

Wolbachia-mosquito interactions and engineered female-specific lethality for *Aedes*-arbovirus control



Jennifer C Molloy
Jesus College
University of Oxford

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“Our own studies are not so complete or so systematic as we could wish,
but pressure of other matters has made it desirable to present at this
time such data as have been obtained.”

Shannon, R. C., & Frobisher, M. (1931). A comparison of the effect
of various substances upon larvae of *Aedes aegypti*. American Journal
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Abstract

Aedes aegypti and *Aedes albopictus* are the primary vectors of dengue (DENV) and chikungunya (CHIKV) viruses, causing millions of infections annually. Novel genetic mosquito control methods are being trialled, including population replacement using disease refractory mosquitoes transinfected with the intracellular bacterial endosymbiont *Wolbachia*, and population suppression by release of male mosquitoes carrying self-limiting genes.

The underlying cause of *Wolbachia*-induced DENV refractoriness is unconfirmed. In Chapter 2, I examine in *Ae. albopictus* the hypothesis that reactive oxygen species (ROS) induced immune activation plays a role, but neither ROS nor innate immune pathways were upregulated. In Chapter 3, an untargeted analysis of the *Ae. albopictus* lipidome showed it is significantly altered by *Wolbachia* infection, highlighting sphingolipids as candidates for future investigations into DENV-refractoriness.

Genetic constructs for population suppression are better characterised than *Wolbachia*-host interactions but incompletely described modes of action and reliance on random genome integration to control expression fails to take full advantage of engineering approaches to design optimal genetic control phenotypes. In Chapter 4, I engineer female-specific, late-acting lethality in *Ae. aegypti* using specific effectors crossed with candidate pupal-stage promoters and a *doublesex* cassette causing sex-specific splicing. In Chapter 5, I transform and characterise *Ae. aegypti* lines producing sex-specific *Drosophila melanogaster* alcohol dehydrogenase (*Adh*) to evaluate potential for early-stage inducible genetic sexing. No effective lines were produced but I suggest future design alterations.

Overall, this thesis contributes to understanding the molecular mechanisms underlying two distinct genetic control strategies in *Aedes* mosquitoes. It promotes the use of that mechanistic knowledge to engineer optimal traits for control and for improving mass-rearing methods to meet the clear need for effective and economical area-wide control of arbovirus vectors.

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Chapter 1

Introduction

1.1 Global burden of mosquito-borne disease

There are approximately 3,200 mosquito species globally (Becker, 2010), of which a small proportion are vectors for human diseases causing more than 1.5 million deaths per year (Hill et al., 2005). Most of these fatalities are caused by malaria, an apicomplexan parasite transmitted by mosquitoes of the *Anopheles* genus. However, diseases such as lymphatic filariasis, West Nile virus, yellow fever, Japanese encephalitis and a wide range of other parasitic and viral pathogens transmitted primarily by *Aedes* and *Culex* mosquitoes also impose a significant global disease burden.

The most prevalent arboviral disease is dengue fever, caused by four serotypes of the dengue RNA *Flavivirus* (DENV-1 to -4; family *Flaviviridae*). 2.5 billion people are at risk across the tropics (Gubler, 2002) (Figure 1.1) and DENV has been estimated to cause 96 million symptomatic cases per year from a total of 390 million infections (Bhatt et al., 2013). While the estimated annual mortality of 20,000 people is far lower than for malaria at over one million (Hill et al., 2005), the disease still causes significant morbidity that is likely to be severely underestimated owing to a lack of reliable data and its non-specific clinical presentation. Symptoms range from mild to severe and include fever, nausea and vomiting, headache, abdominal pain, muscle aches and rashes (WHO, 2009). The most severe and potentially fatal cases are categorised as dengue hemorrhagic fever (DHF), characterised by severe plasma leakage or haemorrhaging accompanied by respiratory distress. This manifestation requires intensive care, although with intravenous fluid administration the DHF death rate can be reduced to 1% (WHO, 2009). The high incidence of dengue, which has seen a 30-fold increase in reported figures across the last 50 years, combined with

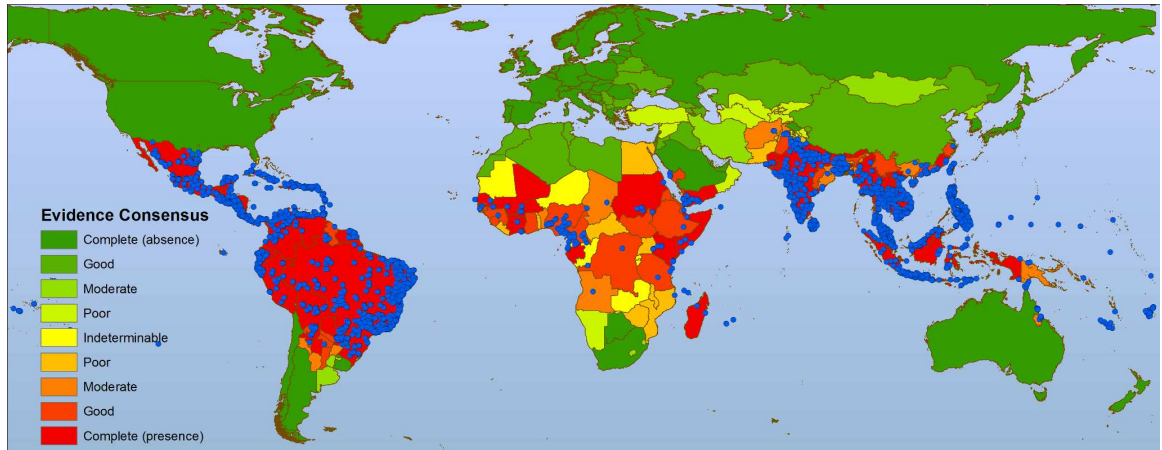


Figure 1.1: Map indicating presence and absence of dengue and quality of evidence for that conclusion in countries worldwide. Blue dots represent locations of experimental field sites. Figure is taken from Brady et al. (2012) and is licensed under CC-BY 2.0.

explosive outbreak dynamics and potentially serious health effects, results in a high social and economic burden for individual citizens as well as the health systems and infrastructure of affected countries (Gubler, 2002).

The true economic cost of dengue outbreaks is challenging to calculate, but Shepard et al. (2013) propose an annual figure of USD 950 million for 12 countries in Southeast Asia, with a demographic and social burden of 372 disability-adjusted life years (DALYs) per million people. Brazil alone is estimated to spend USD one billion annually on controlling the disease and still registered 1.2 million dengue cases in 2010 (WHO, 2012). There is hence a major effort underway to minimise the human and economic costs, with the World Health Organisation (WHO) aiming to reduce dengue related mortality and morbidity by 50% and 25% respectively by 2020 (WHO, 2012).

Both mortality and morbidity can be lessened by improved diagnosis and care, but with no curative clinical interventions available, disruption of transmission is key. There is currently no effective vaccine against all four serotypes; the most advanced candidate to reach phase III clinical trials is a live attenuated tetravalent vaccine (CYD TDV) which gave only 30.2% efficacy overall and failed to protect against infection by DENV-2 (Sabchareon et al., 2012). Other vaccine candidates are in development but the focus of control currently falls on the vectors - *Aedes* mosquitoes. The primary vector is the ‘yellow fever mosquito’ *Aedes aegypti* (Linnaeus, 1762) with the ‘Asian tiger mosquito’ *Ae. albopictus* (Skuse, 1894) acting as an important secondary vector. *Ae. polynesiensis* and other members of the *Ae. scutellaris* com-

plex are also thought to have been responsible for outbreaks in the South Pacific region (Lardeux et al., 2002).

In addition to reducing dengue virus transmission, vector control has the additional benefit of controlling other diseases transmitted by the same mosquitoes, which include yellow fever (YFV; family *Flaviviridae*), another *Flavivirus* causing 30,000 deaths per year, and Chikungunya (CHIKV; family *Togaviridae*), an *Alphavirus* which causes a similar febrile illness to DENV but with severe joint pain and arthritic symptoms lasting for weeks to months after the acute phase of the illness. The opportunity to intervene in transmission of multiple conditions makes vector control an attractive option and a critical focus of the WHO strategy for combating dengue 2012-2020.

“Dengue morbidity can be reduced by implementing improved outbreak prediction and detection through coordinated epidemiological and entomological surveillance; promoting the principles of integrated vector management; deploying locally-adapted vector control measures, including effective urban and household water management; and through communication to achieve behavioural outcomes in prevention programmes.” (WHO, 2012).

1.2 Vector Distribution and Ecology

Ae. aegypti and *Ae. albopictus* (Figure 1.2) are part of the family Culicidae, which contains the genus *Aedes* and its sister genus *Culex*. Both species are part of the subgenus *Stegomyia*, which recent taxonomies have raised to the level of genus (Reinert et al., 2004). This taxonomic designation has not been adopted in the majority of the current literature so *Aedes* will be used throughout this thesis to maintain consistency.

Ae. aegypti originated in Africa while *Ae. albopictus* is native to Southeast Asia and islands in the West Pacific and Indian Oceans. Both species have seen a considerable expansion in their geographic range, facilitated by human activity and globalisation. *Ae. aegypti* likely expanded via trading ships in the 15–17th century with a more recent invasion of Asia in the 19th century (Brown et al., 2013; Christophers et al., 1960; Jansen and Beebe, 2010).

Ae. albopictus has invaded the Americas, Southern Europe and Southern Africa in the last few decades via importation of eggs and larvae in intercontinental shipments of tyres or plants, supported by increasing levels of international air travel (Paupy

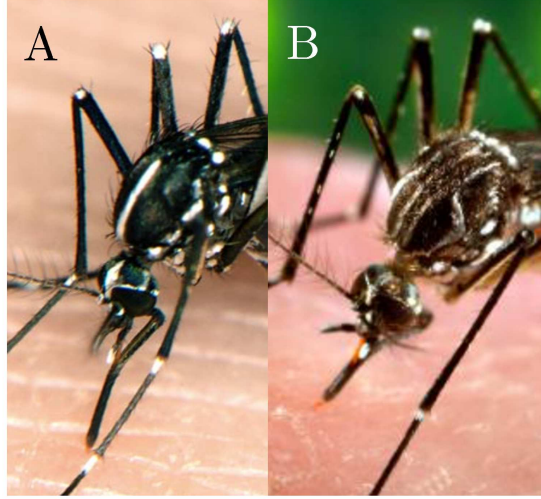


Figure 1.2: A) An adult female *Ae. albopictus* with straight white thoracic markings and typical white-banded legs. B) An adult female *Ae. aegypti* with lyre-shaped thoracic markings, which are the most prominent distinguishing features of the two species. *Ae. aegypti* photo by James Gathney of the United States Centre for Disease Control (US CDC), a Public Domain image sourced from Wikimedia Commons. *Ae. albopictus* photo by Susan Ellis, licensed under CC-BY 3.0 and sourced from Wikimedia Commons.

et al., 2009). There are concerns their range might expand to temperate regions as climate change renders these areas warmer. This is a particular concern with *Ae. albopictus*, as eggs can enter diapause and overwinter, a trait which leads them to be considered a serious risk by the European Environment Agency. In Italy, the species is already prevalent and has been responsible for circulating viruses brought into the country by infected travellers from endemic regions, notably during the 2007 CHIKV outbreak in Ravenna (Bonilauri et al., 2008).

Ae. albopictus is found in sympatry with *Ae. aegypti* in the Americas and elsewhere. However, many studies document interspecies competition, which in some cases has led to displacement or exclusion of *Ae. aegypti*. A decline in Brazilian *Ae. aegypti* populations was documented just 20 years after the introduction of *Ae. albopictus* due to competition at the larval stage (Braks et al., 2004) and this pattern has been mirrored in parts of the United States, although *Ae. aegypti* is still abundant in the south of Florida (O’Meara et al., 1995). There is also evidence of asymmetrical reproductive interference (satyrism) caused by interspecific mating, which leads to rapid evolution of species recognition and mate preference in *Ae. albopictus*-naïve *Ae. aegypti* when the two populations are mixed in laboratory trials (Bargielowski and

Lounibos, 2014). This suggests that interspecific interactions between these two sister species have evolutionary and behavioural consequences which could be relevant to control strategies.

1.2.1 Habitat and Life Cycle

While originally forest-dwelling, both *Ae. aegypti* and *Ae. albopictus* have adapted to urban environments. *Ae. albopictus* was originally zoophilic but has become progressively adapted to feed on domestic animals and humans. It has evolved to breed in water sources in human-inhabited rural and suburban areas, with some identifications in more densely populated cities (Paupy et al., 2009). The domestication of *Ae. aegypti* has progressed further and it is adapted to densely populated tropical cities as well as the peridomestic habitats preferred by *Ae. albopictus*. Genetic studies confirm multiple domestications of *Ae. aegypti* within Africa and independently outside the continent from the ancestral Sub-Saharan forest-dwelling population (Brown et al., 2011). This species is more likely to be found in densely inhabited towns and cities, where it has even been discovered breeding in septic tanks (WHO, 2012).

Both species have highly varied breeding sites depending on availability and this facilitates their invasion of new territory. Eggs are laid just above the waterline in either natural or artificial containers e.g. tree or bamboo stumps, rain water tanks, buckets or stacked car tyres. They typically hatch within a few days although they can survive desiccation for several months and in the case of *Ae. albopictus*, may enter diapause and overwinter. Once hatched, larvae pass through four instars before pupation and eclosion (Figure 1.3), whereupon adult mosquitoes mate and females seek a blood meal to enable egg production.

1.2.2 Biting habits and contribution to disease transmission

Ae. aegypti females are strongly anthropophilic while *Ae. albopictus* obtains blood from a variety of hosts but prefers mammals and specifically humans (Delatte et al., 2010; Richards et al., 2006; Valerio et al., 2010). Host generalism along with other factors including differences in vector competence result in a reduced role for *Ae. albopictus* in DENV and CHIKV transmission compared to the almost exclusively human feeding *Ae. aegypti* (Gratz, 2004; Lambrechts et al., 2010). This is borne out by the decreased severity of outbreaks vectored entirely by *Ae. albopictus* (Gubler, 2002; Lambrechts et al., 2010). Nevertheless, *Ae. albopictus* has been responsible

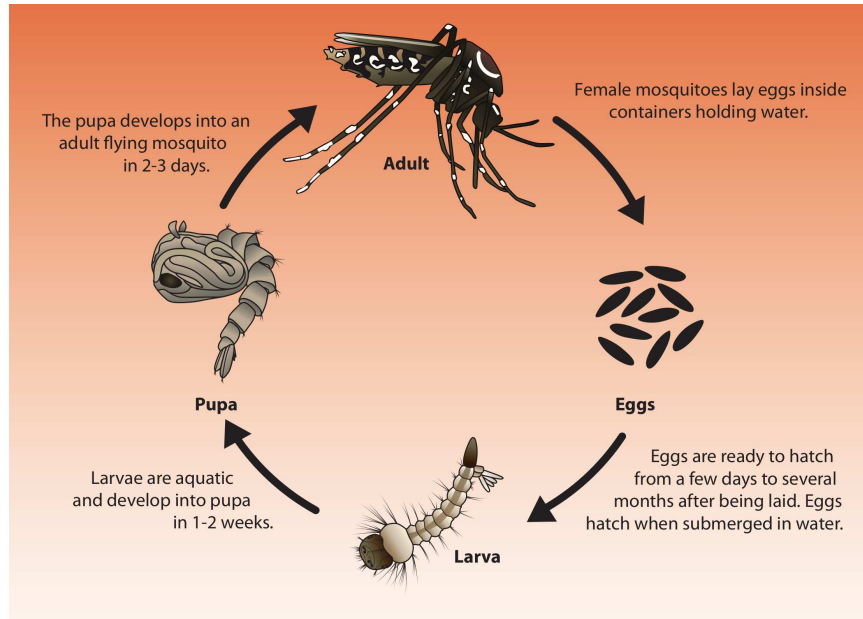


Figure 1.3: Typical life cycle of *Aedes* mosquitoes. Public Domain image generated by US CDC (CDC, 2014).

for major epidemics of DENV and CHIKV, such as the 2005-2006 outbreak, which was particularly severe on the island of Réunion (Reiter et al., 2006) and the 2001 DENV outbreak in Hawaii (Effler et al., 2005). In 2003, *Ae. albopictus* was identified as the primary vector of CHIKV in Dhaka, Bangladesh, despite the presence of *Ae. aegypti* (Ali et al., 2003). This demonstrates that the role of each species in an outbreak is variable and context-dependent.

