres to the same basic principles as the conventional Sterile Insect Technique (SIT) (Knipling, 1955). Conventional SIT utilises gamma radiation to generate large numbers of sterile male insects for release in the field which subsequently mate with wild females (Knipling, 1998; Krafsur, 1998). These sterile matings produce non-viable offspring which leads to the collapse of the wild pest population (Knipling, 1998; Krafsur, 1998). However, irradiation has negative consequences for male performance and virility, which reduces the effectiveness of SIT males, both in the field and in cage trials (Guerfali et al., 2011; Helinski et al., 2008; Hooper, 1972). The benefit of Oxitec's technology over conventional SIT is that released males carry "self-limiting" genetic constructs rather than being irradiated (Asadi et al., 2019; Gorman et al., 2016; Harvey-Samuel et al., 2015), obviating the negative effects of irradiation.

Once a self-limiting strain has been developed, a suite of mating competitiveness and fitness trials are undertaken in cage trials (Asadi et al., 2019; Leftwich et al., 2014). Verified self-limiting males are then released to the field to mate with wild females and the dominant lethality transmitted to offspring by the transgenic males result in non-viable female offspring, which leads to the collapse of the wild pest population (Asadi et al., 2019; Gorman et al., 2016; Harvey-Samuel et al., 2015).

Here I investigated whether the FAW practiced polyandry and last-male sperm precedence, to evaluate this species' compatibility with Oxitec's self-limiting technology. I hypothesised that these characteristics would impact the effect of self-limiting control in the field. My rationale was thus: 1) If FAW were monogamous, non-virgin females spreading into control target areas would be refractory to re-mating with self-limiting males, and the pest population would be propagated by residual, viable sperm obtained from wild-type males, 2) If females exhibited polyandry but did not exhibit last-male sperm-precedence, then secondary matings with self-limiting males would not provide

control, as females would fertilise their eggs with sperm from previous matings. However, it is important to note that, self-limiting technology is predicted to deliver efficient control of the FAW even if wild-type females did not exhibit polyandry or last-male sperm precedence. Under those circumstances the self-limiting population would need to be augmented to provide a sufficient overflooding ratio of transgenics across the landscape.

Investigations into FAW polyandry and last-male sperm precedence were needed because the available data is scant and ambiguous. Simmons et al. (1992) report that FAW females practice polyandry (Simmons et al., 1992) but reports of sperm-precedence have been conflicting (Martin et al., 1989; Snow et al., 1970).

I evaluated the polyandrous behaviour of female FAW by labelling the spermatophores of male FAW with fluorescent rhodamine B dye (Blanco et al., 2006; Johnson et al., 2017; van der Reijden et al., 1997). To mimic the effects of dispersal, females were sequentially mated, first with a labelled male in a primary location then an unlabelled male in a secondary location. I predicted that if females mated with labelled and then unlabelled males in discrete locations, then the bursa copulatrix would contain both stained and unstained spermatophores.

To test last-male sperm precedence, I employed a system of sequential mating in which wild-type females were first mated to a wild-type male, then to a genetically modified OX5382G self-limiting male. OX5382G males used in this study were in the early stages of product development and were therefore hemizygous for a dsRED2 marker gene (Gong et al., 2005). Sperm precedence was calculated via the percentage of offspring in the F1 generation that exhibited fluorescence subsequent to sequential matings (Boorman et al., 1976). According to hemizygous inheritance (Charlesworth et al., 1987), 100% last-male precedence would result in an F1 generation that exhibited 50% fluorescence.

6.3) Methods

6.1) FAW strains utilised for investigations into polyandry and sperm precedence

The experiments to investigate polyandry and sperm precedence were conducted using OX5382G FAW and a wild-type FAW strain, both of which were supplied by Oxitec Ltd. The OX5382G strain had been genetically modified to ubiquitously express a dsRED2 fluorescent marker (Gong et al., 2005).

6.2) Infestation and rearing protocol – Fall Armyworm larvae

OX5382G and wild-type larvae were both reared on equivalent diet (Agar 19.8g/L; Beet Armyworm diet (Frontier, 2019) 161.6g/L), but the diet supplied to OX5382G larvae was supplemented with 0.02 g/L of doxycycline (Sigma) to block expression of the toxic tetracycline-repressible activator variant (tTAV) protein (Gong et al., 2005). Approximately 2ml of larval diet was dispensed to each cell of a 32 well plastic rearing tray (Frontier, 2019) before that cell was "infested" with a single larva (< 24hr old) and enclosed with a clear rearing tray lid (Frontier, 2019). After approximately 16 days the larvae had pupated and were collected with fine forceps to be sexed under a microscope (Figure 6.1)

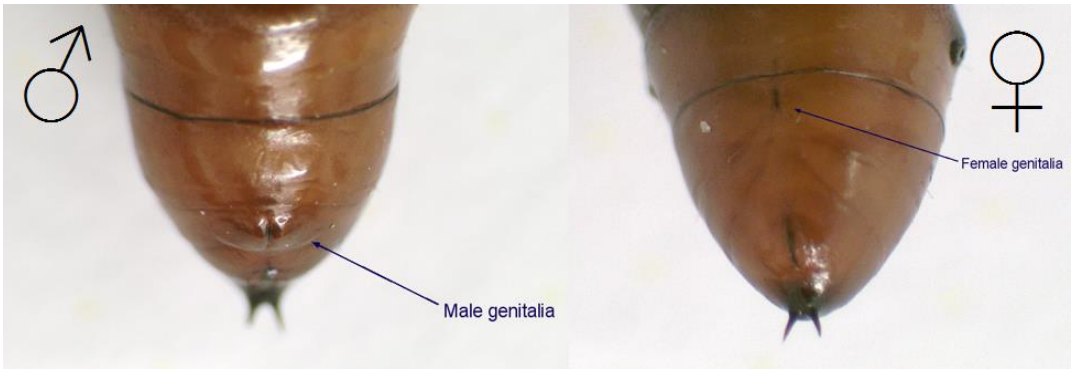

Figure 6.1 The genitals of male and female FAW pupae. The male (left) exhibits two rounded protrusions and a central genital pore on the second segment from the base of the abdomen. The female (right) exhibits a genital pore at the top of the third segment at the end of the abdomen.

6.3) Fall Armyworm adult rearing protocol

Adults were reared in a controlled environment: 25°C ambient temperature, 50% relative humidity and 12L: 12D photoperiod. Twenty-three days after larval infestation, 125 male and 125 female pupae were picked from larval feeding trays and placed in a plastic "bugdorm" (30 X 30 x 30cm) that had netted sides for ventilation. Strips of filter paper were added to the bugdorm to serve as an egg laying substrate. Wild-type insects were provided with sugar water which was prepared by mixing 7.5g of granulated sugar (Tate and Lyle) with 100ml of Milli-Q water (Sigma). OX5382G sugar water was supplemented with 0.2g/L of doxycycline (Sigma) as described above. Both OX5382G and wild-type moths had *ad libitum* access to their respective sugar water which was aliquoted onto two pieces of cotton wool, then placed in separate plastic weigh boats and positioned in the bugdorm. Female moths began to eclose twenty-four days after infestation and male moths began to eclose twenty-five days after infestation. As eggs were laid, filter paper was removed and placed in 600ml deli pots with moistened cotton wool.

6.4) Fluorescence microscopy

The fluorescence of spermatophores and larvae was detected and analysed by using a Leica MSV269 fluorescence stereo-microscope, attached to a pE-300^{WHITE} LED illuminator (CoolLED) with a single-channel, green light filter (520-560 nm). Images were captured with a GXCAM HiChrome-S camera (Vision, 2019) using GX capture software (version 8.5). The GC capture settings were as follows: exposure = 1sec 420ms; gain = 0; gamma = 11; contrast = 8; saturation = 8; red = 0.44; green = 0; blue = 0; denoise = 5; sharpen = 5. Images were analysed with ImageJ version 1.48S (Schindelin et al., 2012).

6.5) Quantification and fluorescence analysis of spermatophores contained in the bursa copulatrix of once-mated and virgin wild-type FAW

Moths were reared in a controlled environment: 25°C ambient temperature, 50% relative humidity and 12L: 12D photoperiod. Day 0 - A recently hatched egg raft was extracted from a wild-type Fall Armyworm bugdorm. Day 23 – Male and female pupae were separated. 125 female pupae were placed in a bugdorm with *ad libitum* access to sugar water (7.5% w/v) and 125 male pupae were placed in a 10-inch gateau dome with *ad*

libitum access to sugar water (7.5% w/v). Day 27 - Numerous male and female moths had eclosed in their respective enclosures. 30 mins before the 12hr dark cycle commenced, male moths were tranquilised with cold exposure (-20° for 3 mins) then transferred to the bugdorm with female moths to begin mating. To efficiently detect all matings, the bugdorm was observed every 30 mins from the beginning of the dark cycle. Thirty mating pairs and thirty unmated females were extracted from the bugdorm on filter paper and transferred to 30ml deli pots. When the matings had ceased, the moths were dissected and spermatophores in the bursa copulatrix of mated and unmated females were quantified and assessed for fluorescence under a microscope.

6.6) Feeding Fall Armyworm larvae with rhodamine B to stain germ cells and generate stained spermatophores in adult males

Moths were reared in a controlled environment: 25°C ambient temperature, 50% relative humidity and 12L: 12D photoperiod. One recently hatched wild-type FAW egg raft was collected on day 0 and another was collected on day 10. Larvae from the day 0 collection (Treatment) were reared on standard larval diet (Agar 19.8g/L; Beet Armyworm diet (Frontier, 2019) 161.6g/L) that had been supplemented with 0.1% w/v fluorescent rhodamine B dye (Sigma). Larvae that were collected on day 10 (Control) were reared on standard larval diet (Agar 19.8g/L; Beet Armyworm diet (Frontier, 2019) 161.6g/L). Day 34 - 125 female pupae from the control feeding regime were placed in a bugdorm and given *ad libitum* access to sugar water (7.5% w/v) and 125 male pupae from the treatment feeding condition were placed in a 10-inch gateau dome with no access to sugar water. Day 35 – Male and female moths began to eclose in their respective enclosures. Day 36 - 30 mins before the 12hr dark cycle commenced, male treatment moths were tranquilised with cold exposure (-20° for 3 mins) then transferred to the bugdorm with female control moths to begin mating. When the dark cycle commenced, the bugdorm was observed every 30 mins and mating pairs were extracted on filter paper, then transferred to 30ml deli pots. When mating had ceased, the bursa copulatrix of mated females were dissected and spermatophores were assessed for fluorescence under a microscope.

6.7.1) Feeding adult male Fall Armyworm moths with rhodamine B to generate labelled spermatophores - 24hr feeding time

Moths were reared in a controlled environment: 25°C ambient temperature, 50% relative humidity and 12L: 12D photoperiod. Day 0 - Two recently hatched egg rafts were extracted from a wild type bugdorm and reared according to standard protocols. Day 23 - Pupae were separated into sex specific cohorts (n=125) and placed in four discrete 10-inch gateau domes (2x female and 2x male gateau domes). Day 25 - Females that had eclosed were subjected to cold shock (-20° for 3 mins) and transferred to individual 600ml deli pots with *ad libitum* access to 7.5% sugar water. Day 26 – The two gateau domes that contained male moths were exposed to -20° for 3 mins to subdue individuals that had eclosed while uneclosed pupae were removed. One male gateau dome (control) was supplied with standard 7.5% w/v sugar water and the other (treatment) was supplied with 7.5% w/v sugar water that had been supplemented with 0.1% w/v fluorescent rhodamine B dye (Sigma). Day 27 – Treatment and control males were tranquilised with cold exposure (-20° for 3 mins) then transferred to individual deli pots with an unstained female. Sugar water was removed from deli-pots to prevent rhodamine B stain from being diluted and intersex pairs were allowed to co-habit for 24hrs. After 24 hrs the bursa copulatrix of females were dissected and spermatophores were quantified and assessed for fluorescence under a microscope.

6.7.2) Feeding adult male Fall Armyworm moths with rhodamine B to generate labelled spermatophores - 48hr feeding time

Moths were reared in a controlled environment: 25°C ambient temperature, 50% relative humidity and 12L: 12D photoperiod. Day 0 - Two recently hatched egg rafts were extracted from a wild type bugdorm and reared according to standard protocols. Day 23 - Pupae were separated into sex specific cohorts (n=125) and placed in four discrete 10-inch gateau domes (2x female and 2x male gateau domes). Day 25 - Females that had eclosed were subjected to cold shock (-20° for 3 mins) and transferred to individual 600ml deli pots with *ad libitum* access to 7.5% sugar water. Day 26 – The two gateau domes containing males were exposed to -20° for 3 mins to subdue individuals that had eclosed males while uneclosed pupae were removed. One male gateau dome (control) was

supplied with standard 7.5% w/v sugar water and the other (treatment) was supplied with 7.5% w/v sugar water that had been supplemented with 0.1% w/v fluorescent rhodamine B dye (Sigma). Day 28 – Treatment and control males were tranquilised with cold exposure (-20° for 3 mins) then transferred to individual deli pots with an unstained female. Sugar water was removed from deli-pots to prevent rhodamine B stain from being diluted and intersex pairs were allowed to co-habit for 48 hrs. After 48 hrs the bursa copulatrix of females were dissected and spermatophores were quantified and assessed for fluorescence under a microscope.

6.8) Using labelled spermatophores to evaluate if Fall Armyworm females exhibit polyandry during dispersal

6.8.1) Assessing whether FAW females exhibit polyandrous behaviour when dispersing under lab conditions

Moths were reared in a controlled environment: 25°C ambient temperature, 50% relative humidity and 12L: 12D photoperiod. Day 0 – A large, recently hatched egg raft was extracted from a bugdorm that contained wild-type Fall Armyworm. Day 23 - Male and female pupae were separated, and 125 female pupae were placed in bugdorm (location 1) with *ad libitum* access to sugar water (7.5% w/v). Two cohorts of male pupae (n =125) were placed in separate 10-inch gateau domes. Day 25 - Eclosed males in gateau domes were tranquilised with cold exposure (-20° for 3 mins) then transferred to 600ml deli pots in groups of 10 (pot-males). Pot-males had *ad libitum* access to sugar water (7.5% w/v) for 48 hrs, that had been supplemented with 0.1% w/v fluorescent rhodamine B dye (Sigma). Day 26/27, eclosed males in in gateau domes were tranquilised with cold exposure (-20°C for 3 mins) and transferred to a second bugdorm (location 2) where they had *ad libitum* access to sugar water (7.5% w/v). Day 27 – Five minutes before the 12hr dark cycle commenced, pot-males had been feeding on rhodamine B for 48 hrs and were transferred to location 1 with unstained females. Location 1 was observed every 30 mins from the beginning of the dark cycle and mating pairs were extracted on filter paper, then transferred to 30ml deli pots. When mating had ceased, males were removed from deli pots. Day 28 - Mated females were split into two groups. One group of mated females were transferred to location 2 with unstained males, and the other group were frozen to

serve as a positive control. The sugar water reservoirs in location 2 were replenished with 7.5% w/v sugar water, and moths were left undisturbed for 96 hours. Day 32 – The bursa copulatrix of female moths were dissected and spermatophores were quantified and assessed for fluorescence under a microscope.

6.8.2) Assessing whether FAW females exhibit polyandrous behaviour when dispersing under semi-natural conditions

This experiment was carried out in Conchal, Brazil in February of 2017 and moths were provided by PROMIP (PROMIP, 2019). Day 0 - Approximately 180 recently eclosed male FAW were placed in three separate bugdorms (Figure 6.2) with *ad libitum* access to 7.5% w/v sugar water that had been supplemented with 0.1% w/v rhodamine B (Sigma). Day 1 - 375 recently eclosed females were placed in three separate bugdorms (location 1) with *ad libitum* access to sugar water (7.5% w/v). Day 2 - Shortly before dusk, males had been feeding on rhodamine B for 48hrs and were tranquilised with cold exposure (-20℃ for 3 mins), then transferred to location 1 with unstained females. The bugdorms were continuously observed and eighty mating pairs were removed on filter paper, then transferred to 30ml deli pots. Once mating had ceased, the male moth was removed and fifty of the mated females were transferred to a bugdorm with *ad libitum* access to 7.5% w/v sugar water. Two more bugdorms were set up containing recently eclosed, virgin male FAW (n=125). Day 3 – 50 once mated females and 250 virgin males were released to a screenhouse (Figure 6.3) where they had *ad libitum* access to sugar water (7.5% w/v). Moths were left undisturbed in the screenhouse for 96 hours. Day 7- Females were recaptured and the bursa copulatrix was dissected so that spermatophores could be quantified and analysed for fluorescence.

Figure 6.2 Budorms containing male moths and rhodamine B labelled sugar water. Three bugdorms were used to supply 180 virgin male moths with fluorescent rhodamine B dye for 48 hrs.

Figure 6.3 The screenhouse used for investigations into the polyandrous behaviours of FAW females. The screenhouse was subject to naturally occurring temperature and humidity conditions but provided an enclosed space and protected moths from wind and rain. Virgin females were mated once with a male that had been labelled with fluorescent rhodamine B dye. Fifty once mated females and 250 virgin, unlabelled males were then brought to the screenhouse in bugdorms (B) and released for 96 hrs before being recaptured. During containment in the screenhouse moths had *ad libitum* access to 7.5% sugar water (A) and corn plants (C/D).

6.9) Analysing the sperm precedence patterns of the FAW

Moths were reared in a controlled environment: 25°C ambient temperature, 50% relative humidity and 12L: 12D photoperiod. Day 0 - Recently hatched wild-type and OX5382G egg rafts were infested on larval diet. Day 23 - Male and female wild-type pupae were separated. 125 wild-type female pupae were transferred to a bugdorm with *ad libitum* access to sugar water (7.5% w/v) and 250 male wild type pupae were placed in two gateau domes with *ad libitum* access to sugar water (7.5% w/v). OX5382G pupae were assessed for fluorescence under a microscope and 125 male pupae that exhibited the fluorescent phenotype were placed in a gateau dome with *ad libitum* access to sugar water (7.5%

w/v) that had been supplemented with 0.2g/L of doxycycline (Sigma). Day 26- Five minutes before the 12hr dark cycle commenced, wild-type male moths were tranquilised with cold exposure (-20°C for 3 mins) and transferred to the bugdorm with female moths. The bugdorm was observed every 30 mins and mating pairs of wild-type moths were removed on filter paper then transferred to 30ml deli pots. When mating ceased the wild-type male was removed. OX5382G moths were tranquilised by cold shock (-20°C for 3 mins) and transferred to 600ml deli pots (3 moths per pot) that with *ad libitum* access to sugar water (7.5% w/v) that had been supplemented with 0.2g/L of doxycycline (Sigma). Day 27 - Mated females that had not laid any eggs subsequent to the first mating were placed individually in deli pots with three OX5382G males. Deli pots were observed every 30 mins and matings that occurred were allowed to progress to completion. When the second mating had finished all three OX5382G males were removed from the pot and females were left undisturbed to lay eggs for six days with *ad libitum* access to sugar water (7.5% w/v). Day 28 to Day 34 - As eggs rafts appeared, they were placed in individual petri dishes that contained larval diet and moistened cotton wool and observed daily until they began to hatch. As egg rafts hatched, groups of larvae (n=30) were assessed for fluorescence under a microscope. To permit accurate detection of the fluorescent phenotype, larvae were allowed to develop for 24 hours so that fluorescent protein could accumulate, then tranquilised with cold shock (-20°C for 2 hrs) and thawed at room temperature for an hour before being assessed for fluorescence.

6.4) Results

6.1) Quantification and fluorescence analysis of spermatophores contained in the bursa copulatrix of once mated and virgin Fall Armyworm

It was hypothesised that a typical wild-type/wild-type FAW cross would produce a single non-fluorescent spermatophore. To investigate this, the bursa copulatrix (Figure 6.4) of virgin and once-mated females were dissected. 100% of the bursas extracted from once-mated females (n=30) contained a single non-fluorescent spermatophore. The bursas of virgin females (n=30) did not contain any spermatophores.

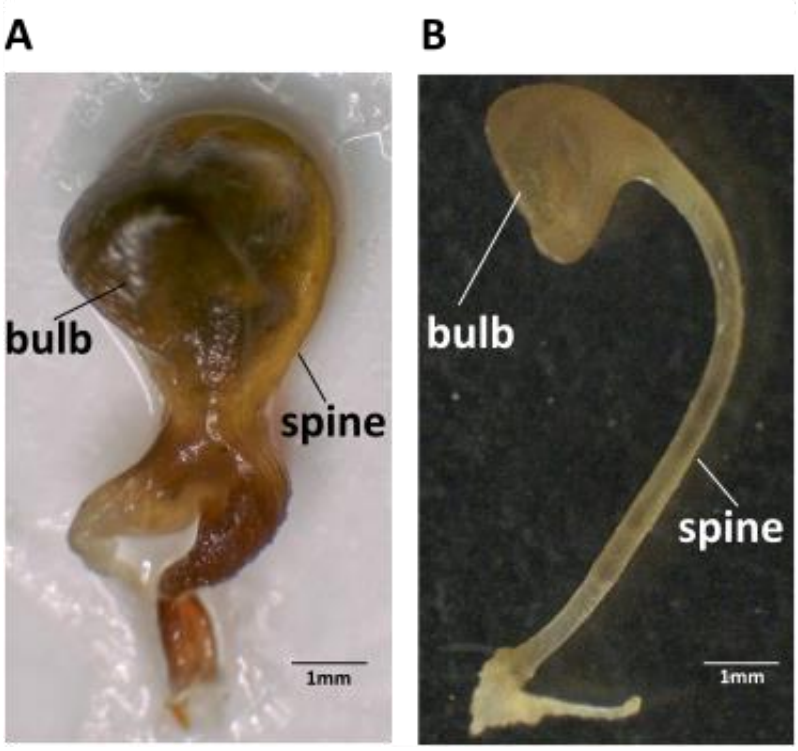

Figure 6.4 Bursa copulatrix of once mated Fall Armyworm. A) Intact bursa copulatrix of once mated female moth. The spine and bulb of a single spermatophore are visible through the bursa membrane, B) A spermatophore that was extracted from the bursa copulatrix in panel A.

6.2) Feeding Fall Armyworm larvae with rhodamine B to fluorescently label the spermatophores of adult males

It was predicted that fluorescent rhodamine B consumed by male FAW larvae would persist throughout all developmental stages and be detectable in the spermatophores of adult moths (Blanco et al., 2006; Johnson et al., 2017; van der Reijden et al., 1997). To test this hypothesis, male larvae were reared on diet containing fluorescent rhodamine B (0.1% w/v) then mated to unstained females as adults. Larvae that consumed rhodamine B exhibited global staining, confirmed by a pink phenotype under white light and fluorescence under green light (Figure 6.5). However, spermatophores that were generated by males reared on rhodamine B as larvae (n=20) did not fluoresce under green light.

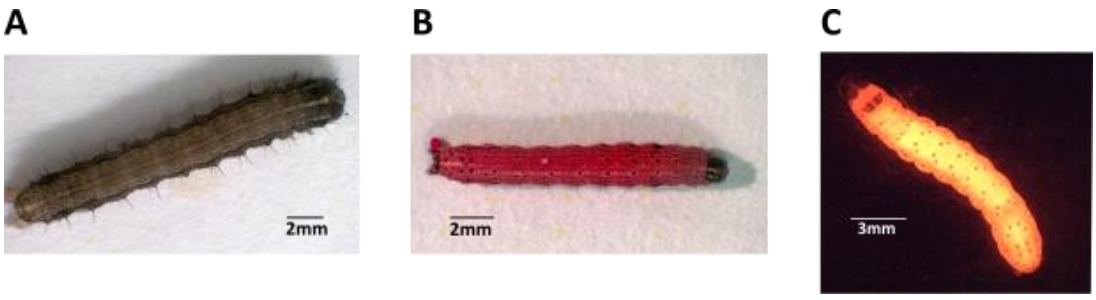

Figure 6.5 Phenotypic variation exhibited by seven-day old Fall Armyworm larvae that were reared on standard or rhodamine B diets. A) Larva reared on standard diet – after 7 days a larva reared on standard diet had an anterior/posterior axis of ≈19mm and green/brown phenotype, B) Larva reared on rhodamine B – after 7 days a larva reared on rhodamine B diet had an anterior/posterior axis of ≈14mm and pink phenotype, C) When viewed under green light the larva reared on rhodamine B (panel B) exhibited obvious fluorescence but this signal was not transmitted to the spermatophores of adult moths.