One particular concern given the generalist host choice of *Ae. albopictus* is that the species could act as a bridging vector and play a role in the transmission and emergence of novel zoonotic diseases. It is already thought that dengue circulation is maintained between human outbreaks in Southeast Asia and the Pacific Islands by a sylvatic cycle involving other primates and monkeys, vectored by *Ae. albopictus* (Paupy et al., 2009). Other viruses known to be maintained in non-human hosts for which *Ae. albopictus* is a competent vector include West Nile (WNV) (Farajollahi and Nelder, 2009; Gubler, 2007; Sardelis et al., 2002), La Crosse (LCV) (Grimstad et al., 1989; Lambert et al., 2010) and Venezuelan Equine Encephalitis (VEEV) (Weaver and Reisen, 2010) viruses in the USA plus YFV in Brazil and Africa (Maciel-de Freitas et al., 2006; Savage et al., 1992). Hence, it is important that both vectors are the subject of control efforts despite disease transmission by *Ae. aegypti* currently constituting the most significant public health risk.

While host preferences differ, other biting habits are similar between the two species. Both are day-biting, with activity peaking in the early morning and late afternoon. This makes measures to limit biting such as bed nets ineffective in contrast to control of nocturnal biting by *Anopheles* mosquitoes, where insecticide treated bed nets have seen considerable success in reducing malaria transmission when used appropriately (Lengeler et al., 2004). Therefore, alternative methods of control have been sought.

1.3 Chemical and biological control measures to reduce dengue transmission

There are two main outcomes for any given vector control measure which might lead to reduced dengue transmission; one is population suppression, a reduction in the absolute numbers of the adult female mosquito population; the other is population replacement, which seeks to introduce and replace wild populations with mosquitoes that are unable to transmit disease. These approaches are not mutually exclusive and can be used in combination as part of an integrated vector management (IVM) programme.

The following section will present examples of both techniques before focusing on genetic control and particularly the mass-release of genetically sterile males for population suppression and the use of the endosymbiotic bacteria *Wolbachia* for population replacement.

1.3.1 Habitat Management and Chemical Population Suppression

Removal of mosquito habitats was one of the earliest forms of vector control and remains effective today. Changes in water usage and storage in both rural and urban environments can reduce opportunities for mosquito oviposition and the growth of larvae e.g. upturning containers to prevent water pooling. Where drainage is difficult, the use of oil or other film-forming chemicals may be used to prevent larvae breathing and developing. However, such chemicals are also hazardous to other organisms, which raises the primary issue with this form of control — non-specificity and the potential for environmental damage and even human health consequences.

Use of insecticides, commonly organophosphate compounds, can be highly effective for both larval water treatment and adult control via ultra low-volume (ULV) spraying ('thermal fogging') of large urban areas and application of long lasting residual insecticide to indoor walls where *Aedes* rest. However, all of the aforementioned disadvantages are amplified by the need to apply large volumes and for long periods to prevent re-invasion. As an example, Dichlorodiphenyltrichloroethane (DDT) based insecticides successfully eradicated *Ae. aegypti* from large parts of North and South America in the 1960s (Gubler, 2005) but re-invasion was rapid when treatment was stopped in the 1970s owing to recognition of the persistence of DDT in the environment and the risk of bio-accumulation in predators (WWF, 1999).

In addition to concerns about ecotoxicity and human health risks, use of chemical controls can force the rapid development of resistance as they exert a high selection pressure on populations and insecticide resistance is a growing problem (reviewed in Hemingway and Ranson, 2000). Resistance has arisen in *Ae. aegypti* to all common chemical families such as organophosphates, pyrethroids, carbamates and organochlorines amid mounting evidence that this is negatively affecting control efforts (reviewed in Vontas et al., 2012). *Ae. albopictus* populations exhibit less resistance than *Ae. aegypti* but this is expected to change as their range expands into heavily treated areas (Vontas et al., 2012).

Chemical treatments offer scalability over wide areas and this gives them an advantage over methods such as adult trapping through use of synthetic lures, which are typically deployed on a local scale. Traps disseminate a mixture of ammonia, fatty acids and lactic acids which mimic human scent and then kill mosquitoes attracted into them. Widespread use can reduce local populations (Kline, 2006; Okumu et al., 2010), but they are prohibitively expensive. Some projects seek to combine trapping with useful tools for economic development such as providing electricity for LED lighting and mobile phone charging (e.g. Okumu, 2014). Even so, these methods remain more suited to local control and vector surveillance rather than area-wide control programmes.

1.3.2 Classical Biological Population Suppression

The need for species specificity, reduction of risk to environmental and human health and area-wide scalability at low cost has led to a focus on biological control. One biological control method differs little from insecticide control except it uses toxins

produced by the bacteria *Bacillus sphaericus* (*Bs*) or *Bacillus thuringiensis israelensis* (*Bti*) rather than synthetic chemicals. While more specific, targeting only the larvae of mosquitoes, black flies and midges (Lacey and Undeen, 1986) and more environmentally friendly (Glare and O’Callaghan, 1998), there is considerable risk of resistance emerging in *Aedes* populations as *Bti* spores persist in the environment (Tilquin et al., 2008) and resistance has been induced multiple times in the laboratory (Paris et al., 2011; Tetreau et al., 2012) as well as being observed in natural populations of other mosquitoes such as *Culex pipiens* (e.g. Saleh et al., 2003).

In contrast, classical biological control uses natural pathogens, parasites or predators of mosquitoes. By introducing a living and co-evolving control agent, resistance is arguably less likely to arise than with chemical insecticides, which typically target a single or limited set of neurotransmitters or vital cellular components. In this case, resistance can evolve following even a single amino acid substitution such as the knockdown resistance *kdr* mutation causing permethrin resistance, which has convergently evolved in multiple independent populations of multiple species (reviewed in Rinkevich et al., 2013).

Larval predators include the mosquito fish *Gambusia affinis* and copepods such as *Mesocyclops*. Larvivorous fish have commonly been used to control *Anopheles* mosquitoes although a systematic review of the literature found insufficient evidence for their effectiveness (Walshe et al., 2013) and most evidence for *Aedes* control is based on lab studies (reviewed in Chandra et al., 2013). Some positive local and national results have been achieved using copepods; notably, a community-based programme in Vietnam used *Mesocyclops* to eliminate *Ae. aegypti* from 40 out of 46 study sites (Nam et al., 2012). Copepod-mediated control of *Ae. aegypti* in artificial water containers in Florida also showed promise (Soumare and Cilek, 2011). However, problems have arisen from biocontrol organisms disrupting the ecosystem to which they are introduced and becoming a pest in their own right. *Gambusia affinis* has itself become the target of biocontrol after displacing native fish species through resource competition and predation of fish eggs (Lacey and Orr, 1993), resulting in a ban from Australia (Department of Employment and Innovation, 2013).

Many pest species have natural parasitoids that have been used for biological control. This is not true of mosquitoes, possibly because of their aquatic oviposition sites and larval environment. However, they do have numerous natural pathogens. Entomopathogenic nematodes (Kaya et al., 1993), protozoa (Lorenz and Koella, 2011;

Solter and Becnel, 2007) and fungi have all been explored as potential vector control agents, with the latter being the closest to field deployment. Several fungi can be lethal to *Ae. aegypti* (Darbro et al., 2011; Scholte et al., 2004), including natural pathogens of mosquito larvae such as *Lagenidium*, *Coelomomyces*, and *Culicinomyces* and others that are rarely seen as natural infections, such as *Beauveria bassiana* and various species of *Metarhizium*.

In addition to being lethal or life-shortening, there is some evidence that certain entomopathogenic fungi can reduce DENV-vectorial capacity in *Ae. aegypti* (Garza-Hernández et al., 2013). Challenges remain in culturing and widely distributing these entomopathogenic microorganisms to larval habitats. While classical biological control addresses some concerns around resistance, issues with environmental protection and species specificity are not fully resolved.

1.4 Genetic Population Suppression

The most direct way to ensure species specificity and minimise off-target effects is to turn the mosquito itself into a biocontrol agent and all genetic techniques share this common approach. This is an advantage compared to the biocontrol methods described above as mosquitoes are highly effective at finding and mating with other mosquitoes, whereas breeding site discovery and even non-specific predation may hamper traditional biocontrol efforts. Genetic control encompasses population suppression or replacement by introgression of desirable traits such as DENV refractoriness into populations.

Sterile insect technique (SIT) describes the strategy of releasing sterile individuals into a wild population, whereupon their matings with wild-type individuals result in inviable offspring and the total population size decreases (Dyck et al., 2005). Historically, sterility is achieved by irradiation of mass-reared males with gamma rays or chemicals to randomly induce major chromosomal aberrations and dominant lethal mutations which impede fertilisation and subsequent viability of offspring. Sterilisation and release of males is optimal as it removes the possibility that sterile males will mate with co-released sterile females rather than seeking wild-type mates (Rendón et al., 2004). In mosquitoes it is also important from a safety and regulatory perspective as females pose a biting nuisance and a disease transmission risk.

SIT has been used successfully for many decades, notably eradicating the livestock pest New World Screwworm *Cochliomyia hominivorax* from North America and controlling the crop pest *Ceratitidis capitata* (Mediterranean fruit fly). All such efforts rely on sustained releases to prevent re-invasion from outside the treated area, for example the Screwworm SIT programme continues across Panama to form a barrier to re-establishment from South America (Klassen and Curtis, 2005; Krafur, 1998).

Trials to control disease vectors such as the trypanosomiasis transmitting Tsetse fly *Glossina austeni* and various mosquitoes including *Culex*, *Anopheles* and *Aedes* species have been attempted over the last five decades, with varying levels of success (reviewed in Dyck et al., 2005). Enough data has been collected to summarize the benefits and challenges of mosquito SIT. SIT relies on high-throughput production of males that are competitive with wild-type, reliable sex separation for male-only releases and high levels of sterility in the release cohort. However, these characteristics have not been easy to achieve. Sufficient mass-production of males was a barrier to some release efforts (Benedict and Robinson, 2003) and much research is now focused on optimising mass-rearing conditions. Sex separation was achieved by pupal size sorting to separate out the larger females in *Aedes* and *Culex* species but there is little size disparity between the sexes in *Anopheles* making this method ineffective (Papathanos et al., 2009).

Penetrance was also sometimes limited, in the sense that levels of sterility were far less than 100%. This is to be expected as not all of the mutations induced randomly by irradiation will be detrimental enough to result in complete sterility (Klassen and Curtis, 2005). Ecological factors such as immigration of mated females from outside the release area reduced effectiveness and emphasised the importance of planning releases using understanding of their geographic and ecological context (Dame et al., 2009). In the 1970s various programmes used irradiation to induce targeted female-lethal chromosomal translocations. These encountered serious problems with male fitness, competitiveness and stability of the translocations, which have since been conflated with the results of classical SIT programmes (Black IV et al., 2011). However, despite the challenges described above, SIT was on balance successful and a major review concluded that ‘there are no fundamental technical barriers to large-scale field use of SIT for many important mosquito species’ (Alphey et al., 2010).

This is not to say that SIT cannot be significantly improved by modern methodology. Many of the challenges with reduced male fitness, incomplete sterility and un-

stable female-lethal phenotypes arising from the irradiation process can be addressed by designed induction of sterility using genetic engineering and genome editing techniques. This approach offers the potential to create more reliably sterile males with minimal loss of fitness. In addition, genetic engineering allows us to move beyond basic sterility traits and design more sophisticated lethal mechanisms based on our knowledge of vector ecology. There is potential to design and introduce one or several desirable traits into populations for the purposes of disease control, including disease refractoriness.

1.4.1 Transgenic approaches to population suppression

Transgenes designed to suppress populations may mirror traditional sterile insect traits. For example, expressing homing endonuclease genes (HEGs) within developing *An. gambiae* spermatids fragments chromosomal DNA and renders sperm unable to viably fertilise ova (Windbichler et al., 2008). However, the flexibility of transgenic techniques allows the introduction of other traits which ultimately result in population suppression such as sex-ratio distortion, lethality through zygotic gene expression and female killing. Common features of these techniques are:

1. Most are self-limiting to an extent. Genes that cause fully penetrant lethality will not persist beyond the first generation while other genes may persist longer but all require repeated release of transgenic individuals into target populations.
2. All require either a way to switch off the lethal phenotype or a distinct compatible population with which to breed in the laboratory or mass-rearing facility.

A range of transgenic approaches to suppression will be briefly described below followed by a more detailed treatment of dominant lethal gene expression in offspring.

1.4.2 Sex-ratio distortion using HEGs

Some transgenic population suppression methods differ from SIT by targeting sex ratio. The HEGs which caused embryonic lethality in *An. gambiae* were initially designed to shred X chromosomes to skew the population ratio towards males and work continues on developing this system (Windbichler et al., 2008). As only females blood-feed and thus vector diseases, skewing the sex ratio reduces the number of

competent vectors in the population, in addition to reducing population size over time.

HEGs originate in bacteria and encode proteins which recognise a specific DNA sequence, typically at the same locus as the HEG. They cut this sequence, causing a double stranded break. The HEG on the homologous chromosome is then used as the template for DNA repair, inserting an additional copy into the genome and creating an identical allele in an action known as ‘homing’ (Figure 1.4). If homing occurs in the germline then a heterozygote becomes a homozygote and transmits the gene to all of their offspring. HEGs are thus selfish genetic elements and form the basis of a gene drive mechanism. One particular form of HEG serendipitously cuts a sequence which is found repetitively along the *An. gambiae* X chromosome. This results in shredding because male cells are heterogametic (XY) and hence there is no homologous X chromosome from which to repair the breaks. Where the X targeting HEG is located on an autosome the effect is semi-invasive but if it is located on the Y chromosome this produces a meiotic drive effect and could lead to a very substantial population reduction (Alphey, 2014; Burt, 2014).

This method is unfortunately not transferable to *Ae. aegypti* as sex determination is autosomal, but other uses for HEGs are being explored, such as engineering HEG proteins to recognise and disrupt a wide range of sequences, including genes linked to vector competence. HEGs may therefore offer a gene drive mechanism for population replacement rather than suppression.

1.4.3 Self-limiting genetic population suppression

Some lethal transgenes are expressed in the zygote or later in the development of the offspring rather than in the parent (Phuc et al., 2007). This can be advantageous as there is greater flexibility of timing and site of expression. On the other hand, there is also a higher potential for interference by environmental effects, gene interactions and genetic background.

The essential elements of a dominant lethal gene system for population control are a method of genome integration, a conditional switch to ensure the gene is turned off in laboratory breeding colonies, a set of regulatory elements to exert control over the spatio-temporal expression of the lethal gene and an effector, the lethal gene itself.