6.3) Feeding adult male Fall Armyworm moths with rhodamine B to generate stained spermatophores

It was predicted that if rhodamine B were consumed by adult male moths, then it would permeate and fluorescently label their spermatophores (Blanco et al., 2006; Johnson et al., 2017; van der Reijden et al., 1997). To investigate this, virgin male FAW (<24hrs post eclosion) were subjected to two discrete feeding conditions (labelled and unlabelled). Unlabelled moths were given *ad libitum* access to 7.5% sugar water, and labelled males

were given *ad libitum* access to 7.5% w/v sugar infused with rhodamine B dye (0.1% w/v). Males were exposed to a specific feeding condition for either 24 or 48 hrs before being placed in an enclosure with a virgin, unlabelled female. After 24hrs of cohabitation with male moths, the bursa copulatrix of females were dissected and spermatophores were analysed for fluorescence. All bursas dissected in this study contained either 0 or 1 spermatophores, indicating that the FAW mated at a maximum frequency of one mating a night. Fluorescent rhodamine B dye was present in spermatophores that had been transferred by labelled males but not unlabelled males (Figure 5.6). However, rhodamine B labelling of spermatophores did not exhibit 100% penetrance unless males were exposed to the dye for 48 hrs (Table 6.1).

Table 6.1 Quantification and fluorescence analysis of spermatophores in the bursa copulatrix of FAW females subsequent to mating with either labelled or unlabelled males. Virgin females were confined in a deli-pot for 24hrs with a male that had previously been exposed to either sugar water (unlabelled), or sugar water that contained rhodamine B (labelled). Males were subjected to a specific feeding conditions for either for 24 or 48 hours prior to cohabitation with females. Approximately thirty male/female pairs of moths were generated for all four feeding treatment conditions. After 24 hours of cohabitation, female moths were dissected and spermatophores were quantified and analysed for fluorescence under a microscope. All females either contained 0 or 1 spermatophores. Fluorescent spermatophores were only observed in females subsequent to a mating with a labelled male. Males that had been labelled with rhodamine B for 24hrs produced labelled spermatophores at a rate of 50%. 100% of spermatophores passed to females were fluorescent when males were exposed to rhodamine B for 48 hrs.

Feeding condition	Mating pairs (n)	Spermatophores (n)	Fluorescent (%)
labelled (24hrs)	28	8	50
unlabelled (24hrs)	29	7	0
labelled (48hrs)	29	10	100
unlabelled (48hrs)	27	8	0

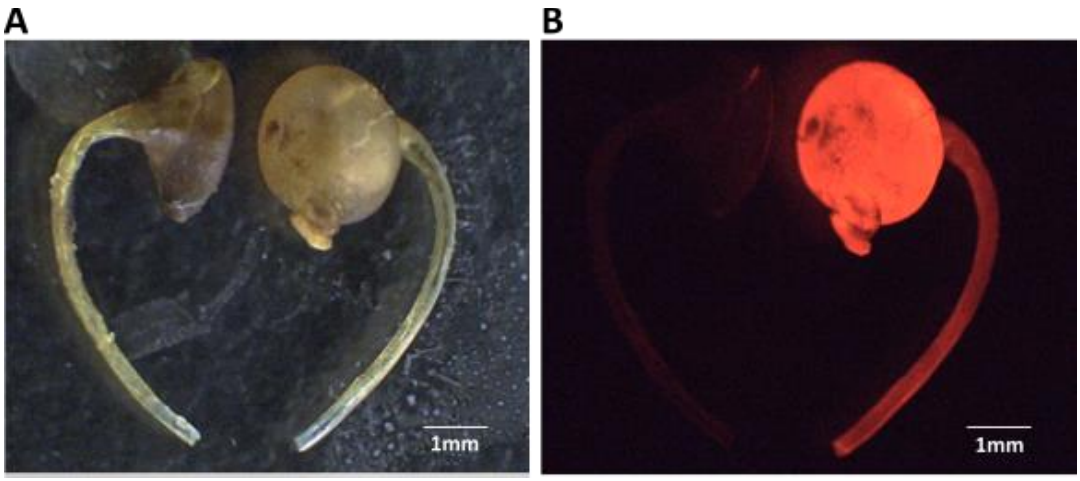

Figure 6.6 Two spermatophores that were transferred to discrete females during independent matings with unlabelled and labelled males. A) Spermatophores viewed under white light – a spermatophore that was extracted from the bursa of a female that mated with an unlabelled male (left) and a spermatophore extracted from the bursa of a female that mated with a labelled male (right). The phenotypes of the labelled and unlabelled spermatophores were similar when observed under white light. B) Spermatophores viewed under green light – the two spermatophores presented in panel A are displayed identically in this image but only the labelled spermatophore was visible under green light due to the presence of fluorescent rhodamine B dye.

6.4) Using fluorescently labelled spermatophores to track the polyandrous behaviour of FAW females in discrete locations – lab trials

Female moths were subjected to a sequential mating assay to test the hypothesis that they practice polyandry when dispersing in the field. In a primary location, virgin females were mated with FAW males that had been exposed to rhodamine B for 48 hrs, with a prediction that this mating would produce a single fluorescent spermatophore (Blanco et al., 2006; Johnson et al., 2017; van der Reijden et al., 1997). Females that had mated with rhodamine B labelled males were split into two treatment groups (re-mating and once mated). The re-mating group was transferred to a second location with unlabelled males and the once mated group was frozen. Females from the re-mating treatment were left undisturbed in the second location with the population of unlabelled males for 96hrs, then recaptured and the bursa copulatrix was dissected. It was predicted that any matings that occurred in the second location would produce a single spermatophore, but, spermatophores obtained by females in the second location would not fluoresce as males in that location had not been labelled with rhodamine B. Therefore, the presence of

multiple spermatophores of both fluorescent and non-fluorescent phenotypes would serve as evidence that females had mated with at least two males in discrete locations. Twenty-five females were recaptured, and all contained a single fluorescent spermatophore and between one and three unlabelled spermatophores, supporting the hypothesis that, in principle, FAW practice polyandry while dispersing (Table 6.2). The majority of the rhodamine B dye had drained from the labelled spermatophores, but a small amount was retained in the lower spine and was easily detected (Figure 6.7).

Table 6.2 Quantification and fluorescence analysis of spermatophores contained in the bursa copulatrix of FAW females subsequent to mating with labelled then unlabelled males in the lab. Virgin females were mated with a male that had been fluorescently labelled with rhodamine B. Once the mating had concluded, females were either frozen (once mated) or introduced to a second location that contained unlabelled males (re-mating). After 96 hrs in the re-mating location, females were recaptured and the contents of the bursa copulatrix was examined. A total of 25 females were recaptured from the re-mating location and all contained at least two spermatophores of distinct fluorescent and non-fluorescent phenotypes, demonstrating that female moths had successfully mated in both locations. Assuming that all matings had resulted in successful passage of a single spermatophore to the female, ten females had re-mated once, twelve females had re-mated twice and three females had re-mated three times. 30, once mated females were dissected and 29 contained a single fluorescent spermatophore. A single once mated female did not contain any spermatophores, demonstrating that the observed mating had been unsuccessful.

Treatment	Fluorescent spermatophores	Non-fluorescent spermatophores	Total matings	Incidence
re-mating	1	1	2	10
re-mating	1	2	3	12
re-mating	1	3	4	3
once mated	1	0	1	29

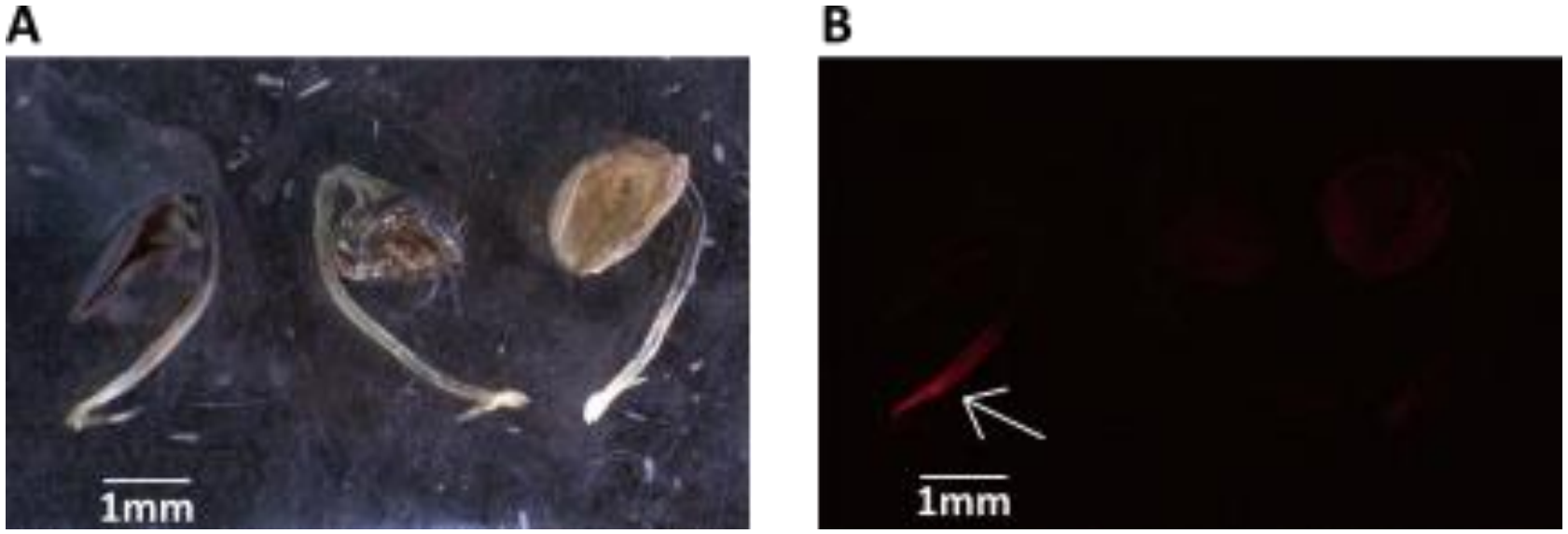

Figure 6.7 Spermatophores extracted from a single female moth subsequent to mating with labelled and unlabelled males in discrete locations. A) Three spermatophores viewed under white light – The labelled spermatophore obtained during the initial mating is located on the left and exhibits a slight pink stain on the spine. Two spermatophores obtained during unstained matings are located in the centre and to the right of the image. B) Spermatophores viewed under green light – The spermatophores presented in panel A are displayed identically in this image but under green light. The labelled spermatophore fluoresced in the lower part of the spine (indicated by an arrow) where some rhodamine B had become trapped.

6.5) Using fluorescently labelled spermatophores to track the polyandrous behaviour of FAW during dispersal - cage trials

A second investigation into the polyandrous behaviours of FAW females during dispersal, was carried out in a screenhouse (Figure 6.3) in Conchal, Brazil. The experiment took place in February when the FAW naturally occurs in Conchal. The screenhouse setup made it possible to model field conditions experienced by FAW more realistically than had been possible in the lab, for three reasons: 1) The size of the screenhouse (≈270 m^3) meant that the population density was reduced, giving females the opportunity to avoid male contact if desired, 2) The experiment took place within the FAW's natural geographical range and the key environmental variables of temperature and humidity were not manipulated and 3) Corn plants were added to the screenhouse to provide the FAW with natural refugia/breeding substrates. Fifty female moths that had mated once with a fluorescently labelled male were released to the screenhouse with approximately 250 unlabelled males and left undisturbed for 96 hours before being recaptured. A total of twenty-eight females were recaptured for dissection. Nineteen recaptured females contained two or more spermatophores of distinct fluorescent and non-fluorescent phenotypes, demonstrating that they had mated in both locations (Table 6.3). Nine females contained a single fluorescent spermatophore, demonstrating that they did not re-mate in the screenhouse. One female contained a single non-fluorescent spermatophore, indicating that the first labelled mating must have been unsuccessful for that individual but that she had successfully mated in the screenhouse.

Table 6.3 Quantification and fluorescence analysis of spermatophores contained in the bursa copulatrix of FAW females subsequent to mating with labelled males in the lab and unlabelled males in a screenhouse. Virgin females were mated with a fluorescently labelled male in the lab. Once the mating had concluded, females were either frozen (once mated) or introduced to a 270 m3 screenhouse that contained unlabelled males (re-mating). After 96 hrs in the screenhouse, females were recaptured and the contents of the bursa copulatrix was examined. A total of 28 females were recaptured from the screenhouse and 19 contained at least two spermatophores of distinct fluorescent and non-fluorescent phenotypes, demonstrating that those moths had successfully mated in both locations. Nine females contained a single fluorescent spermatophore indicating that those individuals had not re-mated in the screenhouse. One female contained a single non-fluorescent spermatophore, demonstrating that the initial labelled mating had not been successful but that she had successfully mated in the screenhouse. Thirty once mated females were dissected, and all contained a single fluorescent spermatophore.

Treatment	Fluorescent spermatophores	Non-fluorescent spermatophores	Total matings	Incidence
re-mating	0	1	1	1
re-mating	1	0	1	9
re-mating	1	1	2	14
re-mating	1	2	3	4
re-mating	1	3	4	1
once mated	1	0	1	30

6.6) Sperm precedence patterns exhibited by FAW females that had been sequentially mated to wild-type then hemizygous OX5382G males

A sequential mating assay was implemented to verify the rate at which females exhibit last-male sperm precedence, as previous studies had drawn opposing conclusions on this topic (Martin et al., 1989; Snow et al., 1970). Wild-type females were first mated to a wild-type male, then subsequently to a transgenic male. The transgenic OX5382G moths used in this study were a prototype self-limiting strain (Asadi et al., 2019; Gorman et al., 2016; Harvey-Samuel et al., 2015) and were hemizygous for a fluorescent dsRED2 marker gene (Gong et al., 2005). According to hemizygous inheritance (Charlesworth et al., 1987), it was predicted that if the FAW exhibited 100% last-male sperm precedence (Snow et al., 1970) then 50% of the F1 generation would be fluorescent. 20 females

(FEMs) mated to both a wild-type male, then a transgenic male and subsequently laid viable eggs that gave rise to larvae for analysis (Table 6.4). When eggs laid by a FEM began to hatch (Day 0) they were monitored for four days. FEMs produced viable egg rafts at a mean rate of 4.65 (± 0.49 s.e) per individual over the four day observation period, with a maximum capacity of five egg rafts in a single day. Each day, a group of larvae (n=30) was extracted from every raft that had hatched to be assessed for fluorescence under a microscope (Figure 5.8). The rate of fluorescence exhibited by these groups of larvae served as a proxy for the rate of fluorescence of the egg raft population, and thus the F1 generation. If a FEM laid multiple egg rafts that hatched on the same day, the rate of fluorescence for that day was judged to be equal to the mean rate of fluorescence for all hatched egg rafts. Over the four day observation period, the F1 generation inherited the fluorescent gene from FEMs at a mean rate of 49.17% (± 0.73 s.e) which supported the hypothesis that FAW exhibit robust last-male sperm precedence (Figure 6.9). However, the mean rate of fluorescence exhibited by larvae dropped from 51% on Day 0 to 43% on Day 3 indicating that females may have reverted to stored wild-type sperm once transgenic sperm had become depleted. Therefore, a one-way ANOVA was performed to test if the rate of fluorescence in the F1 generation varied significantly over time. The rate of fluorescence was not normally distributed throughout the F1 generation across all four observation days (Shap.Wilk, $W=0.89$, $p<0.0001$), so a transformation (Transformation = x ^ 2.9) was performed to produce a normalised dataset for parametric analysis. The variance of fluorescence was homogenous over all four days of observation (Bartlett's $K^2= 2.39$, df = 3, p-value>0.05) but no significant differences were detected in the rate of fluorescence exhibited by groups of larvae on different days (ANOVA, $F(3,50) =1.05$, $p>0.05$).

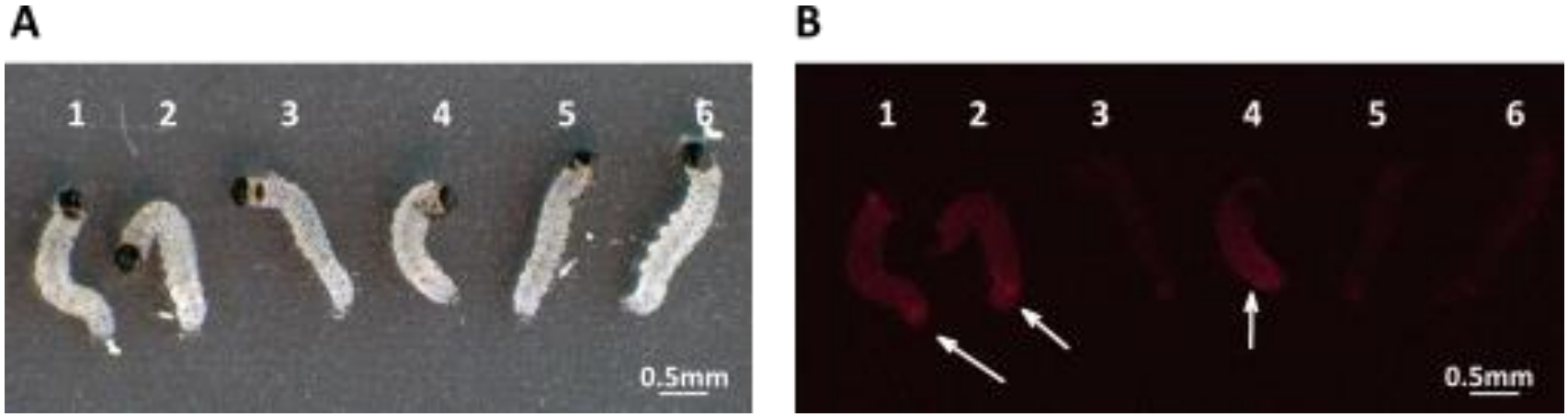

Figure 6.8 The phenotypes of six L1 FAW larvae that emerged from a single egg raft that had been laid by a wild-type female subsequent to sequential matings with a wild-type male then a hemizygous transgenic male. A) Larvae visualised under white light - Under these conditions the larvae exhibit very similar light green phenotype. B) larvae visualised under green light – the same larvae are displayed here as in panel A, in the same configuration. Individuals 1, 2 and 4 fluoresce under these conditions (shown by white arrows) but individuals 3, 5 and 6 do not. Therefore, half of this group of six larvae had inherited the transgenic, fluorescent gene which supported the hypothesis that the FAW exhibits last-male sperm precedence**.**

FEM	Day 0 rafts (n)	Day 0 Fluorescence (%)	Day 1 rafts (n)	Day 1 Fluorescence (%)	Day 2 rafts (n)	Day 2 fluorescence (%)	Day 3 rafts (n)	Day 3 fluorescence (%)	Total egg rafts	Mean fluorescent phenotype of F1 gen (%)
1	4	43.33	2	53.33	1	50.00	0	n/a	7	48.90
2	2	50.00	2	51.67	1	36.67	0	n/a	5	46.11
3	2	61.67	2	50.00	2	48.28	1	50	7	52.49
4	1	50.00	0	n/a	0	n/a	0	n/a	1	50.00
5	3	50.00	1	48.67	1	50.00	1	36.67	6	46.33
6	5	48.67	2	50.00	1	50.00	0	n/a	8	49.56
7	3	54.44	1	50.00	0	n/a	0	n/a	4	52.22
8	3	46.67	2	55.00	2	42.86	0	n/a	7	48.17
9	3	47.78	2	56.67	0	n/a	0	n/a	5	52.22
10	1	53.33	1	53.33	0	n/a	0	n/a	2	53.33
11	2	51.67	2	40.32	0	n/a	0	n/a	4	45.99
12	1	46.67	1	50.00	0	n/a	0	n/a	2	48.33
13	2	56.67	1	53.33	0	n/a	1	43.33	4	55.00
14	1	46.67	1	56.67	1	66.67	1	46.67	4	54.17
15	2	61.67	1	40.00	3	62.22	1	25.00	7	47.22
16	2	46.67	1	46.67	0	n/a	0	n/a	3	46.67
17	2	45.00	0	n/a	0	n/a	0	n/a	2	45.00
18	2	53.33	0	n/a	1	50.00	2	51.67	5	51.67
19	1	50.00	3	48.89	0	n/a	2	45.00	6	45.00
20	2	56.67	0	n/a	2	33.33	0	n/a	4	45.00

Table 6.4 The phenotypes of FAW larvae that developed from egg rafts laid by wild type female moths following sequential matings with a wild-type then a transgenic male. Twenty females (FEMs) laid viable eggs following sequential matings with a wild-type male and then a transgenic male (the transgenic male was hemizygous for a fluorescent marker gene). When eggs laid by FEMs began to hatch (Day 0), all eggs that had been laid by that female were monitored for four days and developing larvae were assessed for fluorescence. When multiple egg rafts that had been laid by the same FEM hatched on the same day, the rate of fluorescence for that day was judged to be equal to the mean rate of fluorescence for all hatched egg rafts. FEMs laid a mean total of 4.65 (± 0.49 s.e) egg rafts that hatched during the observation period and larvae that developed from those rafts fluoresced at a mean rate of 49.17% (± 0.73 s.e). 20 FEMs produced larvae on Day 0, 16 FEMs produced larvae on Day 1, 10 FEMs produced larvae on Day 2 and 7 FEMs produced larvae on Day 3. Only four FEMs laid eggs that produced larvae on four consecutive days.

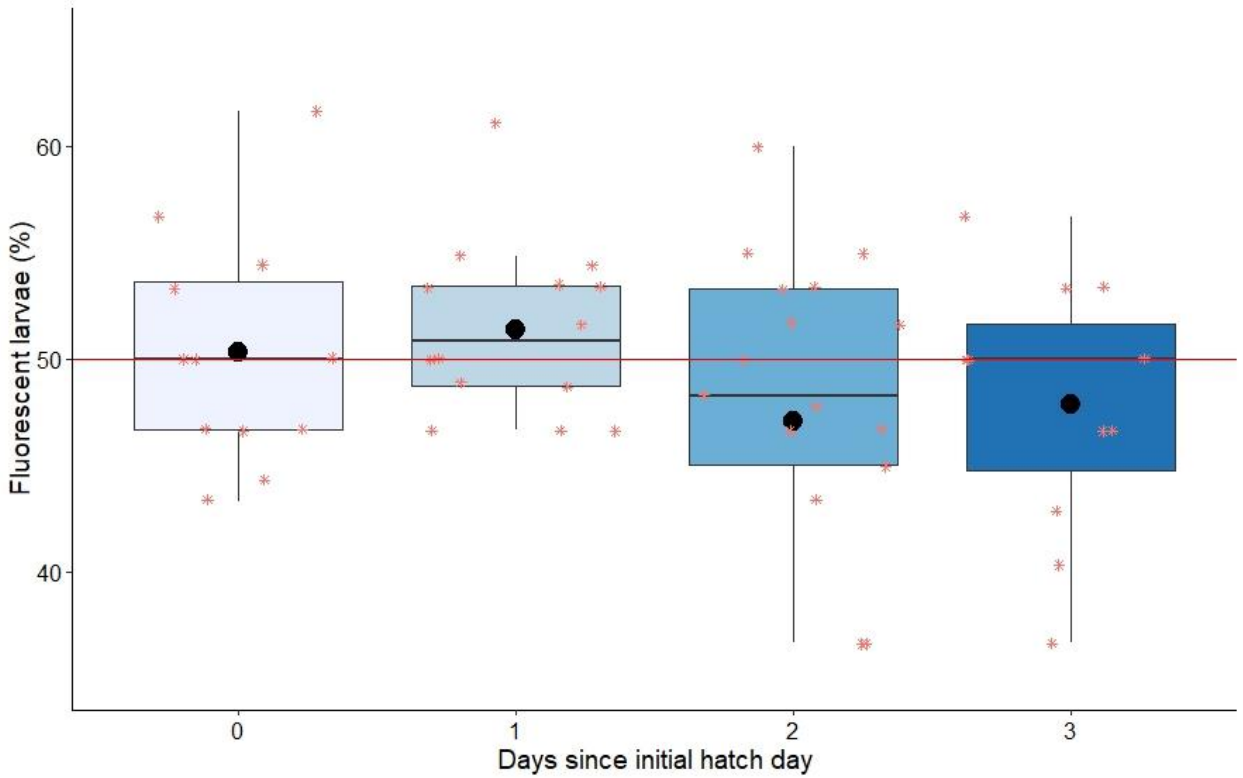

Figure 6.9 The percentage of fluorescent larvae in populations of FAW that developed from eggs laid by wild-type females following sequential matings with a wild-type male and then a transgenic male. The rate of fluorescence exhibited by 93 groups of larvae (n=30) that developed from 20 discrete maternal lineages are represented on the y-axis. Each discrete group of 30 larvae is represented by a red star. 50% fluorescence is represented by a horizontal red line. The x-axis represents a time series that began when eggs from each specific lineage began to hatch (Day 0). The mean rate of fluorescence was lower on Day 3 than on Day 0 (51 vs 43%) but no significant differences were detected in the rate of fluorescence exhibited by groups of larvae on different days (ANOVA, $F(3,50) = 1.05$, $p > 0.05$).