The basic design of the conditional switch system used in self-limiting genetic population suppression, also known as Release of Insects carrying a Dominant Lethal

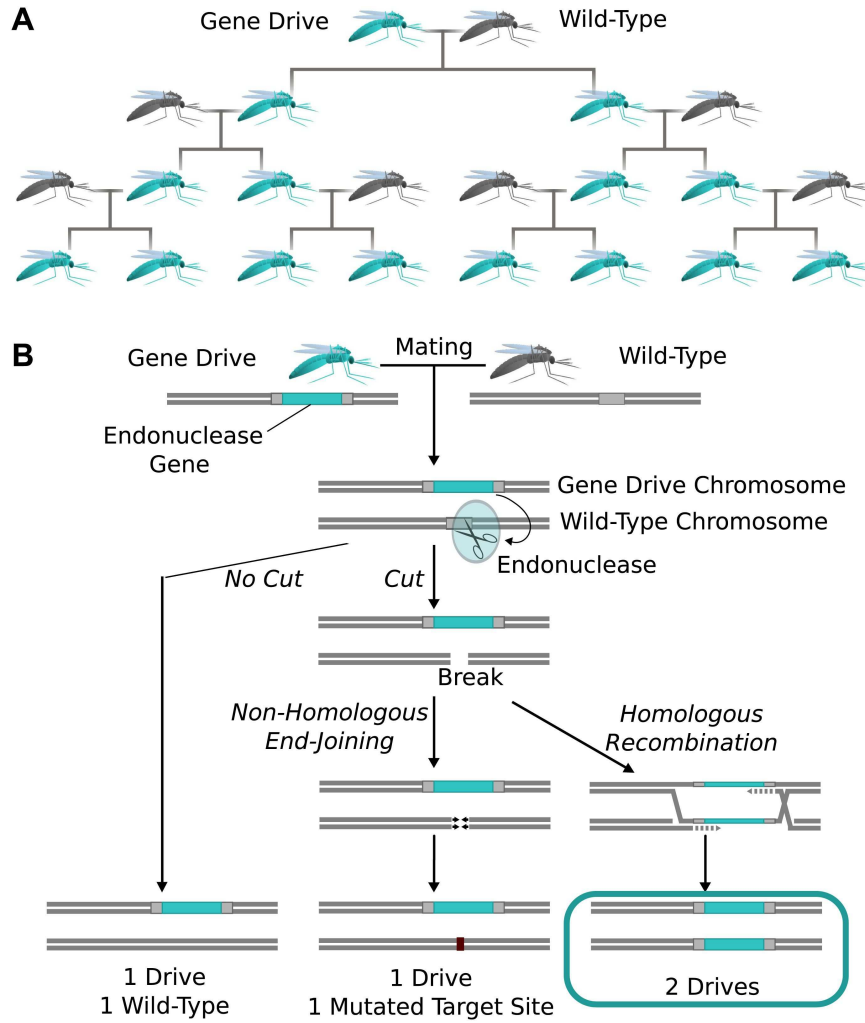


Figure 1.4: A) HEG-carrying organisms (blue) mating with wild-type organisms (grey) will result in >50% offspring inheriting the HEG and thus allow the gene to spread to fixation or near-fixation even if it is mildly deleterious. B) HEGs spread because they cut the homologous chromosome and are then used as a template during homologous recombination to repair the double strand break. In cases where the competing non-homologous end-joining repair mechanism is used, the HEG is not copied and if there is no homologous template from which to repair the damage then the chromosome likewise undergoes end-joining or is not repaired, which is the mechanism underlying X-chromosome shredding (Windbichler et al., 2008). Figure is taken from Esvelt et al. (2014) and is licensed under CC-BY 4.0

(RIDL), derives from work by Thomas et al. (2000) and Heinrich and Scott (2000) on tetracycline-repressible gene expression in bacteria (Alphey and Thomas, 2000). The two-component system consists of a promoter controlling a tetracycline-repressible transactivator tTA (Gossen and Bujard, 1992) and a lethal gene under the control of a minimal promoter and tetO, the tTA response element. Tetracycline has an affinity to tTA, preventing it from binding to tetO, where it would enhance expression from

the minimal promoter and increase transcription of the lethal gene. Insects can therefore be reared normally on a tetracycline-supplemented diet. In the absence of tetracycline, no tetO binding inhibition occurs and the lethal gene is expressed. This results in the death of all offspring carrying the lethal gene in the wild (Figure 1.5).

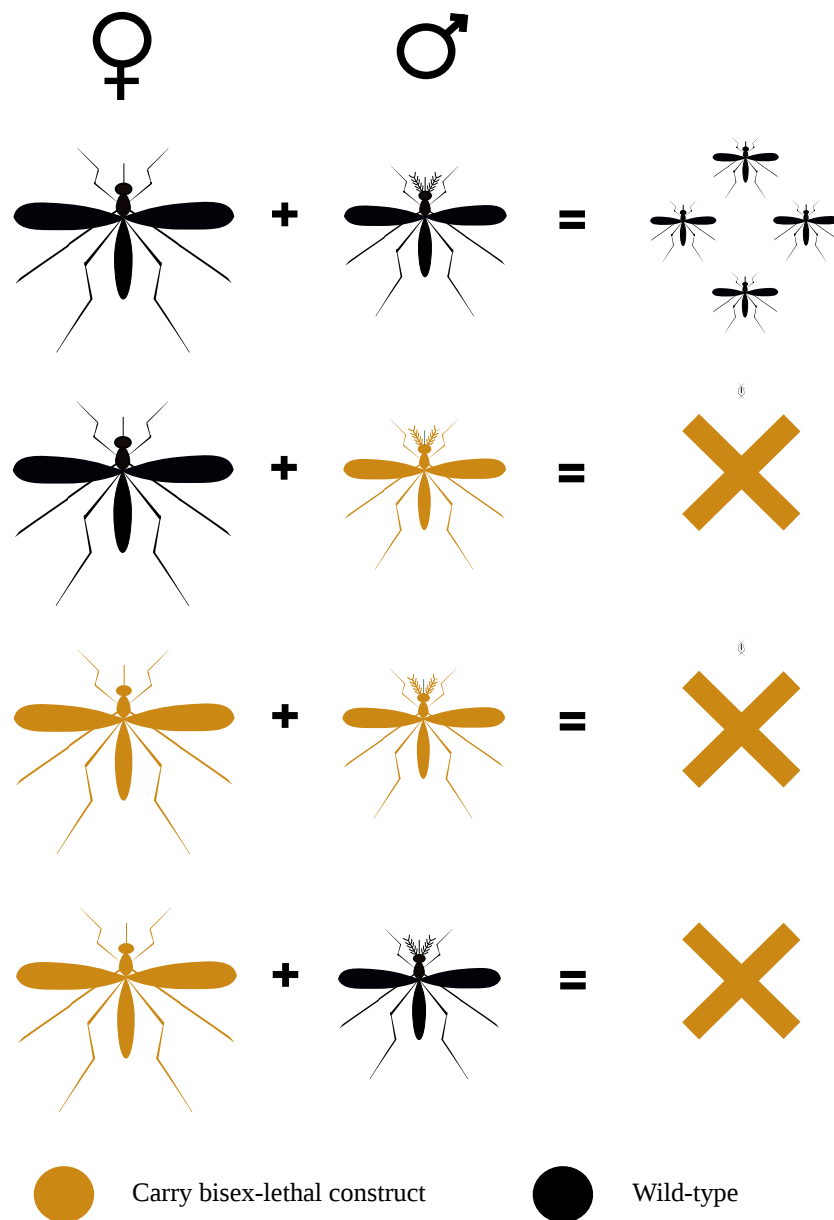


Figure 1.5: Transgenic males used in self-limiting genetic population suppression carry a copy of the dominant lethal transgene, which is suppressed in the laboratory by inclusion of the antibiotic tetracycline in the diet. In the wild the gene is active and expressed zygotically, killing all offspring which inherit it.

This system is flexible in terms of the lethal effector used but the requirement

for two components adds complexity, so a simplified one-component system using modified tTA as the effector was designed by Gong et al. (2005) and inserted into mosquitoes by Phuc et al. (2007). In this case, tTA is situated adjacent to tetO under the control of a minimal promoter and in the absence of tetracycline the minimally transcribed tTA binds to tetO producing a positive feedback loop. tTA is a fusion of tetR and a viral VP16 domain, which is thought to bind to key transcription factors. An accumulation of tTA via the positive feedback loop thus results in cell death by ‘transcriptional squelching’. Phuc et al. (2007) recoded the tTA tetR section to increase expression by codon optimisation and elimination of cryptic splice sites, creating tTAV.

Early transgenic lines using this system include the OX513A strain, which produces a late-acting, bisex-lethal phenotype killing over 95% offspring of released homozygous transgenic males. Lethality at late-larval or pupal stage rather than in embryos or early larvae is preferred because larval competition is maintained, and larval density is thought to strongly influence mosquito population dynamics (Phuc et al., 2007). OX513A has undergone field trials in the Cayman Islands, Malaysia and Brazil with each trial reporting 80–96% suppression of the local target population (Harris et al., 2012; Lacroix et al., 2012; Malavasi, 2014). This demonstrates clearly that transgenic males were able to seek out and mate with wild-type females and perform adequately in competition with wild-type males. The major challenge is now honing mass production and release strategies for maximum effectiveness and efficiency over increasingly large areas.

However, improvements could be made to the underlying molecular technology to improve mass-rearing and the reproducibility of strain phenotypes. The *piggyBac* transposon system is typically used for genome insertion, which means that docking sites are random and therefore the late-acting phenotype in OX513A was produced by the regulatory elements surrounding the insertion site rather than being specifically engineered, making it challenging to recreate. Exerting more precise control over induced traits through use of specific regulatory elements would therefore be attractive to regulators of genetic technologies and is increasingly possible due to advances in genetic sequencing of mosquito species and greater understanding of gene regulation. Control over timing of expression and sex-specific transgene activity are of most immediate interest.

OX513A is bisex-lethal but female-specific dominant lethal strains have been developed in various insects which produce a functional tTAV or lethal effector transcript in females only, due to the inclusion of *doublesex* (*dsx*) gene fragments that are alternatively spliced between the sexes (Fu et al., 2007). Female-specific dominant lethal expression produces genetic sexing strains whose female offspring die during development while male offspring survive. Male offspring therefore transmit the lethal transgene to further generations, although without sustained release it would only persist for a few generations, particularly accounting for associated fitness costs (Harvey-Samuel et al., 2014). The use of alternative splicing to control sex-specific expression of effector genes also offers the possibility of pre-release genetic sexing, which would remove the need for costly manual sorting.

Chapters 4 and 5 explore these concepts further, respectively aiming to build an alcohol-driven female-specific sexing system for release generations and a reliably late-acting and female-specific lethal system using specific and well-characterised lethal effectors.

1.4.4 Genetic Population Replacement

Replacing mosquito populations with disease-refractory strains is another strategy for reducing DENV transmission. Only one DENV resistance transgene has so far been engineered and successfully introduced into *Ae. aegypti*, an RNAi construct derived from the pre-membrane protein coding region of DENV-2 (Franz et al., 2006). RNAi is one of the major antiviral immune pathways in mosquitoes, although research is ongoing to identify other genes involved in DENV resistance or that are essential for DENV replication e.g. Chauhan et al. (2012) and Fansiri et al. (2013). The use of recently developed genome editing techniques that enable targeted mutagenesis, such as transgenic expression of Transcription Activator-Like Effector Nucleases (TALENs), or RNA-guided mutagenesis using the Cas9/CRISPR system is increasingly of interest. Thus far TALENs have been used successfully in *Ae. aegypti* (Aryan et al., 2013, 2014) but Cas9/CRISPR applications in mosquitoes remain in development (Esvelt et al., 2014; Kistler et al., 2014), with a recent success reported by Dong et al. (2015).

Regardless of the method of conferring genetic resistance, one major challenge is ensuring the transgene spreads through the population. It is unlikely to confer the strong advantage that normally drives alleles to fixation on ecologically relevant timescales and may even confer fitness costs (Lambrechts et al., 2008; Marrelli et al.,

2006). Gene drive systems and selfish genetic elements are therefore of great interest as under certain conditions these genes can spread through populations even when they confer a fitness cost and there is natural selection against them. Unlike the self-limiting genetic population suppression techniques, these approaches are invasive and self-sustaining, requiring even greater consideration of safety, confinement and the ability to reverse effects or replace introduced genes.

There are several required features of gene drive mechanisms for control of mosquito-borne disease:

- The drive mechanism must be strong enough to reach at least near-fixation on a timescale that is relevant for inhibiting disease transmission.
- There must be tight linkage between the drive mechanism and the desired transgene to prevent separation.
- There must be flexibility to replace the effector gene if resistance arises and ideally transmit a multigene system to slow the development of resistance.
- There must ideally be no off-target negative consequences and the ability to inhibit or remove the gene from the population in the event of unforeseen negative consequences.

Early attempts to use transposable elements (TEs) as gene drive mechanisms in mosquitoes were largely unsuccessful as highly active TEs were both difficult to introduce and also largely immobile post-integration (Sethuraman et al., 2007). The hope that a transposon as active as the P-element in *Drosophila* could be found thus appeared unlikely and attention was diverted to gene drive systems from other taxa, which have since been reverse engineered or re-purposed for use in mosquitoes.

Maternal-effect dominant embryonic arrest (Medea) is a gene drive system discovered in the flour beetle *Tribolium castaneum*. Females deposit a factor in their eggs which is lethal unless the offspring have inherited the embryonically expressed Medea antidote from one parent, resulting in all offspring who go on to reproduce being carriers of the gene. The *Tribolium* medea genes remain unknown but the effect was reverse-engineered in *Drosophila*. There are currently no functioning Medea systems in mosquitoes, but Medea-derived designs comply with many of the requirements above, including ways to increase the strength of association between the necessary

genes and the ability to release new elements to replace the population should resistance evolve against a transgene should it need to be ‘recalled’ (Marshall and Hay, 2011, 2014).

Homing endonuclease genes, aforementioned in the context of population suppression, may also be used to spread resistance genes or disrupt genes essential for viral replication and transmission, but this application remains theoretical and brings initial challenges of re-engineering HEG proteins to cut the DNA sequence of interest (Thyme et al., 2014). A recent publication by Gantz and Bier (2015) recreates a HEG effect in *D. melanogaster* using the Cas9/CRISPR system by integrating Cas9/CRISPR into the double-strand break that it makes, creating a self sustaining system that generates homozygotes from heterozygotes.

Another technique which has not yet been successfully deployed in mosquitoes is underdominance, also known as negative heterosis. Hybrids have lower fitness than either of two possible homozygous forms and under random mating conditions the least abundant form is disadvantaged because it is more likely to mate with the more abundant form, producing low fitness offspring, while the abundant form is more likely to mate with its own type and produce offspring with higher than average fitness.

This instability leads to dominance of the abundant form and eventual fixation under many conditions (Davis et al., 2001). Therefore, underdominance enables accelerated introgression of alleles without the need for sustained release of engineered insects once the abundant allele frequency reaches the threshold for invasion. Typically, underdominance systems are designed using two pairs of toxins and antidotes which are positioned on separate chromosomes (Akbari et al., 2013b; Matzen, 2012), although recent designs introduce underdominance at a single genetic locus (Akbari et al., 2013b; Reeves et al., 2014). Proof of principle has been demonstrated in *Drosophila* by the respective authors and these technologies continue to be developed.

To date, no functioning transgenic system for disease refractoriness and population replacement has been developed in *Ae. aegypti* but there are promising results from the introduction of microorganisms, which may offer a solution based on existing genes and interactions.

1.5 Genetic population control using microbes

Paratransgenesis is the introduction of transgenes by proxy, through transforming native symbionts or attenuated pathogens of a disease vector to express genes which exert control over the life cycle or disease susceptibility of the host. Wild vector populations must then be infected with the transformed organisms — usually bacteria, fungi or viruses. In *An. gambiae*, proof-of-concept paratransgenesis has been demonstrated using viruses (Ren et al., 2008) and entomopathogenic fungi (e.g. Fang and Leger, 2012; Fang et al., 2011; Kamareddine et al., 2013). These approaches have not yet succeeded in *Aedes*, but bacteria found in the gut microbiota have been evaluated as potential paratransgenesis agents (Hamady et al., 2010) and much work has focused on the endosymbiotic bacteria *Wolbachia* (Rickettsiales, Anaplasmataceae).