6.5) Discussion

The FAW is a destructive agricultural pest (Cruz et al., 1999; Luginbill, 1928; Sparks, 1979) that can reduce crop yields by up to 57% (Cruz et al., 1999). Historically the FAW had been restricted to the Western Hemisphere (Ashley et al., 1989; Luginbill, 1928) but a recent introgression into Western Africa (Goergen et al., 2016) has facilitated its spread throughout the sub-Saharan continent (Day et al., 2017) and into India (Deshmukh et al., 2018), South-East Asia (CABI, 2019) and China (Liu et al., 2019). In the Western Hemisphere the FAW has developed resistance to insecticides (Burtet et al., 2017; Carvalho et al., 2013; Nascimento et al., 2016) and Bt crops (Farias et al., 2014; Omoto et al., 2016 Huang et al., 2014; Storer et al., 2010). Therefore, novel modes of control such as Oxitec's self-limiting technology (Thomas et al., 2000) are required to prevent the FAW from dispersing further and to limit the damage it causes in areas that it already occupies. I hypothesised that self-limiting control would be optimised if the FAW practiced both polyandry and last-male sperm precedence when dispersing in the field. These hypotheses were based on the premise that non-virgin females would be able to transport fertilised eggs to control sites while dispersing and propagate pest populations at those locations, unless they subsequently re-mated with Oxitec males.

To test if the FAW practiced polyandry while dispersing I labelled the spermatophores of male moths with fluorescent rhodamine B (Blanco et al., 2006; van der Reijden et al., 1997). My goal was to mate females with a labelled male in a primary location and then with an unlabelled male in a secondary location, as spermatophores contained in the bursa copulatrix could then be traced back to discrete environments via the presence or absence of the fluorescent dye. Two fundamental assumptions of this system were that typical wild-type X wild-type matings would result in the transfer of a single non-fluorescent spermatophore and also that virgin females would not contain any spermatophores. These assumptions were verified, as I set-up 30 wild-type X wild-type crosses which all resulted in the transfer of a single non-fluorescent spermatophore and I collected 30 virgin females did not contain any spermatophores at all.

Although rhodamine B had previously been used to stain the spermatophores of *Lepidoptera* and *Coleoptera* (Blanco et al., 2006; van der Reijden et al., 1997) and the seminal fluid proteins of *Diptera* (Johnson et al., 2017) this was the first time that this compound had been used to stain the spermatophores of the FAW. Therefore, different feeding treatment conditions were initially imposed on male FAW to optimise transfer of rhodamine B into the spermatophore.

van der Reijden et al. (1997) report that consumption of rhodamine B reduced the mean lifespan of *Photinius* fireflies from 11 to 7 days. It was critical therefore that the feeding conditions imposed on male FAW were selected and administered in a manner that avoided deleterious side effects. As Blanco et al. (2006) had labelled the spermatophores of *Heliothis virescens* with 0.1% w/v rhodamine B and had found that this did not affect the lifespan, fecundity or mating competitiveness of that model, I began my investigations by feeding FAW larvae with the dye at 0.1% w/v. I found that FAW males did not produce fluorescent spermatophores if larvae were reared on standard diet that had been supplemented with 0.1% rhodamine B. Supplementation of larval diet with rhodamine B slowed the larva to adult development time of FAW males from 25 to 35 days, indicating that the compound was toxic but not lethal for larvae at this concentration. I subsequently supplied FAW adults with sugar water that had been supplemented with 0.1% w/v rhodamine B. I found that 100% of spermatophores that were transferred to females were fluorescently labelled, if males were given *ad libitum* access to sugar water that had been supplemented with 0.1% w/v rhodamine B for 48 hours. This established a reliable method for labelling FAW spermatophores that I continued to use in subsequent investigations into polyandry.

Simmons et al. (1992) report that female FAW exhibit polyandry. I performed two experiments to confirm the original Simmons et al. (1992) report. These experiments followed a common procedure; I first mated females with a labelled male in one location, then transferred the mated female to a second location with unlabelled males for a period of 96 hrs. Initially the experiment was performed in the lab, where a group of 25 females were successfully recaptured from the second mating location. The bursa copulatrix of the 25 recaptured females were dissected and all contained between two

and four spermatophores, indicating that all females had all mated at least twice. Critically, spermatophores in the bursa copulatrix of the 25 recaptured females exhibited both fluorescent and non-fluorescent phenotypes which demonstrated that matings had occurred with discrete males in both locations. These data served as evidence that females practiced polyandry in the lab but did not necessarily model the behaviours of females in the field. Therefore, in a second experiment, the labelled/unlabelled mating method was used to investigate polyandry in cage trials in Brazil, where population densities and environmental conditions better mimicked those experienced by FAW in the field. As for the lab experiment, females did practice polyandry in cage trials. However, polyandry occurred at reduced frequency in cage trials relative to the lab. 31% of females did not re-mate in the cage trials which may be more representative of behaviours in the field.

Finally, I investigated whether females exhibited last-male sperm precedence. I employed a system of sequential mating in which females were first mated to a wild-type male, then to a transgenic male that hemizygously carried a fluorescent dsRED2 marker gene (Gong et al., 2005). I predicted that if the FAW exhibited last-male sperm precedence as reported in Snow et al. (1970), then the F1 generation would exhibit fluorescence at a rate of 50% (Boorman et al., 1976; Charlesworth et al., 1987). The data gathered during this experiment supported the hypothesis that FAW practice last-male sperm precedence, as the F1 generation exhibited fluorescence at a rate of 49.17% (± 0.73 s.e). However, it was possible that some wild-type F1 larvae had been fertilised with sperm stored from the initial wild-type X wild-type mating and that last-male sperm precedence was not as robust as it appeared. To validate these data, a reciprocal experiment should be carried out in which females are first mated to a transgenic and then to a wild-type male, with a prediction that 100% of the F1 generation will be non-fluorescent if the FAW practices last-male sperm precedence.

In conclusion, I developed a method for labelling spermatophores with fluorescent dye and was able to use that method to track female matings. Those data demonstrated that females exhibit polyandry in the lab. Furthermore, I was able to provide evidence that females had practiced polyandry in cage trials, demonstrating that multiple matings in

the lab had not simply been a consequence of elevated population density. In addition, I performed a sequential mating assay which indicated that FAW practice last-male sperm precedence. These data will serve as preliminary evidence that the FAW is a suitable candidate for control with Oxitec's self-limiting technology, as non-virgin females would be susceptible to matings with self-limiting males when dispersing in the field.

6.6) References

Asadi, R., Elaini, R., Lacroix, R., Ant, T., Collado, A., Finnegan, L., . . . Koukidou, M. (2019). Preventative releases of self-limiting *Ceratitis capitata* provide pest suppression and protect fruit quality in outdoor netted cages. *International Journal of Pest Management*, 1-12. doi:10.1080/09670874.2019.1601293

Ashley, T. R., Wiseman, B. R., Davis, F. M., & Andrews, K. L. (1989). The Fall Armyworm: A bibliography. *The Florida Entomologist, 72*(1), 152-202. doi:10.2307/3494982

Blanco, C. A., Perera, O., Ray, J. D., Taliercio, E., & Williams, L., 3rd. (2006). Incorporation of rhodamine b into male tobacco budworm moths (*Heliothis virescens*) to use as a marker for mating studies. *Journal of insect science (Online), 6*, 1-10. doi:10.1673/1536-2442

Boorman, E., & Parker, G. A. (1976). Sperm (ejaculate) competition in *Drosophila melanogaster,* and the reproductive value of females to males in relation to female age and mating status. *Ecological Entomology, 1*(3), 145-155. doi:10.1111/j.1365-2311.1976.tb01217.x

Burtet, L. M., Bernardi, O., Melo, A. A., Pes, M. P., Strahl, T. T., & Guedes, J. V. (2017). Managing fall armyworm, *Spodoptera frugiperda (Lepidoptera: Noctuidae),* with Bt maize and insecticides in southern Brazil. *Pest Management Science, 73*(12), 2569-2577. doi:10.1002/ps.4660

CABI. (2019). Invasive species compendium. Retrieved from https://www.cabi.org/isc/fallarmyworm

Carvalho, R. A., Omoto, C., Field, L. M., Williamson, M. S., & Bass, C. (2013). Investigating the molecular mechanisms of organophosphate and pyrethroid resistance in the Fall Armyworm *Spodoptera frugiperda*. *Plos One, 8*(4), e62268. doi:10.1371/journal.pone.0062268

Charlesworth, B., Coyne, J. A., & Barton, N. H. (1987). The relative rates of evolution of sex chromosomes and autosomes. *The American Naturalist, 130*(1), 113-146.

Cruz, I., Figueiredo, M. L. C., Oliveira, A. C., & Vasconcelos, C. A. (1999). Damage of *Spodoptera frugiperda* (smith) in different maize genotypes cultivated in soil under three levels of aluminium saturation. *International Journal of Pest Management, 45*(4), 293-296. doi:10.1080/096708799227707

Day, R., Abrahams, P., Bateman, M., Beale, T., Clottey, V., Cock, M. J. W., . . . Witt, A. (2017). *Fall armyworm: Impacts and implications for africa* (Vol. 28).

Deshmukh, S., Kalleshwaraswamy, C., Asokan, R., Swamy, H. M. M., Maruthi, M. s., H. B, P., . . . Goergen, G. (2018). *First report of the fall armyworm, Spodoptera frugiperda (j e smith) (Lepidoptera: Noctuidae), an alien invasive pest on maize in India* (Vol. 24).

Farias, J. R., Andow, D. A., Horikoshi, R. J., Sorgatto, R. J., Fresia, P., dos Santos, A. C., & Omoto, C. (2014). Field-evolved resistance to Cry1f maize by *Spodoptera frugiperda* (*Lepidoptera: Noctuidae*) in Brazil. *Crop Protection, 64*, 150-158. doi:10.1016/j.cropro.2014.06.019

Frontier. (2019). Frontier agriculture sciences. Retrieved from https://insectrearing.com/

Goergen, G., Kumar, P. L., Sankung, S. B., Togola, A., & Tamò, M. (2016). First report of outbreaks of the fall armyworm *Spodoptera frugiperda* (j e smith) (*Lepidoptera, Noctuidae*), a new alien invasive pest in West and Central Africa. *Plos One, 11*(10), e0165632-e0165632. doi:10.1371/journal.pone.0165632

Gong, P., Epton, M. J., Fu, G. L., Scaife, S., Hiscox, A., Condon, K. C., . . . Alphey, L. (2005). A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly. *Nature Biotechnology, 23*(4), 453-456. doi:10.1038/nbt1071

Gorman, K., Young, J., Pineda, L., Marquez, R., Sosa, N., Bernal, D., . . . Caceres, L. (2016). Short-term suppression of *Aedes aegypti* using genetic control does not facilitate *Aedes albopictus*. *Pest Manag Sci, 72*(3), 618-628. doi:10.1002/ps.4151

Gouin, A., Bretaudeau, A., Nam, K., Gimenez, S., Aury, J.-M., Duvic, B., . . . Fournier, P. (2017). Two genomes of highly polyphagous lepidopteran pests (*Spodoptera frugiperda, Noctuidae*) with different host-plant ranges. *Scientific Reports, 7*(1), 11816. doi:10.1038/s41598-017-10461-4

Groot, A. T., Marr, M., Schöfl, G., Lorenz, S., Svatos, A., & Heckel, D. G. (2008). Host strain specific sex pheromone variation in *Spodoptera frugiperda*. *Frontiers in Zoology, 5*(1), 20. doi:10.1186/1742-9994-5-20

Guerfali, M. M., Parker, A., Fadhl, S., Hemdane, H., Raies, A., & Chevrier, C. (2011). Fitness and reproductive potential of irradiated mass-reared Mediterranean fruit fly males *Ceratitis capitata* (*Diptera: Tephritidae*): Lowering radiation doses. *Florida Entomologist, 94*(4), 1042-1050.

Hardke, J. T., Lorenz, G. M., III, & Leonard, B. R. (2015). Fall armyworm (*Lepidoptera: Noctuidae*) ecology in southeastern cotton. *Journal of Integrated Pest Management, 6*(1). doi:10.1093/jipm/pmv009

Harvey-Samuel, T., Morrison, N. I., Walker, A. S., Marubbi, T., Yao, J., Collins, H. L., . . . Alphey, L. (2015). Pest control and resistance management through release of insects carrying a male-selecting transgene. *Bmc Biology, 13*(1), 49. doi:10.1186/s12915-015-0161-1

Health, E. Panel o. P., Jeger, M., Bragard, C., Caffier, D., Candresse, T., Chatzivassiliou, E., . . . MacLeod, A. (2018). Pest risk assessment of *Spodoptera frugiperda* for the European union. *EFSA Journal, 16*(8), e05351. doi:10.2903/j.efsa.2018.5351

Helinski, M. E., & Knols, B. G. (2008). Mating competitiveness of male *Anopheles arabiensis* mosquitoes irradiated with a partially or fully sterilizing dose in small and large laboratory cages. *J Med Entomol, 45*(4), 698-705. doi:10.1603/0022-2585(2008)45[698:mcomaa]2.0.co;2

Hooper, G. H. S. (1972). Sterilization of the Mediterranean fruit fly with gamma radiation: Effect on male competitiveness and change in fertility of females alternately mated with irradiated and untreated males. *Journal of Economic Entomology, 65*(1), 1.

Huang, F., Qureshi, J. A., Meagher, R. L., Jr., Reisig, D. D., Head, G. P., Andow, D. A., . . . Dangal, V. (2014). Cry1f resistance in fall armyworm *Spodoptera frugiperda:* Single gene versus pyramided Bt maize. *Plos One, 9*(11), e112958. doi:10.1371/journal.pone.0112958

Johnson, B. J., Mitchell, S. N., Paton, C. J., Stevenson, J., Staunton, K. M., Snoad, N., . . . Ritchie, S. A. (2017). Use of rhodamine b to mark the body and seminal fluid of male *Aedes aegypti* for mark-release-recapture experiments and estimating efficacy of sterile male releases. *Plos Neglected Tropical Diseases, 11*(9), e0005902. doi:10.1371/journal.pntd.0005902

Johnson, S. J. (1987). Migration and life-history strategy of the fall armyworm, *Spodoptera frugiperda* in the western hemisphere. *Insect Science and Its Application, 8*(4-6), 543-549.

Knipling, E. F. (1955). Possibilities of insect control or eradication through the use of sexually sterile males. *Journal of Economic Entomology, 48*(4), 459.

Knipling, E. F. (1998). Role of parasitoid augmentation and sterile insect techniques for area-wide management of agricultural insect pests. *Journal of Agricultural Entomology, 15*, 273-301.

Kost, S., Heckel, D. G., Yoshido, A., Marec, F., & Groot, A. T. (2016). A z-linked sterility locus causes sexual abstinence in hybrid females and facilitates speciation in *Spodoptera frugiperda*. *Evolution, 70*(6), 1418-1427. doi:10.1111/evo.12940

Krafsur, E. S. (1998). Sterile insect technique for suppressing and eradicating insect populations: 55 years and counting. *Journal of Agricultural Entomology, 15*(4), 303-317.

Leftwich, P. T., Koukidou, M., Rempoulakis, P., Gong, H.-F., Zacharopoulou, A., Fu, G., . . . Alphey, L. (2014). Genetic elimination of field-cage populations of Mediterranean fruit flies. *Proceedings of the Royal Society B-Biological Sciences, 281*(1792). doi:10.1098/rspb.2014.1372

Liu, H., Lan, T., Fang, D., Gui, F., Wang, H., Guo, W., . . . Liu, X. (2019). Chromosome level draft genomes of the fall armyworm, *Spodoptera frugiperda* (lepidoptera: Noctuidae), an alien invasive pest in China. *bioRxiv*, 671560. doi:10.1101/671560

Luginbill, P. (1928). *The fall army worm*. Retrieved from https://EconPapers.repec.org/RePEc:ags:uerstb:156281

Machado, V., Wunder, M., Baldissera, V. D., Oliveira, J. V., Fiúza, L. M., & Nagoshi, R. N. (2008). Molecular characterization of host strains of *Spodoptera frugiperda (Lepidoptera: Noctuidae)* in Southern Brazil. *Annals of the Entomological Society of America, 101*(3), 619-626. doi:10.1603/0013-8746(2008)101[619:mcohso]2.0.co;2

Martin, J. A., Pashley, D. P., & Mason, L. J. (1989). Sperm use patterns of individual fall armyworm (*Lepidoptera: Noctuidae*). *Annals of the Entomological Society of America, 82*(2), 177-180. doi:10.1093/aesa/82.2.177

Nagoshi, R. N. (2019). Evidence that a major subpopulation of fall armyworm found in the western hemisphere is rare or absent in Africa, which may limit the range of crops at risk of infestation. *Plos One, 14*(4), e0208966. doi:10.1371/journal.pone.0208966

Nagoshi, R. N., Goergen, G., Tounou, K. A., Agboka, K., Koffi, D., & Meagher, R. L. (2018). Analysis of strain distribution, migratory potential, and invasion history of fall armyworm populations in Northern sub-saharan Africa. *Scientific Reports, 8*(1), 3710. doi:10.1038/s41598-018-21954-1

Nagoshi, R. N., Meagher, R. L., & Hay-Roe, M. (2012). Inferring the annual migration patterns of fall armyworm (*Lepidoptera: Noctuidae*) in the United States from mitochondrial haplotypes. *Ecology and evolution, 2*(7), 1458-1467. doi:10.1002/ece3.268

Nagoshi, R. N., Silvie, P., & Meagher, R. L. (2007). Comparison of haplotype frequencies differentiate fall armyworm (*Lepidoptera: Noctuidae*) corn-strain populations from Florida and Brazil. *J Econ Entomol, 100*(3), 954-961. doi:10.1603/0022-0493(2007)100[954:cohfdf]2.0.co;2

Nascimento, A. R. B. d., Farias, J. R., Bernardi, D., Horikoshi, R. J., & Omoto, C. (2016). Genetic basis of *Spodoptera frugiperda (Lepidoptera: Noctuidae)* resistance to the chitin synthesis inhibitor lufenuron. *Pest Management Science, 72*(4), 810-815. doi:10.1002/ps.4057

Omoto, C., Bernardi, O., Salmeron, E., Sorgatto, R. J., Dourado, P. M., Crivellari, A., . . . Head, G. P. (2016). Field-evolved resistance to Cry1ab maize by *Spodoptera frugiperda* in Brazil. *Pest Management Science, 72*(9), 1727-1736. doi:10.1002/ps.4201

Pashley, D. P. (1986). Host-associated genetic differentiation in fall armyworm (*Lepidoptera: Noctuidae*): A sibling species complex? *Annals of the Entomological Society of America, 79*(6), 898-904. doi:10.1093/aesa/79.6.898

Pashley, D. P. (1988). Current status of fall armyworm host strains. *The Florida Entomologist, 71*(3), 227-234. doi:10.2307/3495425

Pashley, D. P., Johnson, S. J., & Sparks, A. N. (1985). Genetic population structure of migratory moths - the fall armyworm (*Lepidoptera, noctuidae*). *Annals of the Entomological Society of America, 78*(6), 756-762.

PROMIP. (2019). Promip - integrated pest management. Retrieved from https://promip.agr.br/

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., . . . Cardona, A. (2012). Fiji: An open-source platform for biological-image analysis. *Nat Methods, 9*(7), 676-682. doi:10.1038/nmeth.2019

Schofl, G., Heckel, D. G., & Groot, A. T. (2009). Time-shifted reproductive behaviours among fall armyworm (*Noctuidae: Spodoptera frugiperda*) host strains: Evidence for differing modes of inheritance. *Journal of Evolutionary Biology, 22*(7), 1447-1459. doi:10.1111/j.1420-9101.2009.01759.x

Simmons, A. M., & Marti, O. G., Jr. (1992). Mating by the fall armyworm (*Lepidoptera: Noctuidae*): Frequency, duration, and effect of temperature. *Environmental Entomology, 21*(2), 371-375. doi:10.1093/ee/21.2.371

Snow, J. W., Young, J. R., & Jones, R. L. (1970). Competitiveness of sperm in female fall armyworms mating with normal and chemosterilized males. *Journal of Economic Entomology, 63*(6), 1799-1802. doi:10.1093/jee/63.6.1799

Sparks, A. N. (1979). A review of the biology of the fall armyworm. *The Florida Entomologist, 62*(2), 82-87. doi:10.2307/3494083

Sparks, A. N. (1986). Fall armyworm (*Lepidoptera: Noctuidae*): Potential for area-wide management. *The Florida Entomologist, 69*(3), 603-614. doi:10.2307/3495397

Storer, N. P., Babcock, J. M., Schlenz, M., Meade, T., Thompson, G. D., Bing, J. W., & Huckaba, R. M. (2010). Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda (Lepidoptera: Noctuidae)* in Puerto rico. *Journal of Economic Entomology, 103*(4), 1031-1038. doi:10.1603/ec10040

Thomas, D. D., Donnelly, C. A., Wood, R. J., & Alphey, L. S. (2000). Insect population control using a dominant, repressible, lethal genetic system. *Science, 287*(5462), 2474.

van der Reijden, A., D. Monchamp, J., & Lewis, S. (1997). *The formation, transfer, and fate of spermatophores in Photinus fireflies (Coleoptera: Lampyridae)* (Vol. 75).

Vision, G. (2019). Gxcam hichrome-s camera. Retrieved from https://www.gtvision.co.uk/

Westbrook, J. K., Nagoshi, R. N., Meagher, R. L., Fleischer, S. J., & Jairam, S. (2016). Modeling seasonal migration of fall armyworm moths. *Int J Biometeorol, 60*(2), 255-267. doi:10.1007/s00484-015-1022-x

Chapter 7 – General discussion

Pest insects can inflict immense social, environmental and economic harm (Aukema et al., 2010; Berté et al., 2019; Bradshaw et al., 2016; Costa et al., 2019; D'Ambrosio et al., 2019; Marklewitz et al., 2019; Matetovici et al., 2019). Conventional approaches to insect control that rely on chemical insecticides can result in considerable damage to off-target organisms and the environment (Carson, 1962; Karmakar et al., 2016; Řezáč et al., 2019; Sánchez-Bayo et al., 2019; Sarkar et al., 2017) and irradiation of insects for the SIT has significant cost for insect fitness (Guerfali et al., 2011; Lutrat et al., 2019; Toledo et al., 2004). Hence, there is a continuing demand for novel, complementary, environmentally friendly, yet reliable modes of insect control.