Wolbachia was initially considered to be a very good prospect for classical paratransgenesis. It is a maternally inherited, intracellular, symbiotic bacterium infecting nematode worms and a large number of arthropods. It was originally thought that greater than 65% of arthropod species could be infected (Hilgenboecker et al., 2008), but a more recent review of the available data has revised this proportion down to 40% (Zug and Hammerstein, 2012). These arthropods feature several important human disease vectors including some *Aedes* mosquitoes. To date, there has been no successful attempt to transform *Wolbachia*. However, it was discovered that when novel *Wolbachia* strains are transinfected into *Aedes* mosquitoes they express endogenous genes inducing desirable traits in the host that could reduce disease transmission: life shortening and viral refractoriness. Novel introductions of *Wolbachia* strains to vector populations have shown considerable promise for both population suppression and population replacement. Although not canonical paratransgenesis, this process still involves the introduction of genes of interest into populations through the introduction of a symbiont and can therefore be considered analogous in many respects.

1.5.1 *Wolbachia* phylogeny and transmission

Wolbachia form a monophyletic branch of the Rickettsiales α -proteobacteria group and are vertically transmitted via the female germline cytoplasm. Horizontal transmission on an ecological time scale has not been observed in mosquitoes, but the incongruence of the *Wolbachia* and mosquito molecular phylogenies along with the discovery of *Wolbachia*-derived genes in the genome of currently aposymbiotic hosts such as *Ae.*

aegypti suggests that cross-species transmission must have occurred over evolutionary time (Klasson et al., 2009). This proposal is further supported by the presence of *Wolbachia* uninfected, singly infected and super-infected mosquitoes even within the *Aedes Stegomyia* subgenus (Table 1.1).

Natural populations of *Ae. aegypti* are uninfected while *Ae. albopictus* has a dual infection with *wAlbA* and *wAlbB*. Several novel, stable transinfections of *Aedes* and transient, somatic infections of *An. gambiae* have been created in the laboratory via microinjection of naive or *Wolbachia*-cured mosquito eggs with purified *Wolbachia* or direct cytoplasm transfer from another species harbouring the desired infection. This strategy has also generated novel super-infections (Table 1.1). An illustration of the sub-cellular location of *Wolbachia* is provided in Figure 1.6.

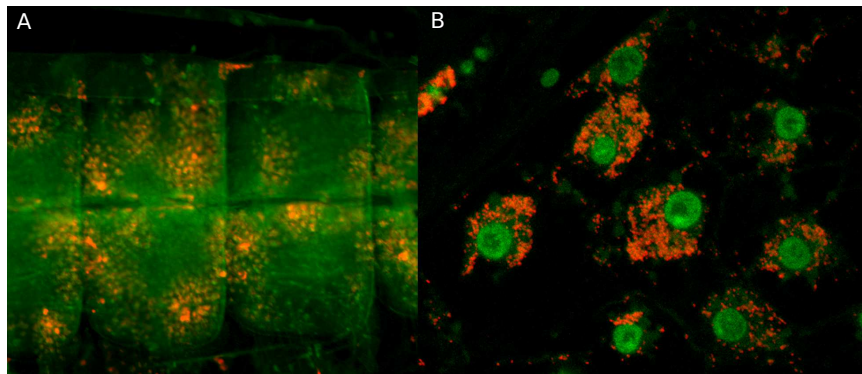


Figure 1.6: Illustrative whole mount fluorescence *in situ* hybridisation of *Wolbachia*-infected *An. gambiae*. A) *Wolbachia* present in the abdomen from ventral view. B) Intracellular *Wolbachia* infecting cells. Red, *Wolbachia*. Green, mosquito cell nuclei. Figure from Hughes et al. (2011), licensed under CC-BY 4.0.

Vertical transmission of bacteria via the maternal cytoplasm can be highly efficient as demonstrated by >96% transmission of *wAlbA* in *Ae. albopictus* populations in Thailand, rising to >99% for the denser *wAlbB* infection (Kittayapong et al., 2002). The bacteria use multiple strategies to increase transmission in different insect species including repression of sterility and apoptosis in the host germline, reduction of the proportion of male offspring generated by infected females and increasing the relative fecundity of infected females (reviewed in Werren et al. (2008)). In mosquitoes it causes an effect called ‘Cytoplasmic Incompatibility’ (CI), which is of interest both for population suppression and as a gene drive mechanism for population replacement.

1.5.2 *Wolbachia* and Population Suppression

CI causes inviable offspring to arise from matings between *Wolbachia*-infected males and uninfected females, while all other combinations of sex and infection status are compatible (Figure 1.7). This means that infected females can mate with all males in the population regardless of infection status, thus increasing their reproductive success and resulting in increased transmission of *Wolbachia*. CI can be both unidirectional, between uninfected females and infected males, or bidirectional, where females infected with one *Wolbachia* strain cannot reproduce with males infected with a different strain either singly or as part of a super-infection.

The most widely accepted model of CI hypothesises that modification to the sperm of infected males leaves them unable to viably fertilise eggs except when ‘rescued’ by a factor in an infected female (reviewed in Serbus et al. 2008; Sinkins 2004). This modification and rescue factor must be sufficiently diverse among *Wolbachia* strains that they are not cross-compatible (Werren, 1997). The exact molecular basis of the effect has not yet been determined, but histone modification and impaired histone deposition (Landmann et al., 2009) along with delayed nuclear envelope breakdown in the male pronucleus and failure of paternal chromosome condensation have all been demonstrated (Tram et al., 2006; Tram and Sullivan, 2002). Several authors have reported genes which could be involved in the process, although there is currently no clear functional verification of their role (Beckmann et al., 2013; Pinto et al., 2013; Yamada et al., 2011; Zheng et al., 2011).

Releasing uni- or bi-directionally incompatible male mosquitoes in large numbers would function as a variation on SIT for population suppression. While many studies have examined CI between natural and novel infections in the laboratory, application of the ‘Incompatible Insect Technique’ (IIT) in the field has been limited. Semi-field trials with *Ae. polynesiensis* were undertaken following introgression of *wScu* into an *Ae. reversi* genetic background (Chambers et al., 2011; O’Connor et al., 2012) but exploration of IIT for *Ae. albopictus* remains inside the laboratory (Blagrove, 2013; Moretti and Calvitti, 2013) and no studies have yet been published regarding *Ae. aegypti* IIT.

The problem of how to prevent inadvertent release of females arises with IIT, as in all male-only release strategies. It is even more crucial here than in SIT or self-limiting population suppression techniques because in addition to posing a potential biting nuisance and disease transmission risk, females would spread compatible *Wolbachia*

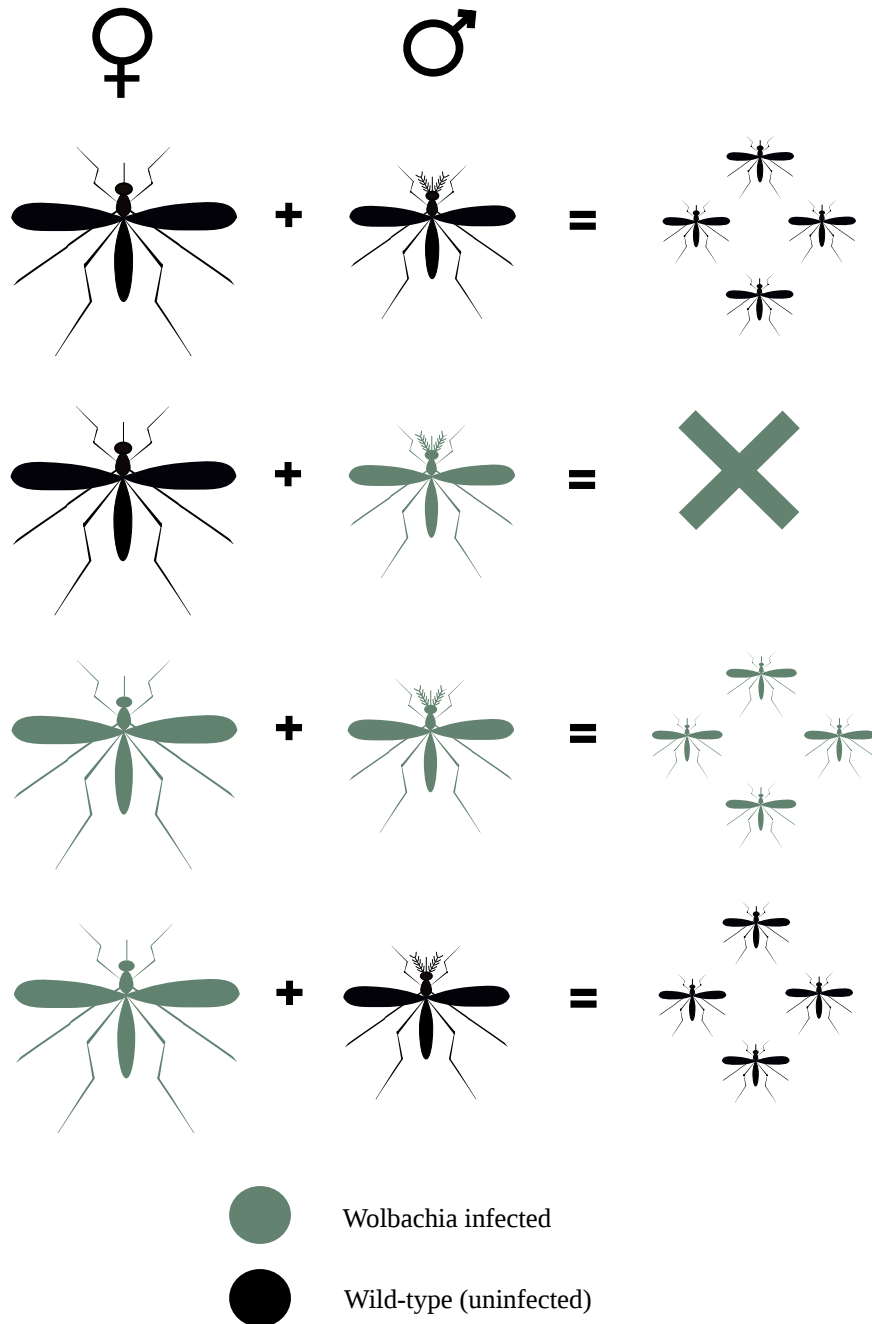


Figure 1.7: *Wolbachia*-infected females are able to produce viable offspring with all males regardless of *Wolbachia* infection status but uninfected females are incompatible with *Wolbachia*-infected males, thus producing no offspring. This phenomenon is known as cytoplasmic incompatibility (CI).

into the wild female population if levels reached a certain threshold, thus invalidating the suppression technique. This threshold is context-dependent but in bidirectional CI is over 50%. Hence, the genetic sexing techniques examined and developed in Chapter 5 are equally relevant to population suppression using *Wolbachia*.

1.5.3 *Wolbachia* and Population Replacement

The mode of bacterial transmission discussed above means that *Wolbachia* are driven through populations under what could be relatively high selection pressure as a selfish genetic element. Thus, by introducing infected females to an uninfected population or super-infected females to a singly infected population at sufficient density, their reproductive advantage could lead to fixation of the new infection and associated traits (Barton and Turelli, 2011; Hancock and Godfray, 2012; Hancock et al., 2011; Jansen et al., 2008; Sinkins and Godfray, 2004; Vavre and Charlat, 2012). Two observed traits of particular interest are life shortening and viral resistance.

Life shortening was observed in *D. melanogaster* infected with *wMelPop*, a mutated, virulent strain of the native *D. melanogaster Wolbachia wMel* (Min and Benzer, 1997). As mosquitoes are only able to transmit viruses following an incubation period of up to two weeks following the infectious blood meal, the demographic of infectious mosquitoes is highly skewed to long-lived females. Hence, any reduction in lifespan could limit both the number of infectious mosquitoes and the period for which they are infectious. The potential for transmitting *wMelPop* and the life shortening trait to disease vectors was thus explored (Brownstein et al., 2003).

Novel infections of *wMelPop* were generated in *Ae. aegypti* and it transpired that the phenotype was transferrable between species — a reduction in life span of up to a half was demonstrated (McMeniman et al., 2009). In addition, *wMelPop* caused a lessening of blood meal intake as mosquitoes aged, potentially facilitating a reduction in disease transmission (Turley et al., 2009). Not all traits were positive, for instance the observed reduction in viability of desiccated eggs would be detrimental for mosquito populations but also for mass release programmes (McMeniman and O'Neill, 2010). Population modelling later showed that *wMelPop* associated fitness costs could be a barrier to population invasion (Turelli, 2010; Yeap et al., 2011) but work continues to assess feasibility (Ross et al., 2014; Yeap et al., 2014). Techniques such as combining *Wolbachia* infection with insecticide resistance may increase selection for the infection and overcome fitness costs (Hoffmann and Turelli, 2013).

The later discovery of viral resistance arose partly as a result of research exploring the driving force behind the spread of *Wolbachia* in *D. melanogaster* populations, which unlike mosquitoes display only weak CI, leading researchers to seek additional fitness advantages. Hedges et al. (2008) and Teixeira et al. (2008) discovered that *Wolbachia* also confers protection against common *D. melanogaster* viruses such as

Drosophila C Virus (DCV), which reduce life expectancy in the wild. Native *wMel* may also play a role in metabolic provisioning in wild *Drosophila* populations (Brownlie et al., 2009). These host benefits are reviewed by Saridaki and Bourtzis (2010). While their adaptive nature and ecological relevance are unproven and expected to differ depending on the strength of *Wolbachia* reproductive parasitism, the possibility of transferring the antiviral phenotype to disease vectors such as *Ae. aegypti* and *Ae. albopictus* led to the generation of several novel transinfections and much research activity around potential for arbovirus control (Table 1.1). The *wMel* strain is of particular interest owing to its induction of viral protection without the problematic life shortening phenotype induced by the more virulent *wMelPop* (Walker et al., 2011).

1.5.4 *Wolbachia* and antiviral immunity

Ae. aegypti has been infected with *wMelPop* and *wMel* while *Ae. albopictus* has been cured of its native *Wolbachia* and infected with *wMel*. These infections have been found to induce refractoriness to DENV (Blagrove et al., 2012; Moreira et al., 2009; Van den Hurk et al., 2012), CHIKV (Blagrove et al., 2013; Van den Hurk et al., 2012) and filarial nematodes (Kambris et al., 2009).

Various hypotheses have been proposed for the effect of *Wolbachia* on RNA virus infection and transmission in *Drosophila* and *Aedes*. In order to make optimum use of *Wolbachia* as a population replacement strategy for disease refractoriness, it is important to understand the mechanism by which the bacteria-host interaction mediates this effect. In addition, it is known that *Wolbachia* densities can fluctuate and the endosymbiont can be lost from lineages (reviewed in Hughes and Rasgon, 2014) yet the novel introduced strains must be stable for prolonged periods of time. Therefore, an understanding of the major components of the symbiotic relationship that contribute to stable symbiosis may help in predicting selection pressures on the tripartite relationship of host, symbiont and pathogen. Our understanding of both is currently incomplete (discussed in Bull and Turelli, 2013).

Observations and evidence for the basis of *Wolbachia*-induced DENV refractoriness have been mixed. Initial reports from cell line studies suggested complete exclusion of DENV from *Wolbachia*-infected *Ae. aegypti* (Moreira et al., 2009), suggesting that viral entry was inhibited. However, Frentiu et al. (2010) later reported that reduction in viral titre was *Wolbachia* density-dependent, with complete inhibition

only observed at the highest *Wolbachia* density and lowest DENV multiplicity of infection (MOI). Lu et al. (2012) confirmed this result by modulating *Wolbachia* density in *Ae. albopictus* cell lines using sub-lethal doses of antibiotic to demonstrate a density-dependent relationship between viral titre and *wAlbB* density. *wAlbB* infection does not reduce DENV transmission in adult mosquitoes so somatic densities were measured via qPCR and factored into the linear model, which predicted no viral inhibition at the density seen in adults.