In this thesis I investigated novel genetic tools for insect control by using two serious agricultural pests. In Chapter 2 I administered dsRNAs to OX3864A Mediterranean fruit flies (medflies) within their diet, to elicit environmental RNA interference (eRNAi) of a fluorescent dsRED2 marker gene (Fu et al., 2007). The results showed that medfly adults did not express eRNAi of dsRED2 when fed dsRNA vectored within transgenic HT115 *E. coli* bacteria or naked (unmodified) dsRNA. Larvae did not express eRNAi when fed naked dsRNA or dsRNA that had been vectored within liposomes. However, there was evidence that adult flies expressed eRNAi when fed with dsRNA vectored in liposomes.

HT115 bacteria aggregated in the anterior medfly midgut but did not elicit eRNAi. The anterior medfly midgut is lined by a type 2 peritrophic matrix (PM) (Lehane, 1997) that forms a barrier between the epithelia and the gut lumen. The PM contains a series of pores which sequester microbes and large food particles but permit the passage of small molecules, enzymes and digested food (Edwards et al., 2000). Therefore, it is possible that consumed HT115 cells are sequestered by the PM, while naked and liposomally vectored dsRNAs traverse the PM and contact the gut epithelia (Edwards et al., 2000). If HT115 bacteria and naked/liposomally vectored dsRNA disperse to discrete locations within the gut, it is plausible that eRNAi is neutralised by distinct mechanisms at those locations.

It is possible that the HT115 bacteria need to contact the epithelium in order to transmit eRNAi but that this interaction is obviated by the PM. This is consistent with the fact that a type 2 PM is continually replaced (Lehane, 1997), as bacteria sequestered at the interior lumen might be excreted as the PM is renewed. In addition, numerous *Coleoptera* express eRNAi when fed HT115 bacteria (Baum et al., 2014; Darrington et al., 2017) and several species belonging to that taxa do not produce a PM (Lehane, 1997). This supports the hypothesis that the PM interferes with the ability of insects to express eRNAi.

To investigate further whether the PM attenuates HT115 mediated eRNAi, one could engineer HT115 bacteria which target PM proteins for knockdown. Depletion of the PM via RNAi has been reported in several systems (Abraham et al., 2017; Kelkenberg et al., 2015; Rodgers et al., 2017) and the technique has the potential to be applied for control, as several plants directly target the PM to inhibit insect growth (Fescemyer et al., 2013; Konno et al., 2018). If some HT115 cells contacted the epithelia and silenced PM genes, then the PM integrity could be compromised, making the anterior midgut epithelium more accessible to a second dose of administered bacteria or dsRNA. However, this system would be dependent on some HT115 cells coming into contact with the midgut epithelium and it is not yet clear whether this can occur within the medfly gut.

The mechanisms that facilitate transport of eRNAi effectors across the gut epithelia are yet to be fully resolved (Darrington et al., 2017; Vélez et al., 2018). It is possible that HT115 cells, naked dsRNA and liposomally vectored dsRNA all come into contact with the midgut epithelium when fed to medflies, but that only liposomally vectored molecules are able to penetrate the cell wall to induce eRNAi. To investigate this further, it would be useful to fluorescently label dsRNAs and track their dispersal within the medfly (Shukla et al., 2016). Assuming that liposomal vectoring does facilitate the uptake of dsRNA across the adult midgut epithelium, then liposomally vectored molecules would be located within epithelial cells, while naked molecules would be located at the epithelial margin. Equally, HT115 cells could be located within the gut with Fluorescence In Situ Hybridisation (FISH) (Aharon et al., 2013) to elucidate if they are sequestered by the PM or if they come into contact with the epithelium.

In Chapter 3, I attempted to permeabilise medfly eggs with electroporation, to transfect them with dsRNA and induce eRNAi. I did not find any evidence that eggs had been made permeable to dsRNA by different electroporation devices, nor did I find that eggs were transfectable if the chorion had been removed. I was able to demonstrate, however, that medfly eggs can withstand electric treatment up to a maximum of 150V, if those eggs are laid by flies that are no more than 6 days old. Future investigations should subject eggs to a greater range of pulsing parameters and electroporation buffers, as those variables can influence transfection (Gosse et al., 2017) and were not investigated in my studies described here.

In Chapter 4, I sequenced and annotated the genome of a *Klebsiella* bacterium (Medkleb) that is a resident of the gut microbiome of the medfly. My goal was to test whether features of the Medkleb genome were diagnostic of a co-dependent life-history of Medkleb and its host. I had hypothesised that Medkleb was a strain of *K. oxytoca*, a species previously suggested to be facultative mutualist of the medfly (Behar et al., 2008). I was able to definitively characterise Medkleb as a strain of *K. oxytoca,* but my data provided no evidence that the Medkleb genome exhibited traits associated with facultative mutualism. However, I uncovered two Medkleb loci (GI1 and LCB18) that appear to be recently acquired and may contain genes that provide the medfly host with a fitness benefit. As many genes encoded by GI1 and LCB18 were characterised by Classic-RAST (Overbeek et al., 2014) as "hypothetical proteins", it is difficult as yet to predict with any certainty that they perform functions that benefit the medfly host. Further investigations could use pipelines other than Classic-RAST, such as PGAP (Tatusova et al., 2016) or DFAST (Tanizawa et al., 2017) to annotate GI1 and LCB18, as those programs may call genes that were missed during the original annotation and elucidate potentially mutualistic functions.

In Chapter 5, I dechorionated medfly eggs to prevent vertical transmission of *K. oxytoca.* However, there was no evidence that dechorionation produced *K. oxytoca* deficient (aKox) medflies, so this hypothesis could not be tested with the current set up.

aKox flies would nevertheless be useful, should it be possible to obtain them, to provide a platform to investigate the putative fitness benefits conferred to the medfly by *K.*

oxytoca. Behar et al. (2008) suggest that *K. oxytoca* is pectinolytic and diazotrophic and that the medfly benefits from these traits. As the *pehX* gene is thought to facilitate *K. oxytoca's* ability to degrade pectin (Kovtunovych et al., 2003), modified pehX$^-$ *K. oxytoca* would not be able to provide the medfly with this benefit. pehX$^-$ *K. oxytoca* could be produced using the λ red knockout system (Bachman et al., 2015; Huang et al., 2014), then subjected to a polygalacturonase enzyme assay (Chapter 4 – protocol 4.14) to quantify their ability to degrade pectin. Assuming that pehX$^-$ *K. oxytoca* had lost the ability to degrade pectin (or if it had been significantly diminished) then pehX$^-$ and pehX$^+$ bacteria could then be fed to *aKox* flies. Hypothetically, if medflies benefit from *K. oxytoca's* ability to degrade pectin, then *aKox* flies fed pehX$^+$ bacteria should exhibit improved fitness in comparison to flies fed pehX$^-$ bacteria.

K. oxytoca's capacity to fix nitrogen in the medfly gut could be tested with Stable Isotope Probing (SIP) (Neufeld et al., 2007). SIP requires that flies are reared in an airtight chamber, filled with air that has been supplemented with a heavy nitrogen isotope (15N). After a designated period, DNA and RNA is extracted from fly guts and spun in an ultracentrifuge, to separate the heavy (15N) and light (14N) fractions. The fractions are individually 16S sequenced (Di Salvo et al., 2019; Suenami et al., 2019) and OTUs enriched in the 15N fraction are characterised as diazotrophic. This analysis should elucidate not only if *K. oxytoca* is fixing nitrogen in the medfly gut but also if the gut hosts any organisms other than *K. oxytoca* that are diazotrophic.

In Chapter 6, I performed behavioural assays to assess whether the Fall Armyworm (FAW) could be a useful agent for self-limiting control. I found that the FAW is a good candidate species for self-limiting control, as it practices polyandry and last-male sperm precedence. My experiments were designed to elucidate the mating behaviours of female FAW when they disperse in the field. However, insects were subjected to enforced dispersal, being manually extracted from mating enclosures and moved to new locations. It is possible that females dispersing in the field exhibit a particular phenotype which affects their mating behaviour. For example, Jones et al. (2015) found that *Helicoverpa armigera* upregulate a suite of genes related to long distance flight when dispersing. If the FAW undergoes a similar physiological transition to *Helicoverpa*

armigera when dispersing, then it is unclear how precisely enforced dispersal would capture the behaviour of naturally dispersing females. To resolve this, dispersing females could be collected in the lab from a "phenotyping platform" (Jones et al., 2015), or wild, dispersing females could be caught in nets. However, both the Jones et al. (2015) phenotyping platform and wild capture would subject females to unnatural conditions that could affect their mating behaviour, thus negating the entire operation.

In summary, I have demonstrated that the medfly is refractory to eRNAi when fed naked dsRNA or dsRNA vectored within HT115 bacteria but that it may become sensitive when dsRNA is vectored within liposomes. I have shown that medfly eggs can withstand electrical insult up to a maximum of 150V and may be amenable to electroporation if treatment variables are optimised. I have characterised a bacterium that appears to have developed a co-dependent life-history with the medfly and elucidated certain traits expressed by that bacteria that may confer the medfly with a fitness benefit. Finally, I assessed the mating behaviours of the FAW and found that it is a suitable candidate for self-limiting control as it practices polyandry and last-male sperm precedence. I believe that these studies will serve as a basis for further investigations into the control of insect pests.

References

Abraham, N. M., Liu, L., Jutras, B. L., Yadav, A. K., Narasimhan, S., Gopalakrishnan, V., . . . Fikrig, E. (2017). Pathogen-mediated manipulation of arthropod microbiota to promote infection. *Proceedings of the National Academy of Sciences, 114*(5), E781. doi:10.1073/pnas.1613422114

Aharon, Y., Pasternak, Z., Ben Yosef, M., Behar, A., Lauzon, C., Yuval, B., & Jurkevitch, E. (2013). Phylogenetic, metabolic, and taxonomic diversities shape Mediterranean fruit fly microbiotas during ontogeny. *Appl Environ Microbiol, 79*(1), 303-313. doi:10.1128/aem.02761-12

Aukema, J. E., McCullough, D. G., Von Holle, B., Liebhold, A. M., Britton, K., & Frankel, S. J. (2010). Historical accumulation of nonindigenous forest pests in the continental United States. *BioScience, 60*(11), 886-897. doi:10.1525/bio.2010.60.11.5

Bachman, M. A., Breen, P., Deornellas, V., Mu, Q., Zhao, L., Wu, W., . . . Mobley, H. L. T. (2015). Genome-wide identification of *Klebsiella pneumoniae* genes during lung infection. *MBio, 6*(3), e00775-00715. doi:10.1128/mBio.00775-15

Baum, J. A., & Roberts, J. K. (2014). Chapter five - progress towards RNAi-mediated insect pest management. In S. D. Tarlochan & S. G. Sarjeet (Eds.), *Advances in insect physiology* (Vol. Volume 47, pp. 249-295): Academic Press.

Behar, A., Jurkevitch, E., & Yuval, B. (2008). Bringing back the fruit into fruit fly–bacteria interactions. *Molecular Ecology, 17*(5), 1375-1386. doi:10.1111/j.1365-294X.2008.03674.x

Berté, D., De Meeûs, T., Kaba, D., Séré, M., Djohan, V., Courtin, F., . . . Ravel, S. (2019). Population genetics of *Glossina palpalis palpalis* in sleeping sickness foci of Côte d'Ivoire before and after vector control. *Infection, Genetics and Evolution, 75*. doi:10.1016/j.meegid.2019.103963

Bradshaw, C. J. A., Leroy, B., Bellard, C., Roiz, D., Albert, C., Fournier, A., . . . Courchamp, F. (2016). Massive yet grossly underestimated global costs of invasive insects. *Nature Communications, 7*, 12986. doi:10.1038/ncomms12986

Carson, R. (1962). *Silent spring*: 40th anniversary edition. Boston : Houghton Mifflin, 2002. ©1962.

Costa, T. L., Sarmento, R. A., Araújo, T. A. D., Pereira, P. S., Silva, R. S., Lopes, M. C., & Picanço, M. C. (2019). Economic injury levels and sequential sampling plans for *Bemisia tabaci (Hemiptera: Aleyrodidae)* biotype b on open-field melon crops. *Crop Protection, 125*. doi:10.1016/j.cropro.2019.104887

D'Ambrosio, D. A., Peele, W., Hubers, A., & Huseth, A. S. (2019). Seasonal dispersal of *Lygus lineolaris (Hemiptera: Miridae)* from weedy hosts into differently fragmented cotton landscapes in North Carolina. *Crop Protection, 125*. doi:10.1016/j.cropro.2019.104898

Darrington, M., Dalmay, T., Morrison, N. I., & Chapman, T. (2017). Implementing the sterile insect technique with RNA interference – a review. *Entomologia experimentalis et applicata, 164*(3), 155-175. doi:10.1111/eea.12575

Di Salvo, M., Calcagnile, M., Talà, A., Tredici, S. M., Maffei, M. E., Schönrogge, K., . . . Alifano, P. (2019). The microbiome of the *Maculinea-myrmica* host-parasite interaction. *Scientific Reports, 9*(1), 8048. doi:10.1038/s41598-019-44514-7

Edwards, M. J., & Jacobs-Lorena, M. (2000). Permeability and disruption of the peritrophic matrix and caecal membrane from *Aedes aegypti* and *Anopheles gambiae* mosquito larvae. *J Insect Physiol, 46*(9), 1313-1320. doi:10.1016/s0022-1910(00)00053-6

Fescemyer, H. W., Sandoya, G. V., Gill, T. A., Ozkan, S., Marden, J. H., & Luthe, D. S. (2013). Maize toxin degrades peritrophic matrix proteins and stimulates compensatory transcriptome responses in fall armyworm midgut. *Insect Biochem Mol Biol, 43*(3), 280-291. doi:10.1016/j.ibmb.2012.12.008

Fu, G., Stainton, K., J Epton, M., Gong, P., Jin, L., Condon, G., . . . Alphey, L. (2007). Female-specific insect lethality engineered using alternative splicing. *25*, 353-357. doi:10.1038/nbt1283

Gosse, C., Zhao, X., Migeotte, I., Suárez-Boomgaard, D., Hue, I., Degrelle, S., . . . Mazari, E. (2017). The use of electroporation in developmental biology. In D. Miklavcic (Ed.), *Handbook of electroporation* (pp. 1-35). Cham: Springer International Publishing.

Guerfali, M. M., Parker, A., Fadhl, S., Hemdane, H., Raies, A., & Chevrier, C. (2011). Fitness and reproductive potential of irradiated mass-reared Mediterranean fruit fly males *Ceratitis capitata* (*Diptera: Tephritidae*): Lowering radiation doses. *Florida Entomologist, 94*(4), 1042-1050.

Huang, T.-W., Lam, I., Chang, H.-Y., Tsai, S.-F., Palsson, B. O., & Charusanti, P. (2014). Capsule deletion via a λ-red knockout system perturbs biofilm formation and fimbriae expression in *Klebsiella pneumoniae* mgh 78578. *BMC Research Notes, 7*, 13-13. doi:10.1186/1756-0500-7-13

Jones, C. M., Papanicolaou, A., Mironidis, G. K., Vontas, J., Yang, Y., Lim, K. S., . . . Chapman, J. W. (2015). Genomewide transcriptional signatures of migratory flight activity in a globally invasive insect pest. *Molecular Ecology, 24*(19), 4901-4911. doi:10.1111/mec.13362

Karmakar, S., Patra, K., Jana, S., Mandal, D. P., & Bhattacharjee, S. (2016). Exposure to environmentally relevant concentrations of malathion induces significant cellular, biochemical and histological alterations in *Labeo rohita*. *Pesticide Biochemistry and Physiology, 126*, 49-57. doi:10.1016/j.pestbp.2015.07.006

Kelkenberg, M., Odman-Naresh, J., Muthukrishnan, S., & Merzendorfer, H. (2015). Chitin is a necessary component to maintain the barrier function of the peritrophic matrix in the insect midgut. *Insect Biochemistry and Molecular Biology, 56*, 21-28. doi:10.1016/j.ibmb.2014.11.005

Konno, K., Shimura, S., Ueno, C., Arakawa, T., & Nakamura, M. (2018). Abnormal swelling of the peritrophic membrane in eri silkworm gut caused by mlx56 family defense proteins with chitin-binding and extensin domains. *Phytochemistry, 147*, 211-219. doi:10.1016/j.phytochem.2018.01.005

Kovtunovych, G., Lytvynenko, T., Negrutska, V., Lar, O., Brisse, S., & Kozyrovska, N. (2003). Identification of *Klebsiella oxytoca* using a specific PCR assay targeting the polygalacturonase pehX gene. *Res Microbiol, 154*(8), 587-592. doi:10.1016/s0923-2508(03)00148-7

Lehane, M. J. (1997). Peritrophic matrix structure and function. *Annu Rev Entomol, 42*, 525-550. doi:10.1146/annurev.ento.42.1.525

Lutrat, C., Giesbrecht, D., Marois, E., Whyard, S., Baldet, T., & Bouyer, J. (2019). Sex sorting for pest control: It's raining men! *Trends in Parasitology, 35*(8), 649-662. Doi:10.1016/j.pt.2019.06.001

Marklewitz, M., Dutari, L. C., Paraskevopoulou, S., Page, R. A., Loaiza, J. R., & Junglen, S. (2019). Diverse novel phleboviruses in sandflies from the Panama canal area, central Panama. *Journal of General Virology, 100*(6), 938-949. doi:10.1099/jgv.0.001260

Matetovici, I., De Vooght, L., & Van Den Abbeele, J. (2019). Innate immunity in the tsetse fly (*Glossina*), vector of African trypanosomes. *Dev Comp Immunol, 98*, 181-188. doi:10.1016/j.dci.2019.05.003

Neufeld, J. D., Vohra, J., Dumont, M. G., Lueders, T., Manefield, M., Friedrich, M. W., & Murrell, J. C. (2007). DNA stable-isotope probing. *Nature Protocols, 2*, 860. doi:10.1038/nprot.2007.109

Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J., Disz, T., . . . Stevens, R. (2014). The seed and the rapid annotation of microbial genomes using subsystems technology (RAST). *Nucleic Acids Research, 42*. doi:10.1093/nar/gkt1226

Řezáč, M., Řezáčová, V., & Heneberg, P. (2019). Contact application of neonicotinoids suppresses the predation rate in different densities of prey and induces paralysis of common farmland spiders. *Scientific Reports, 9*(1). doi:10.1038/s41598-019-42258-y

Rodgers, F. H., Gendrin, M., Wyer, C. A. S., & Christophides, G. K. (2017). Microbiota-induced peritrophic matrix regulates midgut homeostasis and prevents systemic infection of malaria vector mosquitoes. *PLoS Pathogens, 13*(5), e1006391. doi:10.1371/journal.ppat.1006391

Sánchez-Bayo, F., & Wyckhuys, K. A. G. (2019). Worldwide decline of the entomofauna: A review of its drivers. *Biological Conservation, 232*, 8-27. doi:10.1016/j.biocon.2019.01.020

Sarkar, S., & Roy, S. (2017). Monitoring the effects of a lepidopteran insecticide, flubendiamide, on the biology of a non-target Dipteran insect, *Drosophila melanogaster*. *Environmental Monitoring and Assessment, 189*(11). doi:10.1007/s10661-017-6287-6

Shukla, J. N., Kalsi, M., Sethi, A., Narva, K. E., Fishilevich, E., Singh, S., . . . Palli, S. R. (2016). Reduced stability and intracellular transport of dsrna contribute to poor RNAi response in Lepidopteran insects. *RNA Biology, 13*(7), 656-669. doi:10.1080/15476286.2016.1191728

Suenami, S., Konishi Nobu, M., & Miyazaki, R. (2019). Community analysis of gut microbiota in hornets, the largest eusocial wasps, *Vespa mandarinia* and *v. simillima*. *Scientific Reports, 9*(1). doi:10.1038/s41598-019-46388-1

Tanizawa, Y., Fujisawa, T., & Nakamura, Y. (2017). Dfast: A flexible prokaryotic genome annotation pipeline for faster genome publication. *Bioinformatics, 34*(6), 1037-1039. doi:10.1093/bioinformatics/btx713

Tatusova, T., DiCuccio, M., Badretdin, A., Chetvernin, V., Nawrocki, E. P., Zaslavsky, L., . . . Ostell, J. (2016). NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Res, 44*(14), 6614-6624. doi:10.1093/nar/gkw569

Toledo, J., Rull, J., Oropeza, A., Hernandez, E., & Liedo, P. (2004). Irradiation of *Anastrepha obliqua (Diptera: Tephritidae)* revisited: Optimizing sterility induction. *J Econ Entomol, 97*(2), 383-389.

Vélez, A. M., & Fishilevich, E. (2018). The mysteries of insect RNAi: A focus on dsRNA uptake and transport. *Pesticide Biochemistry and Physiology, 151*, 25-31. doi:10.1016/j.pestbp.2018.08.005

Appendix – Darrington et al. (2017) literature review

I have attached a review (Darrington et al., (2017) that I co-authored and cited frequently in Chapters 2 and 3. The review evaluates RNA interference as a mode of insect control, focusing on dsRNA uptake and methods of dsRNA delivery. Although I covered these topics in my thesis, I was able to provide more detail in the review and decided to attach it, as it may provide some beneficial context to the reader.

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SPECIAL ISSUE – STERILE INSECT TECHNIQUE

Implementing the sterile insect technique with RNA interference – a review

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Abstract

We review RNA interference (RNAi) of insect pests and its potential for implementing sterile insect technique (SIT)-related control. The molecular mechanisms that support RNAi in pest species are reviewed in detail, drawing on literature from a range of species including *Drosophila melanogaster* Meigen and *Homo sapiens* L. The underlying genes that enable RNAi are generally conserved across taxa, although variance exists in both their form and function. RNAi represents a plausible, non-GM system for targeting populations of insects for control purposes, if RNAi effector molecules can be delivered environmentally (eRNAi). We consider studies of eRNAi from across several insect orders and review to what extent taxonomy, genetics, and differing methods of double-stranded (ds) RNA synthesis and delivery can influence the efficiency of gene knockdown. Several factors, including the secondary structure of the target mRNA and the specific nucleotide sequence of dsRNA effector molecules, can affect the potency of eRNAi. However, taxonomic relationships between insects cannot be used to reliably forecast the efficiency of an eRNAi response. The mechanisms by which insects acquire dsRNA from their environment require further research, but the evidence to date suggests that endocytosis and transport channels both play key roles. Delivery of RNA molecules packaged in intermediary carriers such as bacteria or nanoparticles may facilitate their entry into and through the gut, and enable the evasion of host defence systems, such as toxic pH, that would otherwise attenuate the potential for RNAi.

RNAi and the sterile insect technique (SIT)

Established methods of insect control are under continual review and development in order to keep track of new knowledge, changing legislation, regulatory concerns, and the maintenance of efficacy (e.g., in the face of increased resistance to pesticides) (Gross, 2013; Tabashnik et al., 2014). In this context, the development of new methods for insect control is of key importance and there has been intense interest in the utility of gene silencing methods induced by RNA interference (RNAi). RNAi can induce mortality (Yang & Han, 2014; Cao et al., 2015; Abd El Halim et al., 2016; Christiaens et al., 2016; Hu et al., 2016; Malik et al., 2016), create beneficial phenotypes for insect control (Salvemini et al., 2009; Shukla & Palli, 2012; Peng et al., 2015; Yu et al., 2016), and prevent pesticide resistance in insect pests (Figueira-Mansur et al., 2013; Guo et al., 2015; Wei et al., 2015; Bona et al., 2016; Sandoval-Mojica & Scharf, 2016). Therefore, the potential for RNAi as a basis for future pest management strategies holds great promise (Huvenne & Smagghe, 2010; Gu & Knipple, 2013; Scott et al., 2013; Baum & Roberts, 2014; Kim et al., 2015). The purpose of this review is to summarize the mechanisms by which gene silencing is achieved, describe the ways in which it is currently being used, and to explore the many factors that affect the efficacy of RNAi in this context.

RNAi can be used to achieve knock-down of the level of gene expression in specific target genes. This is done via the introduction, by various means, of double-stranded RNA (dsRNA) into the cells of the target species (Fire et al., 1998). The evidence suggests that RNAi is facilitated by the canonical small interfering RNA (siRNA) pathway, which results in mRNA degradation (Figure 1). In our review of the mechanisms of RNAi in pest insects we draw

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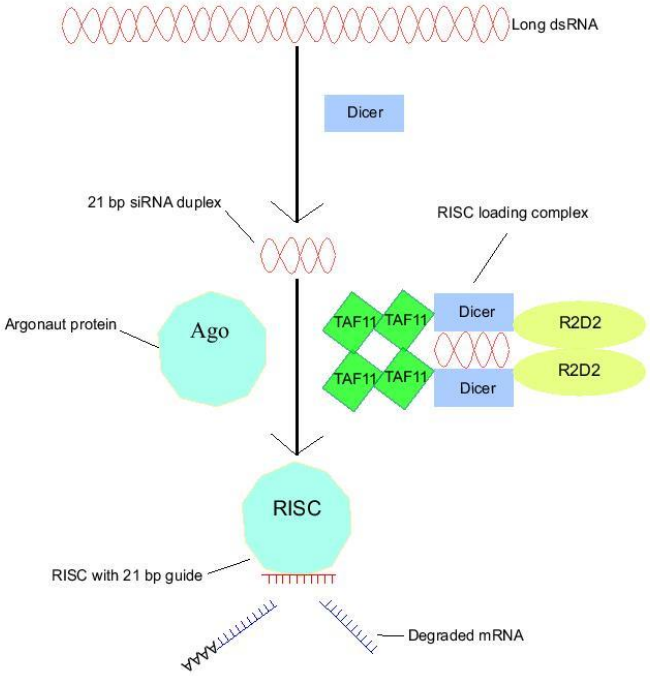

Figure 1 The canonical siRNA pathway. Cytoplasmic long double-stranded RNAs are processed into 21-bp duplex siRNAs by Dicer endonucleases. Dicer then complexes with various molecules to form a RISC loading complex (RLC) (the proposed RLC variant found in *Drosophila melanogaster* is shown here; Liang et al., 2015). The RLC introduces siRNA to an Argonaute protein, which degrades a single 'passenger' strand of the duplex, whilst binding its cognate partner to form an RNA induced silencing complex (RISC). The RISC then utilizes the nucleotide sequence of the bound 'guide' strand to scan cellular mRNAs, which it targets for knockdown via degradation.

strongly from the well-described canonical siRNA pathway in *Drosophila melanogaster* Meigen and *Homo sapiens* L.

It has been increasingly realized that a classic method of insect control, the sterile insect technique (SIT) (Knipling, 1955) could, in principle, be implemented through RNAi (Whyard et al., 2015). The SIT relies upon the production of large numbers of sterile insects for release (usually males) that subsequently mate with wild individuals, resulting in sterile matings and a reduction in the pest population size (Knipling, 1998; Krafsur, 1998). The key to SIT is the effective production of large numbers of sterile individuals. This crucial step is also a potential weakness of the approach. For example, the induction of sterility through irradiation results in well-documented costs to insect performance, and hence control potential (Hooper, 1972; Toledo et al., 2004; Guerfali et al., 2011). Newer developments based on SIT that avoid irradiation, e.g., genetically engineered 'self-limiting' insects (Thomas et al., 2000), can be highly effective (Harris et al., 2011; Carvalho et al., 2015; Gorman et al., 2016) but rely upon the release of genetically engineered insects, which may not be possible in all countries.

The principles by which RNAi might offer an alternative route for the induction of sterility, as well as other potentially useful manipulations for insect control, were recently investigated in a study using *Aedes aegypti* (L.) (Whyard et al., 2015). The scenario envisaged by Whyard et al. (2015) requires knockdown of at least two genes in the target insects. First, females would be targeted through silencing of a gene in the sexual differentiation cascade to turn them into pseudomales, i.e., genetic females which are phenotypically male (Pane et al., 2002; Salvemini et al., 2009; Shukla & Palli, 2012; Liu et al., 2015). Next, genes that could induce male (and pseudomale) sterility would be targeted in order to produce a 100% sterile male release cohort (Whyard et al., 2015). However, two equally important conditions must be met before this technique can be applied in the field, as described below.

The primary condition of RNAi-based SIT is that the sex reversal target must reliably produce a male-only cohort. There are clear benefits of releasing only one sex in SIT programmes, for example it can avoid both assortative mating between released insects and any pest-related damage caused by females. The second condition is to ensure that silencing of neither the sex reversal nor the sterility target unduly reduces insect performance. Evidence suggests that these conditions can be met, although further supporting research is required.

Through RNAi of transformer-2, Salvemini et al. (2009) were able to produce a *Ceratitis capitata*

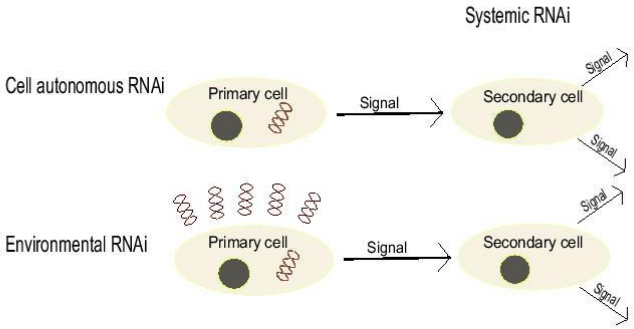

Figure 2 Categories of RNAi response. Cell autonomous RNAi is gene silencing in response to cytoplasmic dsRNA of viral or experimental origin. Non-cell autonomous RNAi occurs in response to an extracellular signal, and is subcategorized by the origin of that signal as either environmental (eRNAi), or systematic RNAi. eRNAi occurs when a cell takes up environmental dsRNA molecules and elicits a gene silencing response. Systemic RNAi is initiated in a secondary cell when a silencing signal is received from a primary cell. Systemic RNAi can be a by-product of either non-cell autonomous RNAi or eRNAi in a primary cell, and if the secondary cell further propagates the signal, this can induce global gene silencing.

(Wiedemann) cohort which was 95.6% phenotypically male. Karyotypic analysis of phenotypically male flies (n = 20) demonstrated that they were 55% genetically female. Most importantly, pseudomales were observed completing male-specific courtship rituals, which should allow them to attract and copulate with females (Briceño & Eberhard, 2003). Gabrieli et al. (2016) report that RNAi of innexin-5 in *C. capitata* produced spermless, sterile males. Spermless males remained sexually competitive with wild-type rivals and were able to induce similar post-mating responses. It is possible that simultaneous RNAi of transformer-2 and innexin-5 (or conserved homologous genes in diverse species) could produce a male-only, sterile cohort that could be used for SIT. However, it is important to note that simultaneous gene silencing is unpredictable (Table 2) and that Gabrieli et al. (2016) and Salvemini et al. (2009) microinjected insect eggs with dsRNA, a technique that is incompatible with large-scale SIT.

Microinjection of dsRNA has been demonstrated to induce RNAi in several insects (Paim et al., 2013; Peng et al., 2015; Xue et al., 2015; Yu et al., 2016). However, SIT programmes may require the production and release of up to a billion insects per week (Alphey et al., 2010) and injection techniques cannot be used to treat insects in such numbers. Therefore, RNAi may provide a useful tool for implementing SIT if gene silencing can be induced via environmental dsRNA (eRNAi) (Whangbo & Hunter, 2008).

Cell autonomous RNAi defines gene silencing in response to intracellular dsRNA of experimental or viral origin. Non-cell autonomous RNAi defines gene silencing in response to an extracellular signal, and is further divided into systemic RNAi or eRNAi based on the nature of that signal. eRNAi describes gene silencing in response to proximal dsRNA molecules, whereas systemic describes RNAi gene silencing in response to a signal received from a proximal cell. Therefore, both non-cell autonomous RNAi and eRNAi occur in a primary cell in direct response to dsRNA, whereas systemic RNAi is initiated in a secondary cell in response to an as yet undefined signal received from a primary cell (Figure 2). eRNAi can be achieved via the introduction of dsRNAs via food (Asokan et al., 2014; Coleman et al., 2015; Li et al., 2015b; Sandoval-Mojica & Scharf, 2016) or through topical delivery (Toprak et al., 2013; Whyard et al., 2015). For reasons that are as yet not entirely clear, the capacity of insects to express systemic RNAi and eRNAi varies both within and between species (Baum & Roberts, 2014; Li et al., 2015a; Shukla et al., 2016; Sugahara et al., 2017).

Many factors affect the efficiency of gene silencing induced by eRNAi. Some are intrinsic properties of the insects themselves (genetic differences, feeding habits, etc.), but others correspond to the nature of dsRNA effector molecules and their state at the point of encounter/entry to the host. In this review, we first describe the mechanisms of RNAi in detail, highlight examples of its use in different pest species, and in the concluding section consider the factors affecting eRNAi, in an attempt to discover whether there are emergent properties that might be useful in the planning of SIT strategies.

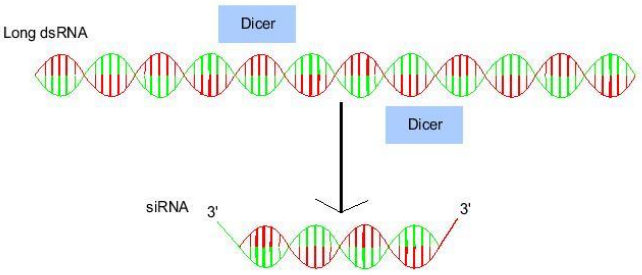

Figure 3 siRNA biogenesis. 21-nt siRNA duplexes are cleaved from long, cytoplasmic dsRNA molecules by Dicer endonucleases. Two cuts are carried out by discrete Dicer RNAase III motifs, leaving short 3′ overhangs on each strand (Tomari & Zamore, 2005).

Mechanism of RNAi

RNAi is facilitated through the canonical siRNA pathway, culminating in the degradation of target mRNA

Small interfering RNAs (siRNAs) are short (ca. 21 nt) double-stranded RNA molecules that are cleaved from long, cytoplasmic dsRNA transcripts (Figure 3). siRNAs belong to a large family of small non-coding RNAs (ncRNAs) that facilitate different modes of gene silencing. ncRNA species include small interfering RNAs (siRNA), microRNAs (miRNA), PIWI-interacting RNAs (piRNA), *trans*-acting RNAs (tasiRNAs), repeat-associated RNAs (rasiRNAs), and small-scan RNAs (scnRNAs) (Kuramochi-Miyagawa et al., 2001; Kim et al., 2009, 2015). While the origin and function of each ncRNA species is distinct (Bartel, 2004; Gasciolli et al., 2005; Babiarz et al., 2008; Kim et al., 2009) their actions are facilitated by homologous molecular mechanisms.

ncRNAs can only initiate gene silencing when bound to an Argonaute protein as part of an RNA-induced silencing complex (RISC). When assembled in a RISC, exo-siRNAs target viral mRNAs for knockdown as part of an immune response (Lan et al., 2016a,b). In contrast, endo-siRNAs target endogenously transcribed mRNAs in order to achieve gene regulation (Babiarz et al., 2008; Okamura et al., 2008). exo-siRNAs function through the canonical siRNA pathway, inducing cleavage of target mRNAs (Elbashir et al., 2001a; Song et al., 2004), while endo-siRNAs inhibit the translation of target molecules (Hannon, 2002).

The canonical siRNA pathway requires the nucleotide sequences of siRNA molecules and their intended mRNA targets to exhibit almost perfect complementarity (Joseph & Osman, 2012). Imperfect homology may result in a mode of gene silencing other than mRNA cleavage (such as translational repression; Hu et al., 2010), which is associated with other ncRNA pathways. Perfect sequence homology is achievable in RNAi, as the target mRNA can usually be used to design effector dsRNA molecules with perfect matching. Therefore, the predominant mechanism of gene silencing induced by RNAi is mRNA degradation.

siRNA biogenesis: Dicer

siRNAs are ubiquitous throughout the Eukaryota (Vaucheret, 2006; Fire, 2007), suggesting that defense to viral infection via the processing of long dsRNA is well conserved. Key effector molecules involved in siRNA biogenesis do vary in both form and function and have been demonstrated to be targets of viral suppression in honeybees (De Smet et al., 2017). Both endo- and exo-siRNAs are cytoplasmically processed by Dicer, a member of the RNAase III endonuclease family (Hammond et al., 2000; Bernstein et al., 2001). RNAase III enzymes are defined as having two RNAase III endonuclease domains and a helicase domain (Sontheimer, 2005). As well as having three conserved RNAase III motifs, the Dicers also contain a conserved RNA-binding PAZ domain (Yan et al., 2003) and a DUF283 domain with unknown function (Dlakić, 2006).

Dicer's PAZ domain binds the 3′ overhangs of long cytoplasmic dsRNA molecules. The captured dsRNA is then brought into contact with Dicer's two RNAase III domains, each of which cleaves (or dices) a particular strand of the molecule. Dicing produces 21-nt siRNA duplexes with short (2 nt) overhangs at the 3′ end on each strand (Elbashir et al., 2001a).

Many organisms (including humans) express a single isoform of Dicer (Zhang et al., 2002). *Drosophila melanogaster* expresses two Dicer variants (Dicer 1 and Dicer 2) that are reported to function in discrete gene silencing pathways (Lee et al., 2004; Tomari et al., 2007), though many details are as yet unclear. Dicer 2 binds and degrades long dsRNA destined to become siRNA, whereas Dicer 1 binds pre-miRNA hairpin loops of ca. 60 nt and cleaves them, creating functional miRNAs duplexes. Dicer 2 is also instrumental for processing of siRNA in the small brown planthopper, *Nilaparvata lugens* (Stål) (Lan et al., 2016a) and the zigzag leafhopper, *Recilia dorsalis* Motschulsky (Lan et al., 2016b).

The RNA-induced silencing complex (RISC)

RISCs are the functional components of all ncRNA-mediated gene silencing pathways (Maniataki & Mourelatos, 2005; Rand et al., 2005; Hartig et al., 2007). RISCs can be defined as an Argonaute protein bound to a single strand of ncRNA. As there are several ncRNA and Argonaute species, the term RISC specifies a diverse group of ribonucleoprotein complexes.

Until complexed in a RISC, siRNAs have no effects upon gene expression. Formation of a RISC requires free siRNA to be captured by a RISC-loading complex (RLC) and introduced to an Argonaute (Tomari et al., 2004; MacRae et al., 2008). After cleaving siRNA from long dsRNA, Dicer complexes with either transactivation response RNA-binding protein (TRBP), or protein activator of PKR (PACT) to form the two human RLC variants (Haase et al., 2005; Lee et al., 2006, 2013; Lau et al., 2009). Variations in the 5′ terminal of TRBP and PACT may bias the binding affinities of human RLC variants toward either siRNA or miRNA, respectively (Lee et al., 2013). Dicer's participation in RISC loading is not required in all mammalian systems, as ΔDicer murine embryonic stem cells remain RLC competent (Murchison et al., 2005).

In *Drosophila* the canonical siRNA pathway utilizes an RLC formed by Dicer 2, R2D2 and TBP-associated factor 11 (TAF11) (Liang et al., 2015). TAF11 is not necessary for RISC loading, as Dicer/R2D2 heterodimers form a competent RLC. Dicer and R2D2 bind opposite poles of siRNA before loading it into a RISC (Tomari et al., 2004). ΔR2D2 and ΔDicer flies are therefore incapable of siRNA-mediated gene silencing (Liu et al., 2006). Liang et al. (2015) suggest that an optimum RLC is formed when R2D2 and Dicer 2 form a tetrameric complex stabilized by TAF11. Tetrameric RLCs that include TAF11 display a 10-fold increase in siRNA binding over Dicer/R2D2 heterodimers.

Dicer's role in siRNA biogenesis and RISC loading elicits potent gene silencing, in a manner which cannot yet be imitated precisely with artificially synthesized siRNA molecules. siRNAs that are enzymatically Diced from 240-bp dsRNA constructs produce more effective gene silencing than artificially synthesized siRNA duplexes of equivalent sequence (Bolognesi et al., 2012). Whyard et al. (2009) also found that synthetic siRNA induced less potent RNAi than did enzymatically diced molecules.

siRNAs associate with Argonaute proteins from the Ago Clade

Based on analysis of nucleotide sequence homology, the Argonaute proteins form two clades. The Ago clade is the largest and took its name from *Arabidopsis Ago1* mutants (Carmell et al., 2002). The Agos are bilobed proteins with a central PIWI endonuclease domain flanked by RNA-binding PAZ (a feature shared with the Dicer enzymes), and MID domains at the N- and C-terminals, respectively (Wang et al., 2008, 2009). A smaller Argonaute subclade (the PIWIs) was named for the *Drosophila* P-element-induced Wimpy testis protein (Aravin et al., 2006). Agos are expressed globally and associate with siRNA and miRNA to form RISCs. Until recently PIWIs (which associate with piRNAs) were thought to be restricted to the germline (Grishok et al., 2001; Morel et al., 2002; Tomari et al., 2007). However, evidence of their additional role in somatic gene silencing is now emerging (Morazzani et al., 2012; Schnettler et al., 2013).

Many organisms express a range of Ago proteins that associate with discrete ncRNA species. In *D. melanogaster*, for example, siRNA complexes with Ago2, miRNA associates with Ago1 (Tomari et al., 2007), and piRNA with PIWI proteins (Vagin et al., 2006; Malone et al., 2009). Although it is generally accepted that Ago2 is required for RNAi in the insects, silencing of Ago1 in *Leptinotarsa decemlineata* Say cells does inhibit gene silencing (Yoon et al., 2016). Humans, on the other hand, express four Ago proteins, all of which bind siRNA. However, only when complexed with Ago2 is siRNA capable of forming a functional RISC (Liu et al., 2004).

Guide strand genesis

Ago2 degrades a single 'passenger' strand of each siRNA duplex presented by an RLC (Matranga et al., 2005; Rand et al., 2005; Leuschner et al., 2006; Wang et al., 2009). In humans and *D. melanogaster* Ago2 initiates the release of the passenger strand by cleavage, creating two single-stranded molecules of 9 and 12 nt (Matranga et al., 2005; Noland & Doudna, 2013). In humans the fragmented passenger strand is then degraded in the cytoplasm by C3PO (Ye et al., 2011). It also appears that C3PO aids passenger strand digestion in *D. melanogaster* and may also enhance gene silencing through RISC activation (Liu et al., 2009). Once the passenger strand has been released, the remaining 'guide' strand complexes with Ago2 to form the RISC.

The thermodynamic properties of duplexed siRNA molecules appear to influence which strand is destined to be integrated into a RISC. The two strands of the siRNA duplex have to be separated from each other by helicases, which try to unwind the duplex from both ends. The ends can have different stability depending on the GC content on the last 3–5 base pairs and the strand that has the 5′ end at the less strongly paired end has a higher chance to become the guide strand (Khvorova et al., 2003; Schwarz et al., 2003; Tomari et al., 2004). Both human RLC variants are capable of sensing the thermodynamics of duplexed siRNA and reorientating the molecule prior to RISC loading (Noland et al., 2011; Noland & Doudna,

2013), which may facilitate strand selection by Argonaute. The Dicer/R2D2 RLC seen in *Drosophila* also configures siRNA according the thermodynamics of the molecule (Liu et al., 2006).

RISCs utilize the guide strand to identify potential mRNA targets

Ago2's N-terminal PAZ domain binds the 3′ end of the guide strand, whereas the C-terminal MID domain binds the 5′ phosphate (Wang et al., 2008). The guide strand is orientated with its phosphate backbone toward Ago2's PIWI domain and the free nucleotides facing outwards. The RISC then utilizes the guide strand to scan cellular mRNA through Watson and Crick base pairing. Cognate mRNA, which base pairs with the guide, is targeted for knockdown (Filipowicz, 2005; Noland & Doudna, 2013). Each RISC is therefore capable of highly selective mRNA targeting based upon the nucleotide sequence of its intrinsic guide.

Cleavage of targeted mRNA

In the final stage of RNAi, cleavage of targeted mRNA occurs in the region bound by the center of the guide strand between residues 10 and 11 (Elbashir et al., 2001b; Haley & Zamore, 2004). The resulting 5′ and 3′ mRNA fragments are then degraded by discrete cytoplasmic enzymes (Orban & Izaurralde, 2005). Within Ago2's PIWI domain is an aspartate-aspartate-glutamate (DDE) motif which is conserved in RNAase-H related enzymes (Song et al., 2004). This motif is critical for mRNA degradation, as mutation of these residues results in loss of slicing ability (Liu et al., 2004).

dsRNA as an experimental gene silencing device

In 1990, Napoli et al. (1990) developed petunias that expressed a hybrid chalcone synthase transgene (CHS). The authors predicted that expression of the transgene would supplement naturally occurring CHS and produce flowers with deep violet colouring (Napoli et al., 1990). Unexpectedly, 42% of the flowers exhibited an unpigmented, white phenotype. This led the authors to hypothesize that the transgene must somehow be inhibiting the expression of its naturally occurring orthologue.

Research into RNAi began following the work of Napoli et al. (1990). The first report of RNA being used to deliberately silence genes in an animal model came in 1995 when Guo & Kemphues (1995) injected *C. elegans* embryos with ssRNA designed to base pair with, and sequester, Par-1 mRNA. Guo & Kemphues (1995) were successful in silencing Par-1, but they were incorrect in their assumption that the underlying mechanism was triggered by ssRNA.

Using improved RNA preparation techniques, Fire et al. (1998) were able to show that Guo & Kemphues (1995) had contaminated their single-stranded antisense RNAs with sense transcripts. Guo & Kemphues's (1995) ssRNAs had therefore base-paired to form duplexes and entered the canonical siRNA pathway. Fire et al. (1998) were able to demonstrate that *C. elegans* when bathed in dsRNA silenced genes up to 100× more efficiently than when bathed in ssRNA. This experiment identified dsRNA as the critical effector molecules in previously described gene silencing experiments and was the first time dsRNA had purposefully been used to implement gene silencing. This finding was the starting point of all subsequent studies of RNAi.

Implementation of eRNAi in pest species

As outlined briefly above, microinjection of dsRNA would not be a viable method for treating the large numbers of insects required for SIT. However, it is thought that exposure to eRNAi might provide a suitable alternative. The susceptibility of target species to eRNAi is critically important and has been reviewed in depth by Baum & Roberts (2014). However, insects that are naturally recalcitrant to eRNAi are not necessarily outside consideration for this type of gene silencing as, although yet to be demonstrated in an insect model, methods such as electroporation can also be used to deliver dsRNA – e.g., as described in tick eggs (Karim et al., 2010; Ruiz et al., 2015), nymphs, and larvae (Lu et al., 2015). Various options are outlined below.

eRNAi delivery methods: larvae

To interrupt the sexual differentiation cascade in a manner that could be useful for SIT, RNAi must be implemented at the relevant critical developmental stages in eggs, embryos (Salvemini et al., 2009; Shukla & Palli, 2012; Liu et al., 2015), or early larvae (Whyard et al., 2015). Larvae are simple to target with eRNAi by ingestion as they eat steadily, volubly, and are generally less mobile than adults (hence can naturally take up dsRNA that is concentrated within local food sources). For aquatic larvae, dissolving dsRNA in solution and bathing the larvae within it, is the most common method of effecting gene silencing via eRNAi (Figueira-Mansur et al., 2013; Singh et al., 2013; Whyard et al., 2015; Bona et al., 2016). dsRNA can be delivered to non-aquatic larvae: (1) topically via droplet feeding (Toprak et al., 2013), (2) by inducing the larvae to feed upon dsRNA-expressing transgenic plants (Xiong et al., 2013; Mamta et al., 2015; Tian et al., 2015; Hu et al., 2016), (3) by feeding larvae dsRNA-expressing transgenic bacteria (Zhu et al., 2011; Yang & Han, 2014; Li et al., 2015c), and (4) by feeding larvae naked dsRNA

overlaid onto an artificial diet (Asokan et al., 2014; Yang & Han, 2014; Hu et al., 2016). Non-aquatic larvae, or those that develop in relatively anoxic conditions, can also be bathed in dsRNA solution, but the timing of exposure is critical to avoid drowning (Whyard et al., 2009). Choi et al. (2012) also report delivery of dsRNA via parental feeding in a study in which nurse ant workers were fed with dsRNA that was then passed to larvae via regurgitation.

eRNAi delivery methods: adults

Genes that can induce sterility when knocked down can be targeted in adult insects for use with SIT. eRNAi has been demonstrated to successfully achieve gene silencing in adults following ingestion of: (1) dsRNA-expressing transgenic plants (Coleman et al., 2015; Tzin et al., 2015; Malik et al., 2016), (2) dsRNA-expressing transgenic bacteria (Li et al., 2011; Taracena et al., 2015; Whitten et al., 2015), (3) dsRNA dissolved in solution (Coy et al., 2012; Ratzka et al., 2013; Shim et al., 2015), and (4) naked dsRNA overlaid on diet (Yi et al., 2014; Zheng et al., 2015). In addition, topical application to adults of dsRNA (Pridgeon et al., 2008; Killiny et al., 2014; Amiri et al., 2015) and infection with transgenic fungi (Chen et al., 2015) are reported. All methods have the potential for use in SIT development. However, the use of transgenic plants may be limited by the feeding habits of target pests, and fungi also need to be tested for their potential to infect unintended secondary targets.