This suggests that DENV inhibition by *wMel* could primarily be a result of increased density rather than an inherent difference in its molecular interaction with the host compared with *wAlb*. Native *Cx. quinquefasciatus* *Wolbachia* strains have been shown to increase resistance to West Nile Virus and lower *Plasmodium* induced mortality (Glaser and Meola, 2010), again suggesting that *Wolbachia* strain and novelty of infection may not be the dominant causal factors in disease refractoriness. These results make it imperative to understand the ways in which *Wolbachia* density is modulated by the host or by external factors. It suggests that a decrease in density during co-evolution of transinfected *Wolbachia* and their novel mosquito hosts could reduce the DENV inhibitory effect, which is important to factor into monitoring of releases.

Once mosquito cells are infected with DENV, the high levels of apoptosis seen in mammalian systems are not observed, although infection does slow cell proliferation (Frentiu et al., 2010). *Wolbachia* appears to prevent this slowing, possibly by reducing cell mortality, and thus confers a net benefit under infected conditions, despite symbiotic cells generally proliferating more slowly than aposymbiotic cells. For instance, the *Wolbachia*- and DENV-positive RML12-*wMelPop*-CLA line showed equal proliferation to *Wolbachia*- and DENV-negative RML12-Tet at four days post-infection. The more densely *Wolbachia*-infected C6/36-*wMelPop*-CLA showed no reduction in proliferation as a result of DENV infection, although it continued to grow more slowly than its *Wolbachia*-cured counterpart at all time points.

There has been no published immunofluorescence study of the time course of infection of *Wolbachia*-infected cells with DENV and therefore it is unclear at exactly which stage DENV infection or replication is being prevented, but there are numerous possibilities within the viral lifecycle, including inhibition of entry, inhibition of replication and inhibition of release.

The major mechanistic hypotheses put forward so far involve modulation of innate immunity and reactive oxygen species in the host (Bian et al., 2010; Kambris et al., 2009; Moreira et al., 2009; Pan et al., 2012; Rancès et al., 2012) or changes in metabolism with the result that compounds essential to viral replication, such as cholesterol, are present in the wrong quantities. Another hypothesis is that, at high density, *Wolbachia* blocks the spatial reconfiguring of organelles and other cellular components required to allow viral replication (Bull and Turelli, 2013; Sim et al., 2014).

1.5.4.1 *Wolbachia* and mosquito innate immunity

The first observation on infection of *Ae. aegypti* with *wMelPop* was that it correlated with upregulation of innate immune pathway genes (Kambris et al., 2009; Moreira et al., 2009), which initially led to investigation of immune pathway involvement in DENV resistance.

The mosquito immune system is composed of physical barriers, such as the peritrophic matrix and midgut epithelium; cellular responses such as phagocytosis and melanisation and humoral responses such as the production of antimicrobial peptides (AMPs) and reactive oxygen or nitrogen species (ROS/RNS) (reviewed in Rolff and Reynolds (2009)). *Wolbachia* infection has thus far only been found to influence the latter two responses. Attention has focused on humoral immunity, although Thomas et al. (2011) demonstrated that *wMel* infected *D. melanogaster* and *Ae. aegypti* showed increased rates of haemolymph melanisation activity.

Humoral responses are typically controlled by a core set of three immune regulatory pathways: Janus kinase- signalling transducer activator of transcription (Jak-STAT) and the nuclear factor- κ B (NF- κ B) pathways Toll and Immune deficiency (Imd). They are homologous to the mammalian Jak-STAT, interleukin-1/Toll-like receptor (IL1/TLR) and Tumour-Necrosis Factor (TNF) pathways respectively and display highly conserved signal transduction mechanisms (reviewed in Rolff and Reynolds 2009; Wang and Ligoxygakis 2006). In *D. melanogaster*, the natural host of *wMel*, there is no induction of any canonical immune system pathways or AMPs (Chrostek et al., 2014; Wong et al., 2011). However, *wMel* in *Ae. aegypti* is associated with significant upregulation of both AMPs and Toll pathway components (Kambris et al., 2009; Moreira et al., 2009; Pan et al., 2012), which is dependent on reactive oxygen species levels as demonstrated by a reduction in Toll activation following RNAi

Species and diseases	<i>Wolbachia</i> infections and transinfections	Phenotypic effects of <i>Wolbachia</i>	Biting behaviour
<i>Ae. aegypti</i> - DENV - CHIKV - YFV	No natural <i>Wolbachia</i> Stable strains established: - <i>wMelPop</i> (McMeniman et al., 2009) - <i>wMel</i> (Walker et al., 2011)	- Complete CI - Life shortening phenotype (<i>wMelPop</i>) (McMeniman et al., 2009) - Immune activation (Kambris et al., 2009) - Limits infection with DENV, CHIKV, <i>Plasmodium gallinaceum</i> (Moreira et al., 2009) and filariasis (of which <i>Ae. aegypti</i> is not a natural vector) (Kambris et al., 2009)	- Bites mammals and mostly humans. (Scott et al., 1993) - Most active at dusk/dawn but also bites during day. (Nelson et al., 1978)
<i>Ae. albopictus</i> - DENV - CHIKV Several arboviruses in lab: - Eastern Equine Encephalitis - WNV - YFV - St. Louis Encephalitis (not natural vector but concerns due to range expansion)	- Two natural strains <i>wAlbA</i> and <i>wAlbB</i> (<i>wAlbB</i> is faster replicator) - <i>wRi</i> strain established in aposymbionts (Xi et al., 2006) - Triple <i>wAlbA/wAlbB/wRi</i> strain established (Fu et al., 2010b) - <i>wPip</i> strain established but unstable (Calvitti et al., 2010) - Experimental infection with <i>wMelPop</i> was unsuccessful (Suh et al., 2009) - <i>wMel</i> (Blagrove et al., 2012)	- Complete CI - Wide tissue distribution - Modulation of CHIKV (Mousson et al., 2010) - Reduction in CI with male age - <i>wAlbA</i> density decreases drastically with male age while <i>wAlbB</i> drives an increase in WO phage density (Tortosa et al., 2010) - Density of <i>Wolbachia</i> highest in ovaries, also found in gut and salivary glands (Zouache et al., 2009) - <i>wMel</i> induces resistance to DENV and CHIKV (Blagrove et al., 2013, 2012)	- Very broad host range including humans - Aggressive outdoor day biter. (Eritja et al., 2005)
<i>Ae. polynesiensis</i> - Lymphatic filariasis (<i>Wuchereria bancrofti</i>) - DENV - Ross River Virus (RRV)	- Naturally hosts <i>wScu</i> group strain (Behbahani et al., 2005) - Experimental infection with <i>wMelPop</i> was unstable (Steven Sinkins, personal communication) - Introgression of genome into <i>Ae. riversi</i> cytoplasmic background (containing Supergroup B <i>Wolbachia</i> from <i>wCon</i> group) (Brelsfoard et al., 2008)	- Complete CI	- Highly anthropophilic - Primarily diurnal, biting peaks early morning and late afternoon. (Belkin, 1962)

Table 1.1: Known *Wolbachia* infections and phenotypic effects in three *Aedes* mosquitoes.

knockdown of genes involved in ROS synthesis (Pan et al., 2012). This has been proposed as the mechanism of DENV resistance because knockdown of two major AMPs led to increased DENV infection in the presence of *wMel* and the Toll pathway has previously been suggested to control DENV infection in *Ae. aegypti* (Pan et al., 2012; Ramirez and Dimopoulos, 2010; Xi et al., 2008). Interestingly, in *Ae. albopictus* neither *wAlb* nor transinfected *wMel* cause significant upregulation of AMPs (Blagrove et al., 2012). Observations and evidence for the role of innate immunity in *Wolbachia*-induced DENV refractoriness are therefore mixed (Rancès et al., 2013, 2012).

1.5.4.2 Reactive Oxygen Species and *Wolbachia*

It has been shown that *Wolbachia* infection in *Ae. aegypti* adults increases expression of reactive oxygen species (ROS) as measured by H_2O_2 production (Pan et al., 2012). This result echoes that of Brennan et al. (2008), who demonstrated increased ROS production coupled with upregulation of antioxidant genes in *Wolbachia*-infected *Ae. albopictus* Aa23 cells.

Wolbachia and its host both have the capacity to generate ROS and antioxidants thus contributing to cellular redox homeostasis. The *Wolbachia* genome encodes a superoxide generating NADH dehydrogenase and an iron-containing superoxide dismutase FeSOD. *Aedes* mosquitoes have a dual oxidase (Duox) and two Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, NOXM and NOX5 as well as a suite of antioxidant genes including superoxide dismutases (SODs), peroxiredoxins and catalase (CAT), which in *An. gambiae* have been shown to operate downstream of the Jnk-induced OXR1 regulator (Jaramillo-Gutierrez et al., 2010). ROS play a major signalling role in multiple pathways involved in immunity and host metabolism as well as basic cellular functions. In terms of effects on gene expression, ROS are inducers of the Toll pathway in *Ae. aegypti* (Pan et al., 2012) and of the Jnk pathway in *D. melanogaster* (Wu et al., 2009), which has been linked to autophagy, apoptosis, AMP expression and stress responses (reviewed in Weston and Davis, 2002).

ROS have been shown to damage bacteria and other pathogens directly and are used as an intracellular defence mechanism (Bogdan et al., 2000; Lindgren et al., 2004; Radtke and O’Riordan, 2006). Therefore redox homeostasis may also impact the survival of *Wolbachia* and stable symbiosis.

1.5.4.3 Role of cholesterol in viral entry and replication

DENV entry into mosquito cells occurs via receptor- and clathrin-mediated endocytosis (Acosta et al., 2008, 2011; Vancini et al., 2013). Mosquito cellular receptors for DENV have not yet been fully elucidated but it is known that the viral envelope glycoprotein E is required for protein interactions at the plasma membrane and during membrane fusion in the late endosome. Once DENV enters the endosomal pathway, an acidic environment is required for virus unshedding and replication (Acosta et al., 2008). CHIKV also shows clathrin-mediated endocytosis (Lee et al., 2013b) and low endosomal pH requirements for successful entry and replication (Gay et al., 2012).

DENV requires high cholesterol content in host cells, most probably to facilitate formation of the lipid rafts necessary for receptor dimerisation (Acosta et al., 2008, 2011). Cholesterol-rich lipid rafts may also play a role in endosomal acidification, for example in mammalian cells they affect the regulation of V-ATPase subunits which control pH (Lafourcade et al., 2008). Accumulation of cholesterol in late endosomes reduced acidification while cholesterol depletion increased it. In Semiliki alphavirus fusion, cholesterol facilitates insertion of the loops of glycoprotein E into the target membrane, thus allowing formation of the functional homotrimers required for membrane fusion (Kaufmann and Rossmann, 2011; Klimjack et al., 1994). DENV shows a similar fusion pattern in mammalian cells and is also affected by lipid composition (Heaton and Randall, 2010; Zaitseva et al., 2010).

Cholesterol is required by both *Wolbachia* and the mosquito host, which may lead to resource competition and modified membrane cholesterol content. Competition would be heightened compared to other lipids or metabolites because insects are unable to synthesise cholesterol and therefore rely on exogenous sources. However, other lipid moieties and overall cell lipid profile are also candidates for being affected by *Wolbachia* and impacting DENV infection pathways.

1.5.4.4 Inhibition of viral replication by manipulation of autophagy and fatty acid availability

Although the majority of work on DENV infection and lipid composition or metabolism has been performed in mammalian cell lines, recent work by Perera et al. (2012) showed that DENV perturbs lipid homeostasis in the mosquito. Considerable alterations in lipid composition and localisation were observed and significantly, inhibition of fatty acid synthase at 4–12 hours post infection resulted in greatly reduced viral

titres, suggesting that fatty acids are vital for viral replication. As *Wolbachia* requires fatty acids and lacks a complete synthesis pathway, there may again be resource competition for these components.

In addition, DENV induces autophagy in mammalian cells, which depletes cytoplasmic lipid droplets (reviewed in Singh et al. (2009)). The released triglycerides are then further metabolised by the upregulated host β -oxidation pathway to generate adenosine triphosphate (ATP) (Heaton and Randall 2010; further work and review by Heaton and Randall 2011b). Suppression of autophagy significantly reduced DENV replication in human cells, an effect which could be reversed by supplementation of media with free fatty acids. Therefore, autophagy appears to enhance viral replication by provision of fatty acids necessary for replication.

Alteration of autophagy is an interesting proposal for the mechanism of *Wolbachia* antiviral effects. Recent work by Voronin et al. (2012) reported that autophagy is also important in regulating *Wolbachia* infection: increased autophagy reduced bacterial density to a similar level as antibiotic treatment while suppression resulted in increased bacterial density. *Wolbachia* may therefore subvert or evade the autophagic response to maintain stable infections. In nematodes, ATG8a protein was observed localised to the *w*Bm-containing vacuole, so Voronin et al. suggest that *Wolbachia* could be modifying or recruiting this protein to assist with evasion of autophagy. If the autophagic response were being actively suppressed by *Wolbachia* this could result in reduced DENV titre by reducing the availability of free fatty acid. This example again illustrates the close link between *Wolbachia*-host interactions that maintain the endosymbiosis and those that have the potential to cause DENV refractoriness — both are important to consider for potential population replacement strains.

1.5.5 Maintaining a stable *Wolbachia* symbiosis

Wolbachia are located inside cytoplasmic vesicles derived from the host membrane, which are Golgi related and concentrated near sites of membrane biogenesis (Cho et al., 2011). These vesicles may offer a degree of protection and environmental control to the bacteria, which are surrounded by two bacterial membranes plus one derived from the host. In order to maintain a symbiosis and be vertically transmitted to the next generation, *Wolbachia* need to replicate and colonise new membrane vesicles as well as spanning cell divisions to ensure both daughter cells are infected. This is particularly vital in the germline, where an uninfected oocyte means an uninfected

offspring. Localisation of vesicles has been shown to rely on cell polarity genes (Cho et al., 2011), microtubules and Dynein/Dynactin (Ferree et al., 2005).

Density also plays a role in ensuring transmission and is a factor of the host age, sex, genetic background and the particular strain of *Wolbachia* (Mouton et al., 2003). Density tends to be far higher in the germline and reproductive organs compared to somatic tissues. It is higher in young females, declining with age and with a particularly rapid decline in ageing males (Tortosa et al., 2010). Heat has also been shown to impact density, with lower titres at lower temperatures (Mouton et al., 2006). The molecular basis of this regulation is unknown but a small number of endogenous and exogenous factors, in addition to autophagy as previously discussed, have been demonstrated to control *Wolbachia* density and maintain a stable symbiosis.

Redox homeostasis is expected to impact *Wolbachia* density because in addition to their aforementioned immune effect, ROS play an important role in basic cellular functions. There is some evidence that *Wolbachia* may manipulate host antioxidant systems to increase survival, for instance the aforementioned increased ROS and antioxidant levels found in infected *Ae. albopictus* cell lines by Brennan et al. (2008). It is unclear whether the ROS increase is due to host production in response to *Wolbachia* infection or to *Wolbachia* aerobic metabolism, although RNAi of *Ae. aegypti* genes involved in ROS production by Pan et al. (2012) suggest that the host component is far more significant. Equally, increased antioxidant production may be a host response to increased ROS, or may be due to manipulation by *Wolbachia* for its own benefit, as it lacks a full complement of bacterial antioxidant genes in its reduced genome (Brennan et al., 2008). Increased antioxidant enzyme production at normal homeostasis has been shown to lead to increased life span in *D. melanogaster* (Orr and Sohal, 1994). However, any survival advantage in the *Wolbachia* infection scenario would be lessened if it is countering a rise in ROS.