An important consideration for eRNAi silencing for insect control is the feasibility of producing and delivering the required amount of dsRNA. Both in vitro and in vivo methods for producing dsRNA for insect control have been tested, as described below.

In vitro dsRNA synthesis

The T7 RNA polymerase (from the T7 bacteriophage) is a highly selective enzyme that enables rapid synthesis of RNA sequences (Tabor, 2001). For in vitro production of dsRNA, linear DNA sequences that code for both sense and antisense RNA transcripts flanked by the 20-nt T7 promoter are transcribed by incubation with T7 polymerase (Singh et al., 2013; Liu et al., 2015; Shim et al., 2015; Whyard et al., 2015). Cognate ssRNA transcripts then base pair to form dsRNA that can be used for eRNAi experiments.

In vivo dsRNA synthesis by bacteria

dsRNA can be synthesized in vivo by bacteria themselves using transgenic HT115 *Escherichia coli* (Migula) Castellani & Chalmers (Kamath et al., 2001). The HT115 genome has been modified to be RNase deficient and to contain a T7 polymerase under the control of lactose regulatory elements. Generally, target sequences flanked by two T7 promoters at each side are introduced to L4440 plasmid vectors by ligation. The plasmid is then transformed into HT115 bacteria and target DNA sequences are transcribed by T7 polymerases induced by the allolactose mimic IPTG (Whyard et al., 2009, 2015; Zhu et al., 2011; Yang & Han, 2014; Taracena et al., 2015). A limitation of this method is that, once introduced to target insects, the effect is transient as the HT115 bacteria fail to colonize the gut and become established in the insect gut microbiome.

Modified symbiotic bacteria have recently been utilized as an alternative to HT115 *E. coli* (Whitten et al., 2015). In this study, microbes from the microbiome of target species were reprogrammed to have similar properties to HT115 (in that they were RNAse-deficient), but RNA sequences were constitutively active rather than being inducible. These symbiotic bacteria were able to repopulate the gut of target insects and induce a long term silencing effect. These results suggest that there is great potential to genetically engineer naturally occurring bacteria in the gut microbiomes of pest species for control purposes.

In vivo dsRNA synthesis by plants

The nuclear genome of plants can be modified using *Agrobacterium tumefaciens* Smith & Townsend (De Block et al., 1984; Horsch et al., 1984) to express non-endogenous dsRNA. dsRNA constructs can be expressed as either a single sequence which forms a long hairpin (hpRNA) (Xiong et al., 2013; Guo et al., 2014; Mamta et al., 2015), or two separate complementary transcripts which base pair in the cytoplasm (Kumar et al., 2012). However, transfer of target sequences into the genome of plant hosts is unpredictable and dsRNA abundance in similarly prepared transgenic plants can vary by up to 900% (Tian et al., 2015).

The genome of plant chloroplasts can also be programmed to synthesize non-endogenous dsRNA (Jin et al., 2015; Zhang et al., 2015a). Due to the prodigious metabolic output of these organelles they are capable of rapid production of large amounts of effector dsRNA molecules. As for the gut microbiota, there is potential for engineering chloroplasts in this manner for application to insects through eRNAi. In the next sections we consider the design features of dsRNAs that may render them useful for control.

Optimum dsRNA construct design: length and GC content

There is a minimum length threshold (MLT) at which dsRNA can induce eRNAi. The MLT has been demonstrated to be ca. 60 bp in several insects (Bolognesi et al., 2012; Miller et al., 2012; Ivashuta et al., 2015), although Miyata et al. (2014) reported an MLT of ca. 100 bp. The

MLT for eRNAi is defined by the minimum length of dsRNA that can be absorbed by the intestine. However, distal tissues may be capable of absorbing shorter transcripts. Ivashuta et al. (2015) report an MLT of 60 bp in *Diabrotica virgifera virgifera* LeConte, and the uptake of a 21-bp siRNAs by the fat body of this insect.

Once the MLT has been met, dsRNA construct length is not an accurate predictor of RNAi potency, as constructs of similar length can elicit diverse silencing effects (Toprak et al., 2013; Asokan et al., 2014). Most RNAi research in insects is carried out using dsRNA constructs of between 200–500 bp (Table 1), although successful silencing has been achieved using constructs of up to 1 800 bp (Baum et al., 2007).

The GC content of dsRNA negatively correlates with eRNAi efficiency (Reynolds et al., 2004; Chan et al., 2009). GC bonds are more stable than AU bonds and less prone to unwinding by Dicer's helicase domain.

Optimum dsRNA construct design: target sequence

Specific nucleotide sequences within targeted mRNAs are intrinsically susceptible to RISC cleavage. Bolognesi et al. (2012) report that a single homologous 21-bp sequence can silence mRNA as efficiently as a 60-bp construct of 100% homology. RNAi potency is therefore governed partly by the quality of 21-nt sequences contained within dsRNA constructs.

Discrete dsRNA constructs targeting different regions of the same mRNA molecule have been demonstrated to elicit variable silencing effects (Xiong et al., 2013; Tian et al., 2015). Neither the pole nor the mid-region of mRNA appears to be intrinsically prone to cleavage, as susceptibility has been reported at various regions of targeted molecules (Mao & Zeng, 2012; Xiong et al., 2013). Asokan et al. (2014) targeted five unrelated mRNAs in *Helicoverpa armigera* (Hübner) and observed variation in silencing efficiency from 21 to 95%.

Sfold software (Ding et al., 2005) is reported to predict the regional susceptibility of mRNA to RISC cleavage prior to construct design, though data from Xiong et al. (2013) showed predictive success to be variable. Regional susceptibility to cleavage is likely to be defined largely by the secondary structure of the target molecule. RNAi potency is inversely proportional to the amount of hydrogen bonds formed between target and non-target sequences (Luo & Chang, 2004). This implies that targets that are tightly bonded to local sequences are unavailable to the RISC complex, rendering gene silencing inefficient.

Optimum dsRNA construct design: off-target effects

Non-target mRNA with precise homology to the guide strand of a RISC will inevitably be targeted and degraded. Hence, it is important to consider the potential for inadvertently silencing any other genes within the same or different species that contain these sequences. Such off-target effects (OTEs) can affect endogenous genes of the experimental target (Kulkarni et al., 2006; Ma et al., 2006; Zhang et al., 2010; Toprak et al., 2013), predators of the target species following consumption (Garbian et al., 2012), and closely related species (Zhu et al., 2012). Singh et al. (2013) found that sequences of continuous homology greater than 19 nt were required to induce OTEs in target (*Aedes*) and non-target (*Drosophila*) species. Ulrich et al. (2015) report that OTEs are equally probable in conserved and non-conserved amino acid sequences.

It is important to minimize the potential for OTEs by careful design of dsRNAs. 'E-RNAi' (Horn & Boutros, 2010) and 'SnapDragon' (Harvard Medical School, 2016) are examples of software that automatically design dsRNAs for use with RNAi and search for OTEs in a selection of well-referenced genomes. If dsRNAs are designed manually then dsCheck (Naito et al., 2005) can be used to predict potential OTEs. However, it is difficult to fully anticipate off-target matching for sequences that have not yet been described.

Genetic attributes which facilitate eRNAi – the *Caenorhabditis elegans* example

The nematode *Caenorhabditis elegans* (Maupas) was the first species in which RNAi was successfully implemented (Fire et al., 1998) and has been used extensively to investigate genetic mechanisms underlying gene silencing. This research has uncovered five systemic interference defective (SID) genes that facilitate RNAi (Feinberg & Hunter, 2003; Jose & Hunter, 2007; Jose et al., 2012). SID2 is an intestinal transmembrane protein that is thought to independently endocytose vesicular dsRNA from the gut lumen, before it is processed in the cytoplasm by Dicer (McEwan et al., 2012). SID2 proteins are essential for eRNAi in *C. elegans* as they allow for passage of ingested dsRNA molecules and enable eRNAi when transgenically expressed in recalcitrant species (Winston et al., 2007). SID1 is a ubiquitously expressed transmembrane protein that is essential for systemic RNAi (Winston et al., 2002; Jose et al., 2009). SID1's precise mode of action is yet to be elucidated, but it transmits silencing signals between cells, either in the form of long dsRNAs, free siRNAs, or siRNA bound by RISCs.

In *C. elegans*, RNAi is propagated by RNA-dependent RNA polymerases (RdRp) (Sijen et al., 2001). RdRps bind primary cytoplasmic RNA transcripts and utilize them to synthesize secondary dsRNAs, which then re-enter the siRNA pathway extending the period of gene knockdown

Table 1 A selection of examples of the effectiveness of eRNAi across diverse insect taxa. The studies included highlight variation in the strength of silencing that can be induced via different methods of dsRNA delivery in various taxa and the differences observed in temporal effects

	Species	dsRNA delivery method	Construct length/bp	Genes targeted	Temporal effects of RNAi	Quantification of gene knockdown	Reference
Hymenoptera	*Camponotus floridanus* (Buckley)	Adults fed dsRNA dissolved in sucrose solution for up to 15 days	≈ 400	Adults: *peptidoglycan recognition protein (PGRP-LB)*	Maximum gene silencing after 5 days	≈ 100%	Ratzka et al. (2013)
	Solenopsis invicta Buren	dsRNA fed to larvae via nurse workers for 12 days	496	*Pheromone biosynthesis activating neuropeptide (PBAN)*	After 21 days mortality increased by 50%	Not known	Choi et al. (2012)
Coleoptera	*Diabrotica virgifera virgifera*	Embryos targeted via parental RNAi; adults fed dsRNA overlaid on artificial diet for 10 days	352, 405	*Brahma (bhm)* and *Hunchback (hb)*	10 days after egg laying hatching rates were 0% (*bhm*) and 2.4% (*hb*)	99% silencing of brahma in eggs; 85.3% silencing of hunchback in eggs	Khajuria et al. (2015)
	Leptinotarsa decemlineata	L2 larvae fed dsRNA or transgenic bacteria overlaid on potato leaves	200–400	5× *Housekeeping* genes	Bacteria-treated larvae exhibit higher mortality than naked dsRNA treatment, 12 days after feeding	Bacteria-treated: 59–91%; dsRNA: 61–93% (both gene-dependent)	Zhu et al. (2011)
	D. v. virgifera	L1 larvae fed dsRNA overlaid on diet for up to 8 days	250, 500, 750, 1000	*Ebony* and *laccase 2 (lac2)*	≈ 90% silencing after 2 days	Lac2 ≈ 100%; ebony ≈ 95%	Miyata et al. (2014)

Table 1 Continued

	Species	dsRNA delivery method	Construct length/bp	Genes targeted	Temporal effects of RNAi	Quantification of gene knockdown	Reference
Hemiptera	*Myzus persicae* (Sulzer)	Adults fed on transgenic plants for up to16 days	Not known	*Receptor of Activated Kinase C (Rack1)*, *MpPIntO2* & *MpC002*	Maximum effect seen after 8 days. Treated insects demonstrate gene silencing for up to 6 days post-feeding. Progeny of treated insects demonstrate gene silencing for up to10 days post-feeding	≈ 70% for all genes	Coleman et al. (2015)
	M. persicae/ *Bactericera cockerelli (Šulc)*	Adults fed on transgenic plants for up to 8 days	250–500	*Aquaporin (AQP)*, *sucrase (SUC)*, and *sugar transporter (ST4)*	*B. cockerelli*: osmotic pressure of haemolymph varied significantly for 8 days; *M. persicae*: gene expression altered for 7 days	*B. cockerelli*: no significant silencing of any genes; *M. persicae*: significant silencing of all genes	Tzin et al. (2015)
	Rhodnius prolixus Stål	Adult females fed ≈ 154.2 ng of dsRNA incorporated in transgenic bacteria	375, 453	*Rhodnius heme-binding protein (RHBP)* and *catalase (CAT)*	*RHBP*: peak silencing after 3 days of feeding, no effect 10 days after feeding; *CAT*: peak silencing after 5 days of feeding	RHBP: 99.6%; CAT: 96% silencing	Taracena et al. (2015)
	Diaphorina citri Kuwayama	Topical application of dsRNA dissolved in solution to adults	Not known	5× *Cytochrome p450* genes	Significant lack of protein 8 days after treatment	50–100% silencing (gene dependent)	Killiny et al. (2014)
	Bemisia tabaci (Gennadius)	Adults fed dsRNA dissolved in sucrose solution for 1 day.	279	*Heat shock protein 70 (hsp70)*	Gene silencing for up to 3 days after feeding.	100% silencing	Shim et al. (2015)

Table 1 Continued

	Species	dsRNA delivery method	Construct length/bp	Genes targeted	Temporal effects of RNAi	Quantification of gene knockdown	Reference
Lepidoptera	*Mamestra configurata* Walker	dsRNA applied topically to L1 or L4 larvae, which are then fed with leaf discs overlaid with dsRNA for up to 2 days	500	*Chitin deacetylase 1 (CDA1)*	Larvae silence *CDA1* 1 (L1) and 1.5 (L4) days after treatment	L1: ≈100% silencing; L4: silencing not quantified	Toprak et al. (2013)
	Helicoverpa armigera	Larvae fed transgenic plants or bacteria for 7 days	400–600	Larvae: *molt regulating transcription factor (HR3)*	2-day latency of effect; larvae fed on transgenic plants have 70% less mass than controls after 6 days	Bacteria: ≈ 80%; plants: ≈ 85%	Xiong et al. (2013)
	H. armigera	Larvae fed (all instars) transgenic bacteria and dsRNA overlaid on artificial diet	562, 450	*Ultraspiracle protein (Ups)* and *ecdysone receptor gene (EcR)*	Surviving larvae were assayed by qRT-PCR 5 days after treatment	60% silencing of USP via continuous bacterial feeding	Yang & Han (2014)
Diptera	*Aedes aegypti*	Topical application of dsRNA to adult females	252, 436, 556	3× *Inhibitor of apoptosis (IAP)* genes	Mosquitos assessed 1 day after treatment	33–87.5% silencing (gene dependent)	Pridgeon et al. (2008)
	Anopheles gambiae Giles	L3 larvae fed dsRNA complexed in chitosan nanoparticles	Not known	*Chitin synthase 1 (CHS1)* and *chitin synthase 2 (CHS2)*	After 4 days of treatment with *CHS1* (but not *CHS2*), larvae silenced both intended target genes	CHS1: 62.8% silencing of CHS1 and 57.9% of CHS2; CHS2: 63.4%	Zhang et al. (2010)
	Bactrocera dorsalis	Adults fed dsRNA overlaid on artificial diet until death	Not known	*Sex peptide receptor (spr)*	Mean life span reduced by 26 days in treated flies	52%	Zheng et al. (2015)
	B. dorsalis	Adults fed dsRNA overlaid on artificial diet for up to 14 days	297, 394	*Odorant receptor (Bdor)* and *odorant coreceptor (Orco)*; targets silenced individually and simultaneously	4-day latency of effect, peak effect after 7 days	70% when both targets applied simultaneously	Yi et al. (2014)
	Drosophila melanogaster	L1 larvae soaked in PBS buffer containing dsRNA bound in liposomal vectors for 1 h	≈ 400	*β-Glucuronidase gene (gus)*	Flies assessed 1 day after feeding	Lipofamectine 2000 vectors: 53%	Whyard et al. (2009)

Table 2 Practical considerations when designing eRNAi-based SIT strategies

eRNAi is dose-dependent.	The potency of gene silencing correlates with dsRNA concentration and period of exposure (Zhou et al., 2008; Tian et al., 2009, 2015; Singh et al., 2013; Asokan et al., 2014; Yu et al., 2014; Li et al., 2015b; Ulrich et al., 2015; Whyard et al., 2015; Rebijith et al., 2016). A period of latency between dsRNA administration and gene silencing is common in larvae and adults, which is consistent with a threshold effect. Reported latency periods include: 12 h in *Helicoverpa armigera* (Tian et al., 2015) and *Aedes aegypti* (Coy et al., 2012), 24 h in *Mamestra configurata* (Toprak et al., 2013) and *Bactrocera dorsalis* (Zheng et al., 2015), 7 days in *Spodoptera exigua* (Tian et al., 2009), and 12 days in *Solenopsis invicta* (Choi et al., 2012). It is of note that Choi et al. (2012) did not feed dsRNA to insects directly but via a secondary worker individual, and this may have diluted dsRNA somewhat leading to an extended latency period.
Taxonomy cannot be used to reliably predict sensitivity to eRNAi or the latency period between dsRNA uptake and gene silencing.	Equivalent eRNAi methods can produce disparate results even when different biotypes (Li et al., 2015a), or subpopulations (Sugahara et al., 2017) of the same species are targeted. Within the Lepidoptera, *H. armigera* is capable of eliciting a robust eRNAi response (Xiong et al., 2013), but *Spodoptera frugiperda* is recalcitrant to eRNAi (Ivashuta et al., 2015). Working on hemipteran and dipteran models, Coleman et al. (2015) and Yi et al. (2014) observed a 4-day period of latency between dsRNA administration and gene silencing. Another dipteran (*A. aegypti*) exhibited gene silencing after 12 h of feeding with dsRNA in solution (Coy et al., 2012). These discrepancies may be eliminated if equivalent feeding protocols are used.
The sensitivity of a gene to RNAi has not been fully assessed unless the entire mRNA molecule has been targeted for knockdown.	When all variables remain constant, variation in RNAi potency is likely to be due to regional susceptibility of mRNAs to cleavage. The strength of gene knockdown can vary greatly when multiple genes are targeted in the same insect using identical methods (Pridgeon et al., 2008; Li et al., 2011; Singh et al., 2013; Toprak et al., 2013; Killiny et al., 2014; Taracena et al., 2015).
Insects may become more tolerant to dsRNA with aging.	A diminution in the efficiency of RNAi with age has been suggested by Tian et al. (2015) and is supported by evidence that silencing appears more efficient in neonates than in late stage larvae (Zhu et al., 2011; Toprak et al., 2013). Furthermore, Coleman et al.'s (2015) observation that silencing is longer lived in nymphs than in adults suggests that fully developed insects are less sensitive to dsRNA.
Optimum eRNAi delivery methods must be determined by trial and error in most insect species.	Yang & Han (2014) found that feeding *H. armigera* with transgenic bacteria induced more efficient eRNAi than feeding with naked dsRNA. However, naked dsRNA elicited more robust gene silencing than did bacterial feeding in *B. dorsalis* (Li et al., 2011). Zhu et al. (2011) report that three of five genes were knocked down more efficiently using a bacterial system in *Leptinotarsa decemlineata*, but that the remaining two were more efficiently silenced by naked dsRNA.
When inducing eRNAi the capacity for systemic RNAi is critical if target genes lie beyond gut tissue.	The systemic RNAi capacity of various insects has been assessed by targeting *chitin synthase* genes specific to the exoskeleton (Tian et al., 2009; Zhang et al., 2010; Singh et al., 2013). Other examples of studies that targeted genes distal to gut tissue include the silencing of *ebony* in *Diabrotica virgifera vigifera* (Miyata et al., 2014) and *Rhodnius* heme binding protein (RHBP) in *Rhodnius prolixus* (Taracena et al., 2015). Ivashuta et al. (2015) suggest that systemic RNAi in *D. v. virgifera* is facilitated by transport of dsRNAs of >60 bp long.
Parental RNAi requires further analysis to determine whether it can be effective for SIT.	A robust systemic RNAi response enables the silencing of genes in germ cells (parental RNAi; pRNAi). When germ cells are affected by pRNAi, gene expression can be limited in zygotes and developing insects (Zwier et al., 2012; Paim et al., 2013; Coleman et al., 2015; Khajuria et al., 2015). A simple application of pRNAi in pest management would be to reduce future insect populations via embryonic lethal gene silencing (Khajuria et al., 2015). For use with SIT, sexual differentiation genes could be targeted in the mothers of target insects (Shukla & Palli, 2012). dsRNA delivery methods may drastically affect the potency of pRNAi. Zheng et al. (2015) report that eRNAi silencing of *sex peptide receptor* in *B. dorsalis* limited eclosion rates of their progeny, whereas Peng et al. (2015) describe that silencing the *transformer* gene by microinjection in this species has no effect on progeny. The disparity in pRNAi efficiency between these delivery methods might be due to the fact that eRNAi would have consistently supplied flies with dsRNA during the development of germ cells, whereas expression of *transformer* would have been only transiently reduced by microinjection.

Table 2 Continued

Insects may become less sensitive to dsRNA over time.	Working with *B. dorsalis*, Li et al. (2015b) demonstrated that eRNAi potency was reduced following a series of exposures to dsRNA. The effect was dose-dependent, was not gene specific, and lasted for up to 20 days following primary exposure. Refractoriness only occurred following targeting of endogenous genes, which suggests a role in immune priming. Li et al. (2015b) suggest that flies may become refractory to dsRNA when genes that mediate endocytosis are downregulated. *Bactrocera dorsalis* has also been reported to upregulate the expression of target genes following exposure to dsRNA (Li et al., 2011). Therefore, reduced eRNAi potency may be due to synergistic overexpression of target genes along with downregulation of endocytic mediators.
Endocytic pathways and SID transport proteins may work synergistically in eRNAi.	Both endocytosis and SID mediated dsRNA transport facilitate eRNAi in *L. decemlineata* (Cappelle et al., 2016).
Insects may become more sensitive to eRNAi if dsRNA is vectored in nanoparticles. The performance of various nanoparticle technologies for use with RNAi is reviewed in Liao et al. (2016).	Nanoparticle-based delivery of dsRNA may serve dual purposes: (1) enhancing passage of dsRNA across the gut, and (2) prolonging the effect of RNAi via slow release of dsRNA. Whyard et al. (2009) utilized Lipofectamine 2000 and Cellfectin liposomal nanoparticles to successfully vector dsRNA to *Drosophila melanogaster*, even though this species is reported as eRNAi incompetent. Liposomal vectoring achieved ca. 50% gene knockdown. Recently, *Drosophila suzukii* (Matsumura) has also been reported as recalcitrant to feeding with naked dsRNA, but sensitive if molecules are vectored in liposomes (Taning et al., 2016). Chitosan nanoparticles can vector molecules to *Anopheles gambiae* and *A. aegypti* (Zhang et al., 2010, 2015b; Mysore et al., 2015), although mosquito larvae (Figueira-Mansur et al., 2013; Singh et al., 2013; Whyard et al., 2015) and adults (Pridgeon et al., 2008; Coy et al., 2012) also demonstrate eRNAi when exposed to naked dsRNA. Potent eRNAi has been demonstrated in *A. aegypti* larvae using carbon quantum dot (CQD) nanoparticles (Das et al., 2015).
Data regarding the simultaneous knockdown of genes via administration of multiple dsRNAs are conflicting.	Simultaneous silencing of genes has been reported to enhance the potency of RNAi in *A. aegypti* using a bacterial feeding approach (Whyard et al., 2015). A plant feeding study of *Myzus persicae* and *Bactericera cockerelli* also suggested that targeting genes simultaneously may induce a synergistic effect (Tzin et al., 2015). Zhang et al. (2015a) and Ulrich et al. (2015) report simultaneous silencing in coleopteran models actually dilutes the potency of RNAi. When feeding combinations of dsRNAs to *D. suzukii*, Taning et al. (2016) found the potency of RNAi was enhanced for some target gene combinations but not others.

(Pak & Fire, 2007). Many viruses also encode RdRps, which allow for the proliferation of viral RNAs (Pan et al., 2016).

The wealth of information about the mechanism of RNAi gained from the study of *C. elegans* has been of great use for elucidating homologous mechanisms in pest species. The transfer of enabling mechanisms, such as SID1 gene functionality, to species that lack them has also provided insights into gene silencing mechanisms that might exist in some pest species.

Genetic attributes that facilitate eRNAi in the insects

Orthologues of SID genes are found across insect taxa but are absent from the Diptera (Huvenne & Smagghe, 2010), members of which represent some of the world's most significant agricultural and medical pests. An orthologue of SID1 facilitates systemic RNAi in *Apis mellifera* L. (Aronstein et al., 2006) and Miyata et al. (2014) suggest that two SID orthologues are involved in, if not essential to, eRNAi in *D. v. virgifera*. On this basis, the presence or absence of SIDs has been used by some researchers to predict the capacity of different insect species to effect gene silencing via eRNAi (Tomoyasu et al., 2008; Tian et al., 2009). However, the presence of SIDs is not fully predictive of eRNAi potential – several studies have demonstrated that dipterans, which lack SIDs as noted above, are eRNAi competent (Table 1). In contrast, *Bombyx mori* (L.) possesses three SID orthologues, yet is not competent for eRNAi (Li et al., 2015d).

SIDs are not the only molecules that contribute to dsRNA uptake as eRNAi is facilitated by endocytic pathways in some insect species. For example, *Bactrocera dorsalis* (Hendel) (Li et al., 2015b) and *Tribolium castaneum* (Herbst) (Xiao et al., 2015) are refractory to eRNAi following challenge with the endocytic inhibitor Bafilomycin A1 (Xu et al., 2003). Refractoriness to eRNAi can also be induced in *Bactrocera* and *Tribolium* if orthologues of the *chc* (*clathrin heavy chain*) gene (Bazinet et al., 1993) are downregulated (Li et al., 2015b; Xiao et al., 2015). It has recently been suggested that SIDs and endocytic mediators synergistically facilitate eRNAi in *L. decemlineata* (Cappelle et al., 2016). Scavenger receptors have also been demonstrated to facilitate systemic RNAi in *D. melanogaster* (Saleh et al., 2009) and eRNAi in *Schistocerca gregaria* Forsskål (Wynant et al., 2014).

Recent evidence suggests that an intracellular mode of dsRNA degradation, other than the siRNA pathway, may exist in some insect species (Shukla et al., 2016). Shukla et al. (2016) demonstrated that cell lines of *L. decemlineata* and *Spodoptera frugiperda* (JE Smith) were both capable of dsRNA uptake, although only the cells of *Leptinotarsa* produced 21-bp siRNA-like transcripts. Apparently dsRNA was degraded in endosomes within the cells of *Spodoptera*, as demonstrated by pH-induced fluorescence of CypHer5E-labelled molecules. Accordingly, Yoon et al. (2016) report that dsRNA may escape endosomes through acidification in *L. decemlineata*, facilitating induction of the RNAi process in that insect. These types of practical studies, along with comparative transcriptomic analyses (such as Swevers et al., 2013), may help us to understand the divergence in eRNAi potency between insects.

Orthologues of RdRps have not been reported in the Insecta. Several insects can nevertheless exhibit sustained RNAi for prolonged periods (Paim et al., 2013; Coleman et al., 2015; Khajuria et al., 2015), suggesting a system of signal amplification. Hemipterans appear to have a robust RNAi amplification system, as gene silencing has been demonstrated for 4 days (Rebijith et al., 2016), 6 days (Coleman et al., 2015), and 8 days (Tzin et al., 2015) after feeding with dsRNA. Coleman et al. (2015) also report that nymphs born from RNA-treated mothers exhibited RNAi for 10 days post-feeding, suggesting that genetic variation between life-history stages might influence signal amplification.

Insects that produce RNAses in salivary and midgut secretions or in haemolymph can degrade dsRNAs and thus limit the extent of gene silencing (Allen & Walker, 2012; Liu et al., 2012; Garbutt et al., 2013; Yang & Han, 2014; Shukla et al., 2016). Yang & Han (2014) suggest that RNAi is more efficient in *H. armigera* if dsRNAs are encapsulated in bacteria when they traverse the gut, as this bypasses potent digestive RNAases in midgut secretions. Das et al. (2015) present a similar idea, but suggest that vectoring dsRNA in carbon quantum dot nanoparticles provides protection, not from nucleolytic enzymes, but from damage incurred through extreme pH in the alimentary canal of *A. aegypti*.

Conclusion

In this review we have described the detailed mechanisms underlying gene silencing by dsRNA, and considered the use of this approach for use with SIT. Currently, the available data are scant and insufficient to design all aspects of eRNAi studies in pest species in a predictive context. We have emphasized several factors that must be considered in the design and implementation of such techniques (Table 2) in order to try to address these omissions.

Knowledge of eRNAi in key areas, such as the most basic mechanisms that enable insects to acquire dsRNA from

their environment, is lacking. SIDs and endocytosis both play roles individually and synergistically, but overall the picture of their modes of action is far from clear. Packaging of dsRNA in intermediate carriers such as bacteria or nanoparticles may overcome refractoriness to eRNAi in some cases. However, certain insects may remain refractory to eRNAi even if dsRNA is successfully packaged and transported across the gut, as discrete modes of dsRNA degradation such as endosomal acquisition may mitigate the silencing process.

SIT strategies rely on mass rearing, which in some cases has knock-on effects for insect quality and performance (Sørensen et al., 2012). The consequences of mass rearing manifest differently across taxa, which is why Chambers (1977) suggested comprehensive quality control measures for all such programs. SIT individuals generated (by any means) from mass-reared populations are likely to perform differently than untreated controls. All SIT techniques should be judged according to the performance of individuals in the field, which very often will differ to those in the laboratory or factory (Mayer et al., 1998; Carvalho et al., 2015). The goal of eRNAi-SIT is to produce mass-reared insects that are at least equal in quality to the currently available alternatives.

The potential of an eRNAi approach is being increasingly realized and groups such as the SITplus partnership are developing this technology to combat the spread of pest insects (CSIRO, 2015). The production of dsRNA for large-scale eRNAi treatments may be expensive. HT115 *E. coli* (see 'In vivo dsRNA synthesis' section above) may represent the most viable option currently available, but economy of scale does present a challenge that needs to be addressed.

As suggested by the transformer-2/innexin-5 model, eRNAi could be used in the future to successfully implement gene silencing, and create insects for application of SIT in the field. There is evidence that other gene targets could also be utilized in an eRNAi-SIT system. A possible outcome when the sexual differentiation cascade is targeted with RNAi is a combination of arrested phenotypic female development in some individuals, and sex-reversal in others. This has been demonstrated by Shukla & Palli (2012) in *T. castaneum*, where parental RNAi of transformer produced a cohort of 91.1% males, 8.9% pseudomales, and 0% females. This outcome is not optimal for SIT as nearly half the population was lost, but it did produce a compatible male-only cohort. More recently, eRNAi of spermatogenic targets has been demonstrated to induce sterility by up to 60% in *B. dorsalis* while maintaining mating competitiveness (Dong et al., 2016).

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References

Abd El Halim HM, Alshukri BMH, Ahmad MS, Nakasu EYT, Awwad MH et al. (2016) RNAi-mediated knockdown of the voltage gated sodium ion channel TcNa$_v$ causes mortality in *Tribolium castaneum*. Scientific Reports 6: 29301.

Allen ML & Walker WB (2012) Saliva of *Lygus lineolaris* digests double stranded ribonucleic acids. Journal of Insect Physiology 58: 391–396.

Alphey L, Benedict M, Bellini R, Clark GG, Dame DA et al. (2010) Sterile-insect methods for control of mosquito-borne diseases: an analysis. Vector Borne and Zoonotic Diseases 10: 295–311.

Amiri A, Bandani AR & Alizadeh H (2015) Molecular identification of cysteine and trypsin protease, effect of different hosts on protease expression, and rnai mediated silencing of cysteine protease gene in the sunn pest. Archives of Insect Biochemistry and Physiology 91: 189–209.

Aravin A, Gaidatzis D, Pfeffer S, Lagos-Quintana M, Landgraf P et al. (2006) A novel class of small RNAs bind to MILI protein in mouse testes. Nature 442: 203–207.

Aronstein K, Pankiw T & Saldivar E (2006) SID-I is implicated in systemic gene silencing in the honey bee. Journal of Apicultural Research 45: 20–24.

Asokan R, Sharath Chandra G, Manamohan M, Krishna Kumar NK & Sita T (2014) Response of various target genes to diet-delivered dsRNA mediated RNA interference in the cotton bollworm, *Helicoverpa armigera*. Journal of Pest Science 87: 163–172.

Babiarz JE, Ruby JG, Wang Y, Bartel DP & Blelloch R (2008) Mouse ES cells express endogenous shRNAs, siRNAs, and other microprocessor-independent, dicer-dependent small RNAs. Genes and Development 22: 2773–2785.

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281–297.

Baum JA & Roberts JK (2014) Progress towards RNAi-mediated insect pest management. Advances in Insect Physiology, Vol. 47 (eds. by SD Tarlochan & SG Sarjeet), pp. 249–295. Academic Press, London, UK.

Baum JA, Bogaert T, Clinton W, Heck GR, Feldmann P et al. (2007) Control of coleopteran insect pests through RNA interference. Nature Biotechnology 25: 1322–1326.

Bazinet C, Katzen AL, Morgan M, Mahowald AP & Lemmon SK (1993) The *Drosophila* clathrin heavy chain gene clathrin function is essential in a multicellular organism. Genetics 134: 1119–1134.

Bernstein E, Caudy AA, Hammond SM & Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409: 363–366.

Bolognesi R, Ramaseshadri P, Anderson J, Bachman P, Clinton W et al. (2012) Characterizing the mechanism of action of double-stranded RNA activity against western corn rootworm (*Diabrotica virgifera virgifera* LeConte). PLoS ONE 7: e47534.

Bona AC, Chitolina RF, Fermino ML, de Castro Poncio L, Weiss A et al. (2016) Larval application of sodium channel homologous dsRNA restores pyrethroid insecticide susceptibility in a resistant adult mosquito population. Parasites and Vectors 9: 397.

Briceño RD & Eberhard WG (2003) Decisions during courtship by male and female medflies (Diptera, Tephritidae): correlated changes in male behavior and female acceptance criteria in mass-reared flies. Florida Entomologist 85: 14–31.

Cao J, Liu Y, Yang Y, Zhang H, Li Z et al. (2015) Molecular characterization and functional analysis of the ultraspiracle (USP) in the oriental fruit moth *Grapholita molesta* (Lepidoptera: Olethreutidae). Comparative Biochemistry and Physiology B 190: 54–62.

Cappelle K, de Oliveira CF, Van Eynde B, Christiaens O & Smagghe G (2016) The involvement of clathrin-mediated endocytosis and two Sid-1-like transmembrane proteins in double-stranded RNA uptake in the Colorado potato beetle midgut. Insect Molecular Biology 25: 315–323.

Carmell MA, Xuan Z, Zhang MQ & Hannon GJ (2002) The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. Genes and Development 16: 2733–2742.

Carvalho DO, McKemey AR, Garziera L, Lacroix R, Donnelly CA et al. (2015) Suppression of a field population of *Aedes aegypti* in Brazil by sustained release of transgenic male mosquitoes. PLoS Neglected Tropical Diseases 9: e0003864.

Chambers DL (1977) Quality control in mass rearing. Annual Review of Entomology 22: 289–308.

Chan CY, Carmack CS, Long DD, Maliyekkel A, Shao Y et al. (2009) A structural interpretation of the effect of GC-content on efficiency of RNA interference. BMC Bioinformatics 10 (Suppl 1): S33.

Chen X, Li L, Hu Q, Zhang B, Wu W et al. (2015) Expression of dsRNA in recombinant *Isaria fumosorosea* strain targets the TLR7 gene in *Bemisia tabaci*. BMC Biotechnology 15: 64.

Choi MY, Vander Meer RK, Coy M & Scharf ME (2012) Phenotypic impacts of PBAN RNA interference in an ant, *Solenopsis invicta*, and a moth, *Helicoverpa zea*. Journal of Insect Physiology 58: 1159–1165.

Christiaens O, Prentice K, Pertry I, Ghislain M, Bailey A et al. (2016) RNA interference: a promising biopesticide strategy against the African sweetpotato weevil *Cylas brunneus*. Scientific Reports 6: 38836.

Coleman AD, Wouters RHM, Mugford ST & Hogenhout SA (2015) Persistence and transgenerational effect of plant-mediated RNAi in aphids. Journal of Experimental Botany 66: 541–548.

Coy MR, Sanscrainte ND, Chalaire KC, Inberg A, Maayan I et al. (2012) Gene silencing in adult *Aedes aegypti* mosquitoes through oral delivery of double-stranded RNA. Journal of Applied Entomology 136: 741–748.

CSIRO (2015) Partnering to find a solution to Australia's Qfly problem. Available at: http://www.csiro.au/en/Research/BF/Areas/Protecting-Australias-agricultural-industries/Robot-technology/SITplus (accessed 06 October 2016).

Das S, Debnath N, Cui Y, Unrine J & Palli SR (2015) Chitosan, carbon quantum dot, and silica nanoparticle mediated dsRNA delivery for gene silencing in *Aedes aegypti*: a comparative analysis. ACS Applied Materials and Interfaces 7: 19530–19535.

De Block M, Herrera-Estrella L, Van Montagu M, Schell J & Zambryski P (1984) Expression of foreign genes in regenerated plants and in their progeny. EMBO Journal 3: 1681–1689.

De Smet L, Ravoet J, Wenseleers T & de Graaf DC (2017) Expression of key components of the RNAi machinery are suppressed in *Apis mellifera* that suffer a high virus infection. Entomological Science 20: 76–85.

Ding Y, Chi YC & Lawrence CE (2005) RNA secondary structure prediction by centroids in a Boltzmann weighted ensemble. RNA 11: 1157–1166.

Dlakić M (2006) DUF283 domain of Dicer proteins has a double-stranded RNA-binding fold. Bioinformatics 22: 2711–2714.

Dong YC, Wang ZJ, Chen ZZ, Clarke AR & Niu CY (2016) *Bactrocera dorsalis* male sterilization by targeted RNA interference of spermatogenesis: empowering sterile insect technique programs. Scientific Reports 6: 35750.

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K & Tuschl T (2001a) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411: 494–498.

Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W & Tuschl T (2001b) Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. EMBO Journal 20: 6877–6888.

Feinberg EH & Hunter CP (2003) Transport of dsRNA into cells by the transmembrane protein SID-1. Science 301: 1545–1547.

Figueira-Mansur J, Ferreira-Pereira A, Mansur JF, Franco TA, Alvarenga ESL et al. (2013) Silencing of P-glycoprotein increases mortality in temephos-treated *Aedes aegypti* larvae. Insect Molecular Biology 22: 648–658.

Filipowicz W (2005) RNAi: the nuts and bolts of the RISC machine. Cell 122: 17–20.

Fire AZ (2007) Gene silencing by double-stranded RNA. Cell Death and Differentiation 14: 1998–2012.

Fire A, Xu SQ, Montgomery MK, Kostas SA, Driver SE & Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391: 806–811.

Gabrieli P, Scolari F, Di Cosimo A, Savini G, Fumagalli M et al. (2016) Sperm-less males modulate female behaviour in *Ceratitis capitata* (Diptera: Tephritidae). Insect Biochemistry and Molecular Biology 79: 13–26.

Garbian Y, Maori E, Kalev H, Shafir S & Sela I (2012) Bidirectional transfer of RNAi between honey bee and *Varroa destructor*: *Varroa* gene silencing reduces *Varroa* population. PLoS Pathogens 8: e1003035.

Garbutt JS, Bellés X, Richards EH & Reynolds SE (2013) Persistence of double-stranded RNA in insect hemolymph as a potential determiner of RNA interference success: evidence

from *Manduca sexta* and *Blattella germanica*. Journal of Insect Physiology 59: 171–178.

Gasciolli V, Mallory AC, Bartel DP & Vaucheret H (2005) Partially redundant functions of arabidopsis DICER-like enzymes and a role for DCL4 in producing trans-Acting siRNAs. Current Biology 15: 1494–1500.

Gorman K, Young J, Pineda L, Marquez R, Sosa N et al. (2016) Short-term suppression of *Aedes aegypti* using genetic control does not facilitate *Aedes albopictus*. Pest Management Science 72: 618–628.

Grishok A, Pasquinelli AE, Conte D, Li N, Parrish S et al. (2001) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. Cell 106: 23–34.

Gross M (2013) EU ban puts spotlight on complex effects of neonicotinoids. Current Biology 23: R462–R464.

Gu L & Knipple DC (2013) Recent advances in RNA interference research in insects: implications for future insect pest management strategies. Crop Protection 45: 36–40.

Guerfali MM, Parker A, Fadhl S, Hemdane H, Raies A & Chevrier C (2011) Fitness and reproductive potential of irradiated mass-reared mediterranean fruit fly males *Ceratitis capitata* (Diptera: Tephritidae): lowering radiation doses. Florida Entomologist 94: 1042–1050.

Guo S & Kemphues KJ (1995) *par*-1, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell 81: 611–620.

Guo H, Song X, Wang G, Yang K, Wang Y et al. (2014) Plant-generated artificial small RNAs mediated aphid resistance. PLoS ONE 9: e97410.

Guo Z, Kang S, Zhu X, Xia J, Wu Q et al. (2015) The novel ABC transporter ABCH1 is a potential target for RNAi-based insect pest control and resistance management. Scientific Reports 5: 13728.

Haase AD, Jaskiewicz L, Zhang HD, Laine S, Sack R et al. (2005) TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. EMBO Reports 6: 961–967.

Haley B & Zamore PD (2004) Kinetic analysis of the RNAi enzyme complex. Nature Structural and Molecular Biology 11: 599–606.

Hammond SM, Bernstein E, Beach D & Hannon GJ (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature 404: 293–296.

Hannon GJ (2002) RNA interference. Nature 418: 244–251.

Harris AF, Nimmo D, McKemey AR, Kelly N, Scaife S et al. (2011) Field performance of engineered male mosquitoes. Nature Biotechnology 29: 1034–1037.

Hartig JV, Tomari Y & Foerstemann K (2007) piRNAs - the ancient hunters of genome invaders. Genes and Development 21: 1707–1713.

Harvard Medical School (2016) SnapDragon. Available at: www.flyrnai.org (accessed 08 August 2016).

Hooper GHS (1972) Sterilization of the Mediterranean fruit fly with gamma radiation: effect on male competitiveness and change in fertility of females alternately mated with irradiated and untreated males. Journal of Economic Entomology 65: 1–6.

Horn T & Boutros M (2010) E-RNAi: a web application for the multi-species design of RNAi reagents-2010 update. Nucleic Acids Research 38: W332–W339.

Horsch RB, Fraley RT, Rogers SG, Sanders PR, Lloyd A & Hoffmann N (1984) Inheritance of functional foreign genes in plants. Science 223: 496–498.

Hu J, Liu J & Corey DR (2010) Allele-selective inhibition of huntingtin expression by switching to an miRNA-like RNAi mechanism. Chemistry and Biology 17: 1183–1188.

Hu X, Richtman NM, Zhao JZ, Duncan KE, Niu X et al. (2016) Discovery of midgut genes for the RNA interference control of corn rootworm. Scientific Reports 6: 30542.

Huvenne H & Smagghe G (2010) Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. Journal of Insect Physiology 56: 227–235.

Ivashuta S, Zhang Y, Wiggins BE, Ramaseshadri P, Segers GC et al. (2015) Environmental RNAi in herbivorous insects. RNA 21: 840–850.

Jin S, Singh ND, Li L, Zhang X & Daniell H (2015) Engineered chloroplast dsRNA silences cytochrome p450 monooxygenase, V-ATPase and chitin synthase genes in the insect gut and disrupts *Helicoverpa armigera* larval development and pupation. Plant Biotechnology Journal 13: 435–446.

Jose AM & Hunter CP (2007) Transport of sequence-specific RNA interference information between cells. Annual Review of Genetics 41: 305–330.

Jose AM, Smith JJ & Hunter CP (2009) Export of RNA silencing from *C. elegans* tissues does not require the RNA channel SID-1. Proceedings of the National Academy of Sciences of the USA 106: 2283–2288.

Jose AM, Kim YA, Leal-Ekman S & Hunter CP (2012) Conserved tyrosine kinase promotes the import of silencing RNA into *Caenorhabditis elegans* cells. Proceedings of the National Academy of Sciences of the USA 109: 14520–14525.

Joseph TT & Osman R (2012) Convergent transmission of RNAi guide-target mismatch information across Argonaute internal allosteric network. PLoS Computational Biology 8: 1002693.

Kamath RS, Martinez-Campos M, Zipperlen P, Fraser AG & Ahringer J (2001) Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. Genome Biology 2: 1–10.

Karim S, Troiano E & Mather TN (2010) Functional genomics tool: gene silencing in *Ixodes scapularis* eggs and nymphs by electroporated dsRNA. BMC Biotechnology 10: 1.

Khajuria C, Vélez AM, Rangasamy M, Wang H, Fishilevich E et al. (2015) Parental RNA interference of genes involved in embryonic development of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. Insect Biochemistry and Molecular Biology 63: 54–62.

Khvorova A, Reynolds A & Jayasena SD (2003) Functional siRNAs and rniRNAs exhibit strand bias. Cell 115: 209–216.

Killiny N, Hajeri S, Tiwari S, Gowda S & Stelinski LL (2014) Double-stranded RNA uptake through topical application, mediates silencing of five CYP4 genes and suppresses insecticide resistance in *Diaphorina citri*. PLoS ONE 9: e110536.

Kim VN, Han J & Siomi MC (2009) Biogenesis of small RNAs in animals. Nature Reviews Molecular Cell Biology 10: 126–139.

Kim YH, Issa MS, Cooper AMW & Zhu KY (2015) RNA interference: applications and advances in insect toxicology and insect pest management. Pesticide Biochemistry and Physiology 120: 109–117.

Knipling EF (1955) Possibilities of insect control or eradication through the use of sexually sterile males. Journal of Economic Entomology 48: 459–462.

Knipling EF (1998) Role of parasitoid augmentation and sterile insect techniques for area-wide management of agricultural insect pests. Journal of Agricultural Entomology 15: 273–301.

Krafsur ES (1998) Sterile insect technique for suppressing and eradicating insect populations: 55 years and counting. Journal of Agricultural Entomology 15: 303–317.

Kulkarni MM, Booker M, Silver SJ, Friedman A, Hong P et al. (2006) Evidence of off-target effects associated with long dsRNAs in *Drosophila melanogaster* cell-based assays. Nature Methods 3: 833–838.

Kumar P, Pandit SS & Baldwin IT (2012) Tobacco rattle virus vector: a rapid and transient means of silencing *Manduca sexta* genes by plant mediated RNA interference. PLoS ONE 7: e31347.

Kuramochi-Miyagawa S, Kimura T, Yomogida K, Kuroiwa A, Tadokoro Y et al. (2001) Two mouse piwi-related genes: miwi and mili. Mechanisms of Development 108: 121–133.