Wolbachia may also play a role in ferritin expression and iron metabolism, which in turn can affect the regulation of oxidative stress, owing to the role of free iron in producing ROS. The *Wolbachia* bacterioferritin (Bfr) was found to be expressed in infected cell lines (Brennan et al., 2008) and as both *Wolbachia* and the host require iron for production of metalloenzymes, there may be resource competition for the element. Iron may play a role in both parasitic and mutualistic symbioses. In *Asobara tabida* (Hymenoptera), mutualistic *Wolbachia* increases female fecundity in low and high iron environments, purportedly by buffering iron metabolism (Kremer

et al., 2009). Fecundity increases were also observed in *wMel* infected *D. melanogaster* raised on a low iron diet compared to uninfected lines (Brownlie et al., 2009), which suggests that *Wolbachia* may engage in metabolic provisioning of iron (reviewed in Gill et al., 2014). As iron levels in wild populations surveyed were similar to those induced by addition of iron chelators to the food, this could provide infected females with a significant reproductive advantage.

In the filarial nematode *Brugia malayi*, the mutualistic *Wolbachia* strain encodes all the necessary genes for synthesis of heme, while the host encodes very few, indicating that iron metabolism may be a factor in the mutualistic relationship (Brownlie et al., 2007, 2009). In *wMel*-infected *Ae. aegypti* (RML12) cells, bacterioferritin expression in high iron conditions reduced the need for any significant upregulation of host ferritin (Kremer et al., 2009), which was observed in uninfected cells under the same treatment and presumably represents a cost for the host. The case of iron metabolism indicates that different symbiotic relationships might be maintained by the same cellular and molecular processes.

Some specific genes affecting *Wolbachia* density have been identified. The axis determining gene *gurken* (*grk*) was found to regulate *D. melanogaster* oocyte *Wolbachia* titre in a dose dependent manner (Serbus et al., 2011). *Grk* mRNA-protein complexes were found to co-migrate with *Wolbachia* and to be a rate limiting factor for *Wolbachia* replication but not to induce *Wolbachia* mortality. Furthermore, densely infected *D. melanogaster* showed phenotypes similar to those with *grk* complex disruptions suggesting a detrimental effect of the close interaction and possibly forming a self limiting loop.

In transinfected *Ae. aegypti*, the metalloprotease M41 *FtsH* gene was identified after sequencing of infected and uninfected adult females for differentially expressed miRNAs, followed by prediction of their genomic targets and expression analysis of selected genes (Hussain et al., 2011). *Ae. aegypti*-miR-2940 was upregulated by *Wolbachia* infection and in turn found to upregulate expression of the metalloprotease. Transient knockdown of M41 *FtsH* or inhibition of the miRNA both resulted in significantly reduced *Wolbachia*. The function of this gene in the symbiosis is not yet known: *FtsH* are ATP-dependent integral membrane proteins conserved across bacteria but also found in eukaryotes, although largely in the mitochondria or chloroplasts. They degrade misfolded cellular and membrane proteins and possibly also act as chaperone proteins (Schumann, 1999).

1.5.6 The role of stable symbioses and *Wolbachia* titre in transinfection and population replacement strategies

A successful introduction of the *Wolbachia*-induced DENV resistance trait into populations via release of infected mosquitoes is dependent on the maintenance of the infection within the population. Evidence is mounting that *Wolbachia* density can be a factor in disease resistance (Frentiu et al., 2010; Lu et al., 2012) so maintenance of density is also important. Studies of transinfected strains have shown density attenuation where fitness costs are high such as in transfer of the virulent, life shortening strain of *wMelPop* from *D. melanogaster* to *D. simulans* (McGraw et al., 2002) but there is currently no evidence to suggest attenuation in transinfections with less pronounced fitness costs. Recent monitoring results from releases of *wMel* infected *Ae. aegypti* in Australia suggest that there has been no reduction in *Wolbachia* density so far and viral inhibition has been maintained (Frentiu et al., 2014).

Trapping and *Wolbachia* screening suggested almost 100% infection rates two years after the first releases, which resulted in a large increase in infection over a few weeks and then maintained this over time (Hoffmann et al., 2014). This supports earlier modelling and semi-field trials suggesting that population replacement with the *wMel* strain could be rapid given an appropriate starting frequency; for example Walker et al. (2011) reported near-fixation after only a few generations from a starting frequency of 65%.

Population invasion is dependent on initial frequencies and fitness costs of infection as well as ecological factors such as spatial distribution of the target population (Hancock and Godfray, 2012; Hancock et al., 2011; Jansen et al., 2008; Martínez-Rodríguez et al., 2014; Vavre and Charlat, 2012). Yeap et al. (2011) and Turelli (2010) both report that the *wMelPop* infection in *Ae. aegypti* may spread very slowly if at all owing to fitness costs increasing the unstable introductory frequency at which the infection has a high risk of declining. However, the minimal fitness costs observed with *wMel* suggest that infections should invade rapidly at initial frequencies as low as 30–40% of the population (Hoffmann et al., 2011).

1.6 Mass-rearing and release of mosquitoes

Releasing mosquitoes for all population suppression and replacement strategies still constitutes a significant challenge in terms of optimising mass-rearing for production

of high-quality, high-fitness, competitive mosquitoes and their release by efficient and economical means. One of the major costs of mass-rearing and a process that introduces risks to mosquito fitness is the sexing of strains for male-only releases. Several solutions have been developed since the 1950s, both mechanical and biological.

1.6.1 Mechanical and behavioural separation during mass-rearing

Mechanical sex separators were first described in 1959 during early SIT field trials. They are composed of a grid through which pupae are pushed using a stream of water, with the smaller male pupae passing through and females being caught on the grid owing to their larger size (Ansari et al., 1977; Fay et al., 1963; Fay and Morlan, 1959; McCray and Elmo, 1961; Sharma et al., 1972). Owing to the significant size disparity in *Aedine* pupae, this method is highly effective at excluding females, achieving up to 99.9% male release cohorts (Ansari et al., 1977). However, because the grid sizing must be set conservatively to avoid female contamination, up to 60% of males can be retained, representing a large waste of rearing effort and increasing the space required to rear a release population large enough to compensate for this loss (Ansari et al., 1977).

In the first field release of the Oxitec self-limiting suppression strain OX513A in Grand Cayman, pupal separation was achieved with up to 99.5% accuracy (Harris et al., 2011). However, effectiveness is highly contingent on rearing conditions and strain differences can have a significant impact. For example, during field trials in Malaysia OX513A achieved 99.67% sex separation but the Malaysian wild-type control MY1 showed 12.7% female contamination owing to the strain's low adaptation to mass-rearing conditions, which resulted in smaller pupae (Lacroix et al., 2012). An additional consideration is selection against large males in the release cohort. Male size has been shown to affect field longevity; therefore loss of larger males through mechanical sorting could lead to a more short-lived release population (Maciel de Freitas et al., 2007), thereby reducing mating opportunities and potentially the effectiveness of population suppression. Relative size differences between wild-type females, wild-type males and transgenic males could also play a role in mating competition.

A further method of sex separation without the need for genetic modification takes advantage of differences in feeding behaviour, specifically in haematophagous arthropods where blood feeding is female-specific. Yamada et al. (2013b) describe

eliminating female *Anopheles arabiensis* by spiking blood meals with toxicants and propose the use of this method in SIT programmes. While the authors achieved 100% female kill after 4 days with the insecticides spinosad and ivermectin, even slight differences in feeding propensity could have serious consequences for quality of separation. In addition, the method involves increased adult handling and maintenance of adult cages for 4-5 days.

1.6.1.1 Genetic Sex Sorting

Genetic modification could allow a more efficient sex separation technique which would also enable sex separation in species with insufficient pupal size disparity between the sexes for mechanical sorting to be feasible. Genetic alternatives fall broadly into two categories: sex-specific markers to enable sorting and sex-specific conditional lethal traits.

Both have been achieved by sex linked mutations using classical genetics, typically chromosomal translocations to the Y chromosome which feature eye colour markers, insecticide resistance genes or temperature sensitive lethal genes (reviewed in Gilles et al. (2013)). An example of the the latter system is found in *Ceratitis capitata* (Medfly) and allows females to be selectively killed by moderate elevations in temperature (Franz, 2005; Robinson, 2002). In mosquitoes, Yamada et al. (2012) report an *An. arabiensis* sexing strain where a dieldrin resistance gene has been lined to the Y chromosome and female eggs can be killed by exposure to the insecticide, which simplifies the handling steps compared to later treatment but has been shown to lead to bioaccumulation of dieldrin in goldfish during egg releases (Yamada et al., 2013a). Disadvantages of classical techniques include the possibility of the sex-linkage breaking by recombination. This is observed at low frequency in both the *C. capitata* strain mentioned above and in the *An. albimanus* MACHO strain, which exhibits Y-linked male resistance to propoxur.

Genetic engineering using transgenic constructs allows a more targeted approach to the creation of sexing strains, allowing greater control over the markers used and regulation of spatial and temporal expression patterns. For example, sex-specific expression can be obtained via linkage to sex chromosomes as in classical approaches but can also be engineered using sex-specific promoters or alternatively spliced autosomal genes. As constructs can potentially use genes and conserved regulatory elements from across a broad range of taxa this offers the potential for modular design, with

elements being swapped in and out as appropriate to generate similar phenotypes across a range of organisms. Use of these techniques will increase as a synthetic biology approach is applied more frequently to technologies in non-model organisms.

Early transgenic sexing techniques inserted fluorescent markers on the Y chromosome (Condon et al., 2007) or expressed them using the β -tubulin promoter, which is specific to the male germline (Catteruccia et al., 2005; IAEA, 2006; Smith et al., 2007). This enabled manual sorting and automation and was later demonstrated to be possible in *An. stephensi* (Catteruccia et al., 2005). However, removing the need for sorting altogether is preferable for area-wide control systems, where mass-rearing of insects for field release will need to be undertaken in multiple locations using as little space and time, and as few resource as possible. Published transgenic approaches to inducible female lethality in mosquitoes have thus far all used a tetracycline-repressible system to produce flightless females, taking advantage of the sex-specifically spliced *actin-4* gene (Labbé et al., 2012) and the *dsx*-splicing controlled system described in Chapters 4 and 5. Tet-repressible genetic sexing systems have also been described in Medfly (Ogaugwu et al., 2013) and the silkworm *Bombyx mori* (Tan et al., 2013). Thus far, attempts to engineer reliable tet-orthogonal expression systems in insects have not been successful; therefore an approach with sex-specific constitutive expression of a genetic factor that causes susceptibility or resistance to an inducing factor introduced during the rearing of the release generation is an alternative solution, as explored in Chapter 5.

1.6.2 Post-release dynamics

Efficiency gains in mass-rearing need to be weighed against effective population suppression in the field, where population modelling demonstrates that over a sustained release period, bisex lethality reduces the number of adult females significantly faster than female-specific modes of control (Oxitec Ltd., unpublished data). This is particularly important where the technology is deployed in an area already affected by *Ae. aegypti* borne disease. In female-lethal self-limiting population suppression, heterozygous transgenic males continue to transmit female-lethal alleles after the first generation, in contrast to bisex-lethal alleles whose spread is self-limited within a single generation. It is currently unclear whether this persistence will be beneficial, for example by reducing the number of males required to sustain suppression over long release periods.

The balance of efficiency and efficacy also comes into play in the timing of lethality, with a choice between early- or late-acting. Modelling demonstrates that late-acting lethality in the pupal stage is advantageous in the field when the larval population is at or near the carrying capacity of the local environment because it preserves the larval population size for the first generation post-release and thus maintains any density-dependent mortality due to resource competition (Atkinson et al., 2007; Phuc et al., 2007; Yakob et al., 2008). Therefore, models that assume density dependence suggest that late-acting lethality will always cause faster population suppression and require a lower minimum release rate to achieve eradication.

If density dependence plays a major role in stabilising population dynamics, early-acting lethality even runs the risk of destabilising and increasing the wild-type population as it imparts a significant advantage to surviving wild-type larvae. It is not known to what extent density-dependent mortality operates in any given environment as there are few detailed studies on this aspect of *Ae. aegypti* ecology, but work by Legros et al. (2009) and Walsh et al. (2011) suggests that density does significantly impact larval survival. On the basis that at least a degree of density dependence is likely to be operating and to avoid the resulting risk of destabilisation, late-acting lethality has been the default for bisex-lethal *Ae. aegypti* strains such as the major Oxitec Ltd. product line OX513A.

Despite the above, early-acting female-specific lethality in the embryonic or early larval stages would be optimal for mass-rearing because rearing the release generation in the absence of tetracycline would result in autocidal removal of 50% of larvae, thus halving space and food requirements in addition to removing the need for mechanical sex sorting. This would bring about significant cost savings in the current mass-rearing process but also raises the possibility of egg stage releases, avoiding all laboratory- or mass-rearing of the release generation, which may be logistically simpler for control programmes in large areas over prolonged periods (Fu et al., 2010a; Labbé et al., 2012). However, alongside the obvious risks of predation and disease, these inherently heterogeneous and uncontrolled rearing conditions are likely to heighten variation in male size and fitness, all of which could reduce efficacy compared with using mosquitoes from a controlled mass-rearing facility. For these reasons, egg-stage releases are unlikely to be acceptable under the current regulatory and quality control framework surrounding release of genetically modified insects.

1.6.3 Release monitoring

Release strategies and regulatory frameworks are essential as control programmes scale from field trials to on-going releases by commercial and governmental entities. For example, release timing and location must be planned to maximise invasion in a spatially heterogeneous environment (e.g. Hancock and Godfray, 2012). Target populations must be monitored over time for prevalence of *Wolbachia* or transgenes and DENV inhibition where applicable. Nested within these logistical challenges lie scientific questions such as the spatial and age structure of target populations, reliable and quantitative methods to determine quality and fitness as well as further work on validating models and the factors that influence invasion under field conditions. No data have yet been collected to provide evidence of impact on DENV transmission for either technology and trials continue in order to provide this.

Further to the many interesting scientific questions that remain, the social acceptance and political handling of genetically modified insects for disease control are active areas for research, public communication of science and science policy. Establishing the regulatory and economic market framework in which to deploy genetic control interventions also remains a challenge.

It is likely that suppression and replacement methodologies could be integrated because replacement strategies benefit from a suppressed target population, which reduces the absolute number of transinfected mosquitoes required to exceed the unstable initial frequency. Suppression rarely leads to complete eradication whereas fixation of refractory phenotypes may be a more realistic goal, so there is potential to maintain a heavily reduced population of non-transmitting mosquitoes, which tackles both the biting nuisance and public health problems in arbovirus endemic areas. The interplay between all of the vector control techniques discussed above needs to be considered when formulating an IVM strategy for any given context.

1.7 Thesis Aims

This thesis aims to contribute to the further development of female-lethal, self-limiting population suppression and *Wolbachia*-based genetic control strategies in three ways. I explore the molecular mechanisms that might underlie the DENV-refractory phenotype observed in *Ae. albopictus* (Blagrove et al., 2012) and contribute to maintaining a stable symbiosis. Chapter 2 looks at ROS production and innate immune gene

expression in *w*Mel-infected *Ae. albopictus*. These factors have already been observed to differ in *Ae. aegypti* and have been hypothesised as causal factors in DENV refractoriness (Pan et al., 2012). Chapter 3 investigates the general hypothesis that lipids may play a role in the *Wolbachia*-host interaction by profiling the lipidome of *Ae. albopictus* Aa23-T cells and examining gene expression in relevant pathways to generate more specific hypotheses for functional testing.

Studies such as these are required because elucidating the molecular basis of *Wolbachia*-host interactions and the resulting phenotypes is vital to planning monitoring of field releases and formulating strategies for avoiding loss of DENV refractoriness, reduction in *Wolbachia* density or even loss of the strain. Strategies are also required to reverse inadvertent effects, such as increased susceptibility of the mosquito to DENV or increased virulence of DENV circulating in refractory vector populations. These strategies would also be greatly informed by molecular mechanisms because likely scenarios for co-evolution of the tripartite interaction between host, *Wolbachia* and DENV could be modeled and predicted. None of this is possible based on current information so recapture of mosquitoes from trial release areas, *Wolbachia* density assays and DENV replication or transmission testing are the only tools available to monitor the trait.