Lan H, Chen H, Liu Y, Jiang C, Mao Q et al. (2016a) Small interfering RNA pathway modulates initial viral infection in midgut epithelium of insect after ingestion of virus. Journal of Virology 90: 917–929.

Lan H, Wang H, Chen Q, Chen H, Jia D et al. (2016b) Small interfering RNA pathway modulates persistent infection of a plant virus in its insect vector. Scientific Reports 6: 20699.

Lau P-W, Potter CS, Carragher B & MacRae IJ (2009) Structure of the human Dicer-TRBP complex by electron microscopy. Structure 17: 1326–1332.

Lee YS, Nakahara K, Pham JW, Kim K, He Z et al. (2004) Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell 117: 69–81.

Lee Y, Hur I, Park SY, Kim YK, Suh MR & Kim VN (2006) The role of PACT in the RNA silencing pathway. EMBO Journal 25: 522–532.

Lee HY, Zhou K, Smith AM, Noland CL & Doudna JA (2013) Differential roles of human Dicer-binding proteins TRBP and PACT in small RNA processing. Nucleic Acids Research 41: 6568–6576.

Leuschner PJF, Ameres SL, Kueng S & Martinez J (2006) Cleavage of the siRNA passenger strand during RISC assembly in human cells. EMBO Reports 7: 314–320.

Li X, Zhang M & Zhang H (2011) RNA interference of four genes in adult *Bactrocera dorsalis* by feeding their dsRNAs. PLoS ONE 6: e0017788.

Li J, Li X, Bai R, Shi Y, Tang Q et al. (2015a) RNA interference of the P450 CYP6CM1 gene has different efficacy in B and Q biotypes of *Bemisia tabaci*. Pest Management Science 71: 1175–1181.

Li X, Dong X, Zou C & Zhang H (2015b) Endocytic pathway mediates refractoriness of insect *Bactrocera dorsalis* to RNA interference. Scientific Reports 5: 08700.

Li Y, Dou K, Gao S, Sun J, Wang M et al. (2015c) Impacts on silkworm larvae midgut proteomics by transgenic *Trichoderma* strain and analysis of glutathione S-transferase sigma 2 gene essential for anti-stress response of silkworm larvae. Journal of Proteomics 126: 218–227.

Li Z, Zeng B, Ling L, Xu J, You L et al. (2015d) Enhancement of larval RNAi efficiency by over-expressing Argonaute2 in *Bombyx mori*. International Journal of Biological Sciences 11: 176–185.

Liang C, Wang Y, Murota Y, Liu X, Smith D et al. (2015) TAF11 assembles the RISC loading complex to enhance RNAi efficiency. Molecular Cell 59: 807–818.

Liao W, Li W, Zhang T, Kirberger M, Liu J et al. (2016) Powering up the molecular therapy of RNA interference by novel nanoparticles. Biomaterials Science 4: 1051–1061.

Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM et al. (2004) Argonaute2 is the catalytic engine of mammalian RNAi. Science 305: 1437–1441.

Liu X, Jiang F, Kalidas S, Smith D & Liu Q (2006) Dicer-2 and R2D2 coordinately bind siRNA to promote assembly of the siRISC complexes. RNA 12: 1514–1520.

Liu Y, Ye X, Jiang F, Liang C, Chen D et al. (2009) C3PO, an endoribonuclease that promotes RNAi by facilitating RISC activation. Science 325: 750–753.

Liu J, Swevers L, Iatrou K, Huvenne H & Smagghe G (2012) *Bombyx mori* DNA/RNA non-specific nuclease: expression of isoforms in insect culture cells, subcellular localization and functional assays. Journal of Insect Physiology 58: 1166–1176.

Liu G, Wu Q, Li J, Zhang G & Wan F (2015) RNAi-mediated knock-down of transformer and transformer 2 to generate male-only progeny in the oriental fruit fly, *Bactrocera dorsalis* (Hendel). PLoS ONE 10: e0128892.

Lu P, Zhou Y, Yu Y, Cao J, Zhang H et al. (2015) RNA interference and the vaccine effect of a subolesin homolog from the tick *Rhipicephalus haemaphysaloides*. Experimental and Applied Acarology 68: 113–126.

Luo KQ & Chang DC (2004) The gene-silencing efficiency of siRNA is strongly dependent on the local structure of mRNA at the targeted region. Biochemical and Biophysical Research Communications 318: 303–310.

Ma Y, Creanga A, Lum L & Beachy PA (2006) Prevalence of off-target effects in *Drosophila* RNA interference screens. Nature 443: 359–363.

MacRae IJ, Ma E, Zhou M, Robinson CV & Doudna JA (2008) In vitro reconstitution of the human RISC-loading complex. Proceedings of the National Academy of Sciences of the USA 105: 512–517.

Malik HJ, Raza A, Amin I, Scheffler JA, Scheffler BE et al. (2016) RNAi-mediated mortality of the whitefly through transgenic expression of double-stranded RNA homologous to acetylcholinesterase and ecdysone receptor in tobacco plants. Scientific Reports 6: 38469.

Malone CD, Brennecke J, Dus M, Stark A, McCombie WR et al. (2009) Specialized piRNA pathways act in germline and somatic tissues of the *Drosophila* ovary. Cell 137: 522–535.

Mamta B, Reddy KRK & Rajam MV (2015) Targeting chitinase gene of *Helicoverpa armigera* by host-induced RNA interference confers insect resistance in tobacco and tomato. Plant Molecular Biology 90: 281–292.

Maniataki E & Mourelatos Z (2005) A human, ATP-independent, RISC assembly machine fueled by pre-miRNA. Genes and Development 19: 2979–2990.

Mao J & Zeng F (2012) Feeding-based RNA intereference of a gap gene is lethal to the pea aphid, *Acyrthosiphon pisum*. PLoS ONE 7: e48718.

Matranga C, Tomari Y, Shin C, Bartel DP & Zamore PD (2005) Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell 123: 607–620.

Mayer DG, Atzeni MG, Stuart MA, Anaman KA & Butler DG (1998) Mating competitiveness of irradiated flies for screwworm fly eradication campaigns. Preventive Veterinary Medicine 36: 1–9.

McEwan D, Weisman A & Hunter CP (2012) Uptake of extracellular double-stranded RNA by SID-2. Molecular Cell 47: 746–754.

Miller SC, Miyata K, Brown SJ & Tomoyasu Y (2012) Dissecting systemic RNA interference in the red flour beetle *Tribolium castaneum*: parameters affecting the efficiency of RNAi. PLoS ONE 7: e47431.

Miyata K, Ramaseshadri P, Zhang Y, Segers G, Bolognesi R & Tomoyasu Y (2014) Establishing an in vivo assay system to identify components involved in environmental RNA interference in the western corn rootworm. PLoS ONE 9: e101661.

Morazzani EM, Wiley MR, Murreddu MG, Adelman ZN & Myles KM (2012) Production of virus-derived ping-pong-dependent piRNA-like small RNAs in the mosquito soma. PLoS Pathogens 8: e1002470.

Morel JB, Godon C, Mourrain P, Béclin C, Boutet S et al. (2002) Fertile hypomorphic Argonaute (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance. Plant Cell 14: 629–639.

Murchison EP, Partridge JF, Tam OH, Cheloufi S & Hannon GJ (2005) Characterization of Dicer-deficient murine embryonic stem cells. Proceedings of the National Academy of Sciences of the USA 102: 12135–12140.

Mysore K, Sun L, Tomchaney M, Sullivan G, Adams H et al. (2015) siRNA-mediated silencing of doublesex during female development of the dengue vector mosquito *Aedes aegypti*. PLoS Neglected Tropical Diseases 9: e4213.

Naito Y, Yamada T, Matsumiya T, Ui-Tei K, Saigo K & Morishita S (2005) dsCheck: highly sensitive off-target search software for double-stranded RNA-mediated RNA interference. Nucleic Acids Research 33: W589–W591.

Napoli C, Lemieux C & Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. Plant Cell 2: 279–289.

Noland CL & Doudna JA (2013) Multiple sensors ensure guide strand selection in human RNAi pathways. RNA 19: 639–648.

Noland CL, Ma E & Doudna JA (2011) siRNA repositioning for guide strand selection by human Dicer complexes. Molecular Cell 43: 110–121.

Okamura K, Chung WJ, Ruby JG, Guo H, Bartel DP & Lai EC (2008) The *Drosophila* hairpin RNA pathway generates endogenous short interfering RNAs. Nature 453: 803–806.

Orban TI & Izaurralde E (2005) Decay of mRNAs targeted by RISC requires XRN1, the Ski complex, and the exosome. RNA 11: 459–469.

Paim RMM, Araujo RN, Lehane MJ, Gontijo NF & Pereira MH (2013) Long-term effects and parental RNAi in the blood feeder *Rhodnius prolixus* (Hemiptera; Reduviidae). Insect Biochemistry and Molecular Biology 43: 1015–1020.

Pak J & Fire A (2007) Distinct populations of primary and secondary effectors during RNAi in *C. elegans*. Science 315: 241–244.

Pan ZH, Gao K, Hou CX, Wu P, Qin GX et al. (2016) Corrigendum to 'dsRNA interference on expression of a RNA-dependent RNA polymerase gene of *Bombyx mori* cytoplasmic polyhedrosis virus' [Gene (2015) 565: 56–61]. Gene 586: 282.

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

Peng W, Zheng W, Handler AM & Zhang H (2015) The role of the transformer gene in sex determination and reproduction in the tephritid fruit fly, *Bactrocera dorsalis* (Hendel). Genetica 143: 717–727.

Pridgeon JW, Zhao L, Becnel JJ, Strickman DA, Clark GG & Linthicum KJ (2008) Topically applied AaeIAP1 double-stranded RNA kills female adults of *Aedes aegypti*. Journal of Medical Entomology 45: 414–420.

Rand TA, Petersen S, Du FH & Wang XD (2005) Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. Cell 123: 621–629.

Ratzka C, Gross R & Feldhaar H (2013) Systemic gene knockdown in *Camponotus floridanus* workers by feeding of dsRNA. Insectes Sociaux 60: 475–484.

Rebijith KB, Asokan R, Hande HR, Kumar NKK, Krishna V et al. (2016) RNA interference of odorant-binding protein 2 (OBP2) of the cotton aphid, *Aphis gossypii* (Glover), resulted in altered electrophysiological responses. Applied Biochemistry and Biotechnology 178: 251–266.

Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS & Khvorova A (2004) Rational siRNA design for RNA interference. Nature Biotechnology 22: 326–330.

Ruiz N, De Abreu LA, Parizi LF, Kim TK, Mulenga A et al. (2015) Non-invasive delivery of dsRNA into de-waxed tick eggs by electroporation. PLoS ONE 10: e130008.

Saleh MC, Tassetto M, van Rij RP, Goic B, Gausson V et al. (2009) Antiviral immunity in *Drosophila* requires systemic RNA interference spread. Nature 458: 346–350.

Salvemini M, Robertson M, Aronson B, Atkinson P, Polito LC & Saccone G (2009) *Ceratitis capitata* transformer-2 gene is required to establish and maintain the autoregulation of Cctra, the master gene for female sex determination. International Journal of Developmental Biology 53: 109–120.

Sandoval-Mojica AF & Scharf ME (2016) Silencing gut genes associated with the peritrophic matrix of *Reticulitermes flavipes* (Blattodea: Rhinotermitidae) increases susceptibility to termiticides. Insect Molecular Biology 25: 734–744.

Schnettler E, Donald CL, Human S, Watson M, Siu RW et al. (2013) Knockdown of piRNA pathway proteins results in enhanced Semliki Forest virus production in mosquito cells. Journal of General Virology 94: 1680–1689.

Schwarz DS, Hutvágner G, Du T, Xu Z, Aronin N & Zamore PD (2003) Asymmetry in the assembly of the RNAi enzyme complex. Cell 115: 199–208.

Scott JG, Michel K, Bartholomay LC, Siegfried BD, Hunter WB et al. (2013) Towards the elements of successful insect RNAi. Journal of Insect Physiology 59: 1212–1221.

Shim JK, Lee GS, Lee S & Lee KY (2015) Oral ingestion of heat shock protein 70 dsRNA is lethal under normal and thermal stress conditions in the sweetpotato whitefly, *Bemisia tabaci*. Journal of Asia-Pacific Entomology 18: 797–800.

Shukla JN & Palli SR (2012) Sex determination in beetles: production of all male progeny by Parental RNAi knockdown of transformer. Scientific Reports 2: 602.

Shukla JN, Kalsi M, Sethi A, Narva KE, Fishilevich E et al. (2016) Reduced stability and intracellular transport of dsRNA contribute to poor RNAi response in lepidopteran insects. RNA Biology 13: 656–669.

Sijen T, Fleenor J, Simmer F, Thijssen KL, Parrish S et al. (2001) On the role of RNA amplification in dsRNA-triggered gene silencing. Cell 107: 465–476.

Singh AD, Wong S, Ryan CP & Whyard S (2013) Oral delivery of double-stranded RNA in larvae of the yellow fever mosquito, *Aedes aegypti*: implications for pest mosquito control. Journal of Insect Science 13: 6901.

Song JJ, Smith SK, Hannon GJ & Joshua-Tor L (2004) Crystal structure of argonaute and its implications for RISC slicer activity. Science 305: 1434–1437.

Sontheimer EJ (2005) Assembly and function of RNA silencing complexes. Nature Reviews Molecular Cell Biology 6: 127–138.

Sørensen JG, Addison MF & Terblanche JS (2012) Mass-rearing of insects for pest management: challenges, synergies and advances from evolutionary physiology. Crop Protection 38: 87–94.

Sugahara R, Tanaka S, Jouraku A & Shiotsuki T (2017) Geographic variation in RNAi sensitivity in the migratory locust. Gene 605: 5–11.

Swevers L, Huvenne H, Menschaert G, Kontogiannatos D, Kourti A et al. (2013) Colorado potato beetle (Coleoptera) gut transcriptome analysis: expression of RNA interference-related genes. Insect Molecular Biology 22: 668–684.

Tabashnik BE, Mota-Sanchez D, Whalon ME, Hollingworth RM & Carriere Y (2014) Defining terms for proactive management of resistance to Bt crops and pesticides. Journal of Economic Entomology 107: 496–507.

Tabor S (2001) Expression using the T7 RNA polymerase/promoter system. Current Protocols in Molecular Biology Unit16.2. https://doi.org/10.1002/0471142727.mb1602s11

Taning CNT, Christiaens O, Berkvens N, Casteels H, Maes M & Smagghe G (2016) Oral RNAi to control *Drosophila suzukii*: laboratory testing against larval and adult stages. Journal of Pest Science 89: 803–814.

Taracena ML, Oliveira PL, Almendares O, Umaña C, Lowenberger C et al. (2015) Genetically modifying the insect gut microbiota to control Chagas disease vectors through systemic RNAi. PLoS Neglected Tropical Diseases 9: e3358.

Thomas DD, Donnelly CA, Wood RJ & Alphey LS (2000) Insect population control using a dominant, repressible, lethal genetic system. Science 287: 2474.

Tian H, Peng H, Yao Q, Chen H, Xie Q et al. (2009) Developmental control of a lepidopteran pest *Spodoptera exigua* by ingestion of bacteria expressing dsRNA of a non-midgut gene. PLoS ONE 4: e6225.

Tian G, Cheng L, Qi X, Ge Z, Niu C et al. (2015) Transgenic cotton plants expressing double-stranded RNAs target HMG-CoA reductase (HMGR) gene inhibits the growth, development and survival of cotton bollworms. International Journal of Biological Sciences 11: 1296–1305.

Toledo J, Rull J, Oropeza A, Hernandez E & Liedo P (2004) Irradiation of *Anastrepha obliqua* (Diptera: Tephritidae) revisited: optimizing sterility induction. Journal of Economic Entomology 97: 383–389.

Tomari Y & Zamore PD (2005) Machines for RNAi. Genes and Development 19: 517–529.

Tomari Y, Matranga C, Haley B, Martinez N & Zamore PD (2004) A protein sensor for siRNA asymmetry. Science 306: 1377–1380.

Tomari Y, Du T & Zamore PD (2007) Sorting of *Drosophila* small silencing RNAs. Cell 130: 299–308.

Tomoyasu Y, Miller SC, Tomita S, Schoppmeier M, Grossmann D & Bucher G (2008) Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in *Tribolium*. Genome Biology 9: R10.

Toprak U, Baldwin D, Erlandson M, Gillott C, Harris S & Hegedus DD (2013) In vitro and in vivo application of RNA interference for targeting genes involved in peritrophic matrix synthesis in a lepidopteran system. Insect Science 20: 92–100.

Tzin V, Yang X, Jing X, Zhang K, Jander G & Douglas AE (2015) RNA interference against gut osmoregulatory genes in phloem-feeding insects. Journal of Insect Physiology 79: 105–112.

Ulrich J, Dao VA, Majumdar U, Schmitt-Engel C, Schwirz J et al. (2015) Large scale RNAi screen in *Tribolium* reveals novel target genes for pest control and the proteasome as prime target. BMC Genomics 16: 674.

Vagin VV, Sigova A, Li C, Seitz H, Gvozdev V & Zamore PD (2006) A distinct small RNA pathway silences selfish genetic elements in the germline. Science 313: 320–324.

Vaucheret H (2006) Post-transcriptional small RNA pathways in plants: mechanisms and regulations. Genes and Development 20: 759–771.

Wang Y, Sheng G, Juranek S, Tuschl T & Patel DJ (2008) Structure of the guide-strand-containing argonaute silencing complex. Nature 456: 209–U234.

Wang B, Li S, Qi HH, Chowdhury D, Shi Y & Novina CD (2009) Distinct passenger strand and mRNA cleavage activities of human Argonaute proteins. Nature Structural and Molecular Biology 16: 1259–U1276.

Wei Q, Wu SF, Niu CD, Yu HY, Dong YX & Gao CF (2015) Knockdown of the ionotropic γ-aminobutyric acid receptor (gabar) rdl gene decreases fipronil susceptibility of the small brown planthopper, *Laodelphax striatellus* (Hemiptera: Delphacidae). Archives of Insect Biochemistry and Physiology 88: 249–261.

Whangbo JS & Hunter CP (2008) Environmental RNA interference. Trends in Genetics 24: 297–305.

Whitten MMA, Facey PD, del Sol R, Fernández-Martínez LT, Evans MC et al. (2015) Symbiont-mediated RNA interference in insects. Proceedings of the Royal Society B 283: 20160042.

Whyard S, Singh AD & Wong S (2009) Ingested double-stranded RNAs can act as species-specific insecticides. Insect Biochemistry and Molecular Biology 39: 824–832.

Whyard S, Erdelyan CN, Partridge AL, Singh AD, Beebe NW & Capina R (2015) Silencing the buzz: a new approach to population suppression of mosquitoes by feeding larvae double-stranded RNAs. Parasites and Vectors 8: 716.

Winston WM, Molodowitch C & Hunter CP (2002) Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1. Science 295: 2456–2459.

Winston WM, Sutherlin M, Wright AJ, Feinberg EH & Hunter CP (2007) *Caenorhabditis elegans* SID-2 is required for environmental RNA interference. Proceedings of the National Academy of Sciences of the USA 104: 10565–10570.

Wynant N, Santos D, Van Wielendaele P & Vanden Broeck J (2014) Scavenger receptor-mediated endocytosis facilitates RNA interference in the desert locust, *Schistocerca gregaria*. Insect Molecular Biology 23: 320–329.

Xiao D, Gao X, Xu J, Liang X, Li Q et al. (2015) Clathrin-dependent endocytosis plays a predominant role in cellular uptake of double-stranded RNA in the red flour beetle. Insect Biochemistry and Molecular Biology 60: 68–77.

Xiong Y, Zeng H, Zhang Y, Xu D & Qiu D (2013) Silencing the HaHR3 gene by transgenic plant-mediated RNAi to disrupt *Helicoverpa armigera* development. International Journal of Biological Sciences 9: 370–381.

Xu J, Feng HT, Wang C, Yip KH, Pavlos N et al. (2003) Effects of Bafilomycin A1: an inhibitor of vacuolar H (+)-ATPases on endocytosis and apoptosis in RAW cells and RAW cell-derived osteoclasts. Journal of Cellular Biochemistry 88: 1256–1264.

Xue J, Ye Y-X, Jiang Y-Q, Zhuo JIC, Huang H-J et al. (2015) Efficient RNAi of rice planthoppers using microinjection. Protocol Exchange. https://doi.org/10.1038/protex.2015.005.

Yan KS, Yan S, Farooq A, Han A, Zeng L & Zhou MM (2003) Structure and conserved RNA binding of the PAZ domain. Nature 426: 469–474.

Yang J & Han Z-J (2014) Efficiency of different methods for dsRNA delivery in cotton bollworm (*Helicoverpa armigera*). Journal of Integrative Agriculture 13: 115–123.

Ye X, Huang N, Liu Y, Paroo Z, Huerta C et al. (2011) Structure of C3PO and mechanism of human RISC activation. Nature Structural & Molecular Biology 18: 650–U643.

Yi X, Zhao H, Wang P, Hu M & Zhong G (2014) Bdor\Orco is important for oviposition-deterring behavior induced by both the volatile and non-volatile repellents in *Bactrocera dorsalis* (Diptera: Tephritidae). Journal of Insect Physiology 65: 51–56.

Yoon JS, Shukla JN, Gong ZJ, Mogilicherla K & Palli SR (2016) RNA interference in the Colorado potato beetle, *Leptinotarsa decemlineata*: identification of key contributors. Insect Biochemistry and Molecular Biology 78: 78–88.

Yu R, Xu X, Liang Y, Tian H, Pan Z et al. (2014) The insect ecdysone receptor is a good potential target for RNAi-based pest control. International Journal of Biological Sciences 10: 1171–1180.

Yu B, Li DT, Wang SL, Xu HJ, Bao YY & Zhang CX (2016) Ion transport peptide (ITP) regulates wing expansion and cuticle melanism in the brown planthopper, *Nilaparvata lugens*. Insect Molecular Biology 25: 778–787.

Zhang HD, Kolb FA, Brondani V, Billy E & Filipowicz W (2002) Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. EMBO Journal 21: 5875–5885.

Zhang X, Zhang J & Zhu KY (2010) Chitosan/double-stranded RNA nanoparticle-mediated RNA interference to silence chitin synthase genes through larval feeding in the African malaria mosquito (*Anopheles gambiae*). Insect Molecular Biology 19: 683–693.

Zhang J, Khan SA, Hasse C, Ruf S, Heckel DG & Bock R (2015a) Full crop protection from an insect pest by expression of long double-stranded RNAs in plastids. Science 347: 991–994.

Zhang X, Mysore K, Flannery E, Michel K, Severson DW et al. (2015b) Chitosan/interfering RNA nanoparticle mediated gene silencing in disease vector mosquito larvae. Journal of Visualized Experiments 97: e52523.

Zheng W, Liu Y, Zheng W, Xiao Y & Zhang H (2015) Influence of the silencing sex-peptide receptor on *Bactrocera dorsalis* adults and offspring by feeding with ds-spr. Journal of Asia-Pacific Entomology 18: 477–481.

Zhou X, Wheeler MM, Oi FM & Scharf ME (2008) RNA interference in the termite *Reticulitermes flavipes* through ingestion of double-stranded RNA. Insect Biochemistry and Molecular Biology 38: 805–815.

Zhu F, Xu J, Palli R, Ferguson J & Palli SR (2011) Ingested RNA interference for managing the populations of the Colorado potato beetle, *Leptinotarsa decemlineata*. Pest Management Science 67: 175–182.

Zhu JQ, Liu S, Ma Y, Zhang JQ, Qi HS et al. (2012) Improvement of pest resistance in transgenic tobacco plants expressing dsRNA of an insect-associated gene EcR. PLoS ONE 7: e38572.

Zwier MV, Verhulst EC, Zwahlen RD, Beukeboom LW & van de Zande L (2012) DNA methylation plays a crucial role during early *Nasonia* development. Insect Molecular Biology 21: 129–138.