Genetic population suppression is more advanced in this regard because while *Wolbachia* contains an entire genome with multiple potential effects on vector life cycle, reproduction, gene expression and metabolism, the dominant lethal genetic constructs used for self-limiting population suppression are known and largely well characterised. The ways in which they might be disrupted, for example through mutations and deletions, are known and can be closely monitored. The ability to reverse problems with the construct or background strain by reseeded breeding colonies from stocks and only releasing ‘fixed’ mosquitoes is a major advantage of the self limiting nature of gene introgression in this technology.

Despite these advantages, improvements can still be made. The currently deployed lethal dominant genetic construct in *Ae. aegypti* OX513A mosquitoes relies on random genome integration for control of expression and a ‘transcriptional squelching’ killing mechanism which has not been fully elucidated, whereas other more precisely characterised lethal effectors are available. Genetic information and technologies are increasingly allowing regulatory specification of spatio-temporal expression patterns.

Chapter 4 addresses this issue by moving towards a specific promoter-regulated late-expression construct and the use of specific lethal effector genes. As for *Wolbachia*, this approach increases the potential for informed predictions about likely resistance mechanisms.

An immediate problem in moving forward with existing *Aedes* strains for population suppression using both dominant lethal transgenic constructs and *Wolbachia* as etiological agents is improving the mass-rearing process and significant gains in time and resources are possible by removing the need for manual sex-sorting. Chapter 5 trials a genetic sexing system using sex-specific expression of alcohol dehydrogenase (ADH) to increase sensitivity of female larvae to toxic alcohols introduced during the rearing process. Early removal of females not only alleviates the requirement for mechanical sorting but also allows double the number of males to be reared in a given space and for given resources. Chapter 5 thus addresses practical measures to scale up use of genetic control technologies while continuing the theme of promoting well-characterised and predictable regulation of traits that runs throughout this thesis.

Chapter 2

Wolbachia-induced effects on reactive oxygen species (ROS) and innate immune pathways in *Ae. albopictus*

2.1 Introduction

Wolbachia-infected *Ae. aegypti* have shown upregulation of the insect innate immune pathways Toll, Imd and Jak/STAT, which have since been linked to immunity to various RNA viruses, including DENV (Bian et al., 2010; Kambris et al., 2009; Moreira et al., 2009; Pan et al., 2012). Pan et al. (2012) further hypothesised that the increased ROS production they observed in *wMel*-infected *Ae. aegypti* induced the Toll pathway, which has previously been shown to control DENV infection (Ramirez and Dimopoulos, 2010; Xi et al., 2008) and that this was the causal factor in DENV refractoriness. The link between ROS and Toll induction was supported by provision of H₂O₂ supplemented sugar water to *Wolbachia*-uninfected *Ae. aegypti*, whereupon Toll pathway genes were upregulated. Conversely, Toll genes were downregulated following knockdown of ROS-generating enzymes via RNAi. Under this model, DENV refractoriness is thought to be mediated by Toll-induced AMPs such as Cecropins and Defensins. Knockdown of Cecropin D (CECD) and Defensin C (DEFC) did lead to increased DENV viral titres indicating that these proteins inhibit DENV infection, although the mechanism underlying AMP antiviral action is unknown.

I determined the sequence of a broader range of *Ae. albopictus* genes involved in innate immunity and redox homeostasis. To investigate *Wolbachia*-induced modulation of both systems, I measured relative gene expression in adult and cell lines with

different strains and densities of *Wolbachia* infection. H_2O_2 levels in *Ae. albopictus* adults and cell lines were also assayed to determine if ROS production is increased by a range of *Wolbachia* infections. Furthermore, a toolkit of methods and validated primers is presented for further functional experimentation with innate immunity in *Ae. albopictus* and the related DENV vector *Ae. polynesiensis*.

2.1.1 Mosquito innate immune pathways

There are three major mosquito innate immunity pathways: Jak-STAT, Toll and Imd (Section 1.5.4.1 and Figure 2.1).

Observation of AMP upregulation and Toll activation in novel *wMel* and *wMelPop* infections of *Ae. aegypti*, a naive host, originally led to the hypothesis that immune priming was responsible for the observed viral protection (Kambris et al., 2009; Pan et al., 2012). However, no AMP upregulation was found in *Ae. albopictus* (Blagrove et al., 2012; Glaser and Meola, 2010) and Rancès et al. (2012) demonstrated no evidence of immune priming in *D. melanogaster*, the natural host of *wMel* and *wMelPop* (see also Wong et al., 2011). However, both of these species display viral resistance (Blagrove et al., 2012; Hedges et al., 2008; Teixeira et al., 2008), suggesting that immune activation is not a predominant causal factor common to all species. The upregulation in *Ae. aegypti* could be a consequence of the recent introduction of *Wolbachia* into this novel host, whereas native hosts show immune attenuation. This hypothesis is supported by immune upregulation in somatically *Wolbachia*-infected naive *An. gambiae* hosts (Kambris et al., 2010).

While immune priming appears to be unnecessary in every case of *Wolbachia*-induced viral immunity, the aforementioned work of Pan et al. (2012) produced evidence that AMPs do play a part in infected *Ae. aegypti* DENV refractoriness and therefore the impact of *Wolbachia* infections on *Ae. albopictus* immunity warrants further investigation.

Regardless of their role in viral resistance, immune interactions between the mosquito host and *Wolbachia* are intrinsically interesting because immune effectors could have a direct effect on stability or density of the bacterial symbiosis. They could also represent a fitness cost to the host, thereby indirectly impacting *Wolbachia* infection dynamics. As viral refractoriness has been shown to positively correlate with *Wolbachia* density (Frentiu et al., 2010; Lu et al., 2012), *Wolbachia* infections used for

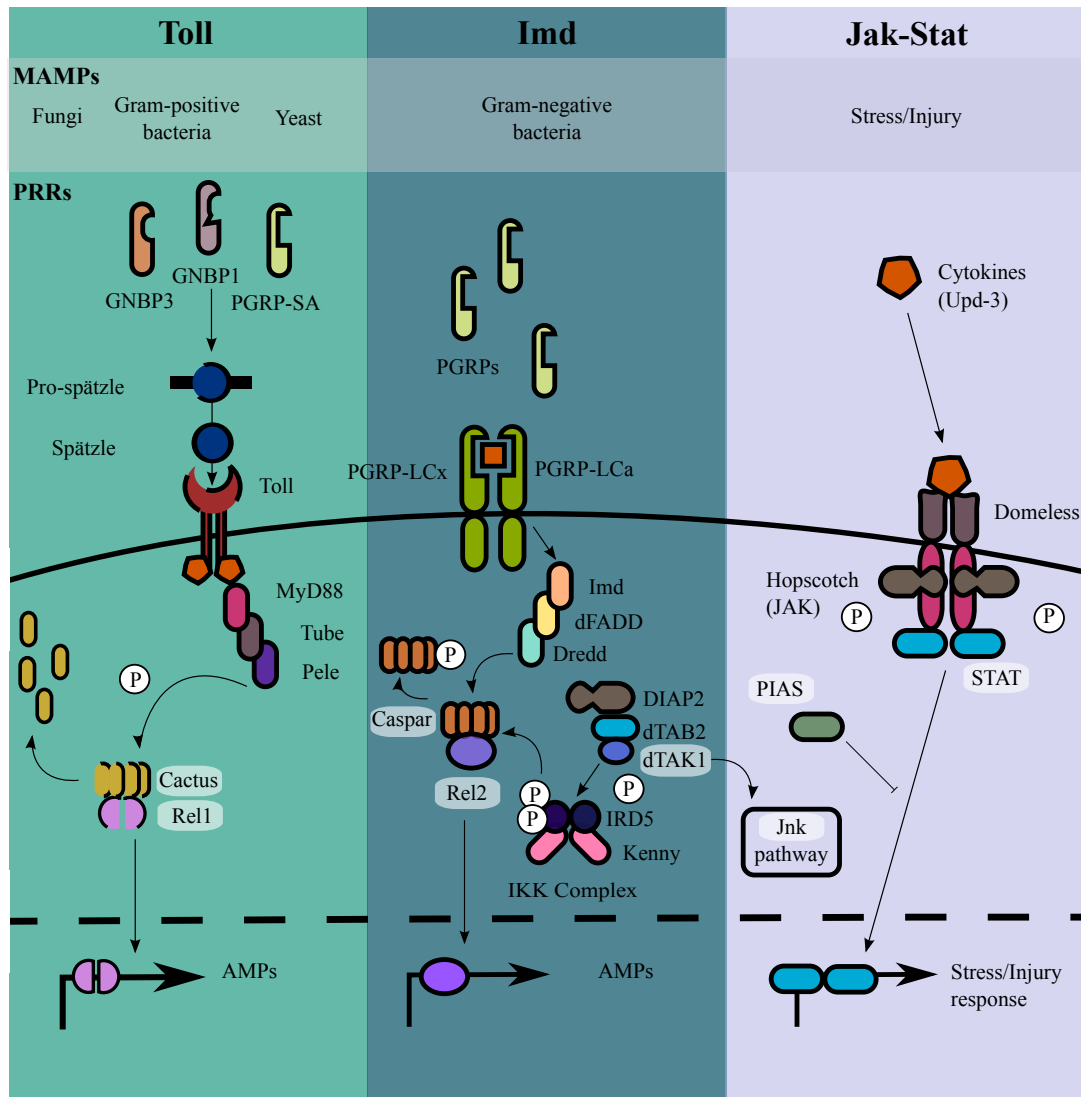


Figure 2.1: Toll and Imd are activated by Pattern Recognition Receptors (PRRs) on exposure to microbe-associated molecular patterns (MAMPs), for example bacterial peptidoglycan cell walls. Secreted Toll pathway PRRs induce proteolytic cascades which cleave the ligand Spätzle allowing it to bind the transmembrane Toll receptor, whereas Imd has membrane bound PRRs which directly regulate the intracellular signal transduction pathway. Both pathways eventually lead to phosphorylation of transcription factor (TF) negative regulators, Cactus (CAC) or Caspar (CSP) respectively, and translocation of the TFs Rel1 or Rel2 to the nucleus where they induce expression of immune effectors such as AMPs or components of cellular immunity pathways (reviewed in Lemaitre, 2004; Lemaitre and Hoffmann, 2007). The activation of Jak-STAT is less well described but is thought to involve induction of a cytokine which binds to the Domeless receptor (Agaisse and Perrimon, 2004). The end result is Jak mediated phosphorylation and dimerisation of STAT proteins, which then migrate to the nucleus as TFs and induce gene expression (reviewed in Arbouzova and Zeidler, 2006). Genes investigated in this chapter are highlighted in white.

population replacement must be stably maintained at a high density, so understanding basic *Wolbachia*-host immune interactions that might influence these factors is important.

2.1.2 The role of ROS in mosquito immunity

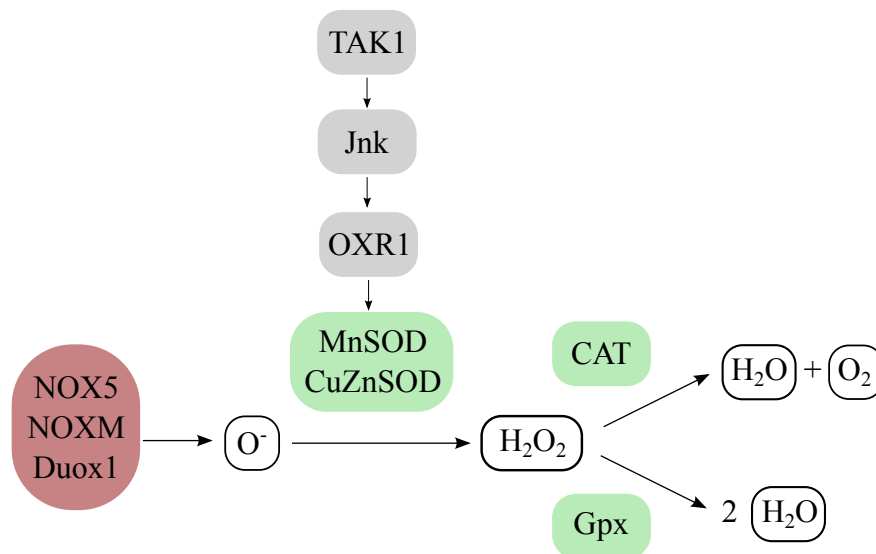


Figure 2.2: ROS are derived from oxygen primarily by the ROS-generating enzymes NOX and DUOX, which produce superoxide anions $O^{\cdot -}$ that are reduced to H_2O_2 and O_2 by SODs and to other intermediates by further redox reactions via enzymes such as CAT and Glutathioneperoxidases (Gpx). ROS-producing enzymes are represented in red, antioxidants in green and regulatory genes in grey. TAK1 is positively activated by the Imd pathway and operates upstream of Jnk signalling, which activates transcription of the broad antioxidant regulator OXR1, promoting expression of all antioxidant genes represented (Jaramillo-Gutierrez et al., 2010).

Innate immune pathway signalling molecules and effectors are distinct proteins, but some small molecules such as ROS moieties play a dual role (Figure 2.2). They are implicated in both immune signalling and direct killing of pathogens in a number of systems (reviewed in Fang, 2004). In both vertebrate macrophages and insect haemocytes, oxidative bursts transiently increase ROS levels in the vicinity of phagocytosed microbes and are believed to directly oxidise microbial targets and inflict DNA damage in conjunction with reactive nitrogen species (RNS). In mosquitoes specifically, there is evidence that ROS modulate immunity against *Plasmodium* and bacteria. For example, *An. gambiae* strains with naturally higher systemic H_2O_2 levels show

higher antibacterial activity which is reduced upon antioxidant treatment (Molina-Cruz et al., 2008) and exogenous ROS from gut microflora can induce *Plasmodium* resistance (Cirimotich et al., 2011).

In terms of indirect antimicrobial action, there is evidence from *An. gambiae* immunity to *Plasmodium* that Duox generated ROS may be involved in peroxidase mediated cross-linking of protective midgut layers (Kumar et al., 2010) and melanotic encapsulation (Kumar et al., 2003), while NOX5 generated ROS mediate the TEP-1 complement response (de Almeida Oliveira et al., 2012). Furthermore, Surachetpong et al. (2011) showed that ROS could directly activate the mitogen-activated protein kinase (MAPK) and Phosphatidylinositol 3-kinase/Akt (PI3K/AKT) signalling pathways, which are thought to modulate innate immune responses, although precise molecular links have not been elucidated.

Aside from work by Pan et al. (2012), there is little evidence of direct ROS interaction with the three canonical immune pathways in mosquitoes despite extensive mammalian literature exploring ROS activation of NF- κ B pathways (reviewed by Morgan and Liu, 2010) and ROS-mediated cross-talk between Jnk and NF- κ B (Nakano et al., 2005).

ROS can be beneficial for hosts due to their immune role, but they can also induce oxidative stress by directly damaging DNA and interfering with signalling and metabolic pathways, reducing longevity and fecundity (DeJong et al., 2007; Jaramillo-Gutierrez et al., 2010). Maintenance of redox homeostasis is also important for controlling mosquito gut microbiota, which proliferate following blood feeding due to ingestion of the antioxidant heme (Oliveira et al., 2011). There is hence a fine balance to be struck by the host in controlling ROS generation and antioxidant production to target pathogens while minimising systemic damage. Upregulation of antioxidant genes has already been reported in *Wolbachia*-infected *Ae. albopictus* (Brennan et al., 2008) and *Ae. aegypti* (Pan et al., 2012). Biochemical ROS measurements therefore need to be interpreted in the context of underlying ROS-modulating gene expression (Figure 2.2). I assayed a subset of relevant genes using qRT-PCR to provide some of this context.

Line	<i>Wolbachia</i> Infection	Notes
Uju.T	None	<i>Ae. albopictus</i> cured by tetracycline treatment (Otsuka and Takaoka, 1997).
Uju.wAlb	wAlbA and wAlbB	wAlbA and wAlbB from <i>Ae. albopictus</i> Ascoli strain were introgressed into an UJU-T nuclear background (Blagrove et al., 2012).
Uju.wMel	wMel	Uju.T transinfected by cytoplasm transfer from wMel-infected <i>D. melanogaster</i> eggs via microinjection (Blagrove et al., 2012).
APM	wScu	Naturally infected <i>Ae. polynesiensis</i> .

Table 2.1: *Ae. albopictus* and *Ae. polynesiensis* lines used in experiments.

2.2 Materials and Methods

2.2.1 Mosquito rearing conditions

All lines were reared at 27°C and 70% relative humidity on a 12 h light/dark cycle. Eggs were hatched in deionised water (dH₂O) and larvae were reared at a density of 200-250 per tray containing 2 L dH₂O. Larvae were fed on liver powder (MP Biomedicals) *ad libitum*, limited to amounts which did not lead to excess bacterial growth in the water. After transfer of pupae to 30 cm x 30 cm x 30 cm plastic mesh cages (Bugdorm Model DP1000), eclosed adults were provided with either 10% sugar solution or a separate sugar cube and moist cotton *ad libitum*. Females were blood fed using defibrinated sheep’s blood (TCS Biosciences Ltd) in a Hemotek membrane feeder (Discovery Workshops) covered with Parafilm or rabbit skin. It was observed that the *Ae. polynesiensis* lines were not attracted to Parafilm alone and even if probing of the feeder occurred they would not become engorged, hence these lines were always fed using rabbit skins.

Egg bowls consisting of small plastic tubs lined with moist cotton wool covered with a piece of filter paper were provided three days post blood meal for oviposition. Eggs were collected after another three days and stored in sealed plastic boxes with slightly damp cotton wool for hatching within the next two weeks or dried for 48–72 h, wrapped in tissue and placed in sealed plastic bags for longer term storage of up to two months.

2.2.2 Cell culture

Cells were grown in 25 cm² or 75 cm² flasks kept at 27°C in Schneiders Medium (Promo-Cell) with 10% fetal bovine serum (FBS) and 1% penicillin-streptidicillin (pen-

Line	Wolbachia Infection	Notes
Aa23-T	None	Tetracycline cured Aa23 cell line (Pinto et al., 2012). Aa23 cell line originally from (O'Neill et al., 1997).
Aa23-T.wAlbB	wAlbB	Michael Petridis, unpublished.
Aa23-T.wMel	wMel	Transinfected from wMel-infected <i>An.stephensi</i> MSQ43 cell line donor (Pinto et al., 2012).
Aa23-T.wMelPop	wPop	Transinfected from wMelPop-infected <i>An.stephensi</i> MSQ43 cell line donor (Pinto et al., 2012).

Table 2.2: *Ae. aegypti* Aa23 cell lines used in experiments.

strep, Gibco). Standard passage time was 5 - 7 days.

2.2.3 RNA Extraction

RNA was extracted from pools of three 7-day old adult females or from cultures of Aa23 in 12-well plates, seeded from 250 k cells and incubated for 72 h.

Samples were homogenised in 100 µl Trizol (Invitrogen) or Tri Reagent (Sigma-Aldrich) per mosquito or 10^6 cells using an electric motor driven pestle (Kimble Chase), or by grinding with borosilicate beads at 40 Hz for 4 min using a Qiagen TissueLyser LT (Qiagen). After incubation for 10 min at room temperature (RT), chloroform was added at 0.25 x initial volume of Trizol and samples were incubated for 3 min at RT then vortexed prior to centrifugation at 20,000 x g for 10 min at 4°C. All subsequent centrifugation steps were also performed at 20,000 x g and 4°C.

The aqueous layer was removed and precipitated with isopropanol at a volume equal to the original volume of Trizol. After washing with 75% ethanol the pellet was dried and resuspended in 20 µl Tris-EDTA (TE) buffer. 1 µl DNase I (2000 U/µl) (NEB) and 3.5 µl 10 x DNase I buffer were added prior to incubation at 37°C for 20 min.

2.2.4 cDNA Synthesis

All cDNA synthesis reactions were made up on ice and reactions were performed in 0.2 ml tubes in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems) using High Capacity RNA-to-cDNA Kit (Applied Biosystems) mixed as per Table 2.3.

The mixture was incubated for 10 min at 25°C, 120 min at 37°C and 5 min at 85°C prior to storage at -20°C. Standard dilution for qRT-PCR was 1:20.

Component	Volume μ l)
RNA template	1 μ g Total RNA
10 RT Buffer	2
25 dNTP Mix (100 mM)	0.8
10 RT Random Primers	2
MultiScribe Reverse Transcriptase	1
Nuclease-free H ₂ O	to 20

Table 2.3: Components of High Capacity RNA-to-cDNA Kit (Applied Biosystems) reaction mixture.

2.2.5 Degenerate PCR

The sequences of six immune pathway genes in *Ae. albopictus* and *Ae. polynesiensis* were obtained using degenerate primers designed against the currently sequenced genomes of *Ae. aegypti*, *An. gambiae* and *Cx. quinquefasciatus* by aligning nucleotide sequences for the relevant genes using ClustalW (Goujon et al., 2010; Larkin et al., 2007). In some cases, primers were designed in highly conserved regions using Primer3 (Rozen and Skaletsky, 1999). If sequences diverged substantially, the alignment was input to Primacade (Gadberry et al., 2005) to design degenerate primers. Primer sequences are listed in Table 2.13.

Different PCR reagents were used to optimise reactions (Table 2.4). Cycling conditions were as per Table 2.5 but in some cases, a touchdown PCR (TD-PCR) cycling sequence was used to increase sensitivity while retaining specificity (Korbie and Mattick, 2008). This typically involved reducing the annealing temperature by one or two degrees every three cycles for at least five increments, followed by 20 cycles at the lowest annealing temperature. Annealing temperatures usually started 5°C above the predicted annealing temperature of the primers and finished 5°C below.

PCR products were run out on a 1% agarose gel (Sigma-Aldrich) made with TAE buffer and 1 μ l ethidium bromide per 100 ml gel. Size and density of bands were estimated against SmartLadder SF (100-1000 bp) or SmartLadder (200-10k bp) (Eurogentec). Bands were imaged using a Uvitec/Uvipro imaging system (Uvitec). Products for gel extraction were run out on a 0.75% low melting point agarose and purified using a PureLink Quick Gel Extraction & PCR Purification Combo Kit (Life Technologies) according to manufacturers' instructions. Where necessary due to the use of a blunt-ended polymerase enzyme such as Phusion Hot Start, products were A-tailed prior to ligation into a pGEM-T Easy Vector. 10x PCR Reaction buffer (with

MgCl₂) was added to 1–7 μ l PCR product followed by dATP to a final concentration of 0.2 mM, 5 units of Taq polymerase and nuclease free H₂O to 10 μ l. The mixture was incubated at 70°C for 30 min. All reagents were sourced from NEB.

Reagents (μ l)	Crimson Taq	Phusion Hot Start	ONEtaq
DNA	2–4	2–4	2–4
Forward Primer	0.5	1	0.5
Reverse Primer	0.5	1	0.5
Enzyme/Master Mix	0.125	0.2	12.5
Supplied Buffer	5	4	NA
10mM dNTPs (NEB)	0.5	0.4	NA
dH ₂ O	to 25 μ l	to 25 μ l	to 25 μ l

Table 2.4: PCR reagents used for degenerate PCR reactions.

	Crimson Taq (NEB)	Phusion Hot Start (NEB)	ONEtaq (NEB)
1 x	95°C	98°C for 30 s	94°C for 30 s
	95°C for 30 s	98°C for 5 s	94°C for 30 s
35 x	45–68°C for 30 s	45–72°C for 10 s	45–68°C for 30 s
	68°C for 60 s per kb	72°C for 30 s per kb	68°C for 60 s per kb
1 x	68°C for 5 min	72°C for 5 min	68°C for 5 min
Hold	4–10°C	4–10°C	4–10°C

Table 2.5: Standard cycling conditions for PCR, where necessary a touchdown PCR reaction was used as described in Section 2.2.5

2.2.6 Cloning of PCR Products

Ligation reactions were set up on ice as per Table 2.6. Reactions were then mixed by pipetting and incubated for 1 h at RT or overnight at 4°C.

Reagent	Volume μ l
PCR	x (up to 3)
2 x RLB	5
pGEM-T Easy	1
T4 DNA Ligase	1
dH ₂ O	10-x

Table 2.6: pGEM-T Easy ligation mixture (Promega)

D5- α competent cells (NEB) were transformed by adding 1 μ l ligation reaction to 10 μ l cells thawed on ice. After incubation on ice for 30 min the cells were heat shocked

at 42°C for 30 s and placed back on ice for 5 min. 190 µl SOC medium was dispensed into each reaction prior to incubation at 37°C for 60-90 min with vigorous shaking at 250 rpm. 50 µl and 10 µl of each reaction were plated on LB/ampicillin/IPTG/X-gal plates and incubated overnight at 37°C. After incubation at 4°C for 30 min to allow X-gal blue colour to deepen in untransformed colonies, white colonies were picked using a sterile pipette tip and dipped in a pre-pipetted 25 µl Taq Polymerase mixture in a 96-well PCR reaction plate, then into 200 µl LB-Amp medium in a 96-well culture plate for further colony growth at 37°C with shaking at 200 rpm.

The colony PCR reaction was run under standard condition with an annealing temperature of 55°C and positive colonies were marked on the resulting gel picture. Relevant colonies were transferred from the 96 well plate to 14 ml tissue culture vials containing 1–5 ml LB-Amp medium, which were then incubated at 37°C for 12–16 h before spinning down cells at 6000 x g for 3 min and removing supernatant ready for mini-prep.

Plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen), designed for purification of high-copy plasmid DNA from overnight incubations in 1–5 ml LB, according to manufacturers' instructions. The resulting 50 µl solution was stored at -20°C. A restriction digest was performed to ensure that the plasmid insertion was the correct size. 4 µl of mini-prep was digested using EcoRI (Table 2.7), with the reaction mixture incubated at 37°C for 1 h followed by deactivation at 65°C for 20 min. The digest was run on a 1% agarose gel to determine insert size. PCR products and plasmids for sequencing were diluted to 10-50 ng µl⁻¹ or 30-100 ng µl⁻¹ respectively in nuclease free H₂O and sent to GATC Biotech for sequencing.

Reagent	Volume µl
Buffer (NEB 1–4)	2
EcoRI (20,000 U/ml)	1
DNA	4
dH ₂ O	13

Table 2.7: Ligation reaction recipe for pGEM-T Easy (Promega, Madison, WI, USA)

2.2.7 Sequence analysis

Ae. albopictus and *Ae. polynesiensis* putative sequences were compared to the *Ae. aegypti* sequence obtained from VectorBase (Lawson et al., 2009) and a BLAST (Altschul

et al., 1990) analysis was performed to further support their homology. On release of the *Ae. albopictus* transcriptome database (Poelchau et al. (2011); located online at <http://www.albopictusexpression.org>), a BLAST search was performed using the partial sequences obtained through degenerate PCR. Where matches were found, these were used in preference for future analysis as their read length was longer and updated as new database releases arose. Sequences used in this chapter derive from the pharate larvae, embryo and oocyte merged assembly, quality filtered contigs.

For immune pathway genes, both novel *Aedes* sequences plus *An. gambiae* and *Cx. quinquefasciatus* were mapped to the reference *Ae. aegypti* gene and trimmed to leave only the CDS or partial CDS. Nucleotide and protein sequences were aligned using Clustal Omega (Sievers et al., 2011), with translation and frame selection via translatorex (Abascal et al., 2010) and MUSCLE (Edgar, 2004). Proteins were then scanned for InterPro domains (Quevillon et al., 2005) in Geneious (version 7.1.5, Biomatters, Available from <http://www.geneious.com> Kearse et al., 2012).

All other sequences from *Ae. albopictus* were aligned to *Ae. aegypti* using the tblastx function on <http://www.albopictusexpression.org> for protein identities and ClustalW (Thompson et al., 2002) though Geneious for nucleotide identities.

2.2.8 qRT-PCR

qRT-PCR primers were designed as per Table 2.11 and 2.12. All primers were tested using a template standard curve and achieved efficiencies of 1.8 - 2.1 (80 - 110%). Standard curves were run on all plates to assess efficiency per run. Quantitative real-time PCR was performed in a MJ Research PTC-200 DNA Engine using a Chromo4 detection system and Opticon Monitor Software v3.1 (all manufactured by Bio-Rad Laboratories). Reagents used were the SYBR Green EXPRESS qPCR Supermix Universal (Life Technologies) and the iQ SYBR Green supermix (Bio-Rad Laboratories), which were used in 10 µl reaction as per manufacturers' instructions. Cycling conditions are described in Table 2.8. Some independent repeats used SYBR Green JumpStart Taq ReadyMix (Sigma Aldrich) on a Stratagene Mx3005P Real-Time PCR System (Agilent Technologies) using the same mix and thermocycler settings.

Ct values were analysed using the R package MCMC.qPCR, which employs a Bayesian approach to data analysis. An informed model was fitted assuming no variation in the control gene S17 and log(2) fold-changes were derived along with Bayesian

Cycles	Temperature	Time
1	95°C	4 min
40	95°C	15 s
	59°C	30 s
Melting curve 55–95°C		Read every 1°C, hold 5 s

Table 2.8: Thermocycling conditions for qPCR and qRT-PCR.

95% credible intervals and pMCMC values. Primer efficiencies were calculated per experiment using a standard cDNA curve and fed into the informed model.

2.2.9 *Wolbachia* Density Assay

As *Wolbachia* density needed to be assessed across multiple mosquito species, it was desirable to find a single copy host and *Wolbachia* gene for which universal primers could be designed targeting the mosquitoes and *Wolbachia* strains of interest. These can then be cloned into a plasmid allowing absolute qPCR quantification of gene copy number based on a plasmid standard curve and hence the ratio of *Wolbachia* genomes to host genomes.

Primers were designed to amplify all *Wolbachia* surface protein (Wsp) sequences in the *Wolbachia* strains included in the study (Sofia Pinto, University of Oxford, unpublished). I sought a single copy nucleotide sequence conserved across all mosquito species and a 93 bp conserved regulatory element upstream of the homothorax (HTH) gene was identified from the literature (Glazov et al., 2005). Primers were designed against HTH using PrimerBLAST (Table 2.9).

Sofia Pinto (University of Oxford), cloned the two PCR products into a pGEM vector. I generated the standard curve and assessed primer efficiency on CTAB extracted gDNA, which was consistently 90 - 100%.

Primer	Sequence	Designed by
Mos_qHTH_F2	TGGTCCTATATTGGCGAGCTA	Jennifer Molloy
Mos_qHTH_R	TCGTTTTTTGCAAGAAGGTCA	Jennifer Molloy
qWSP_all-R1	AAAGTCCCTCAACATCAACCC	Sofia Pinto
qWSP_all-F3	ATCTTTTATAGCTGGTGGTGGT	Moreira et al. (2009)

Table 2.9: Primers used in *Wolbachia* density qPCR, designed to amplify across all sequences Culicidae species and the *Wolbachia* strains *wAlbA*, *wAlbB*, *wMel*, *wMelPop*, *wPip* and *wRi* among others.

DNA was extracted from pools of three 7-day old adult females or from confluent cultures of Aa23-T cell lines in 12-well plates. Samples were homogenised as per Section 2.2.3 in 100 μ l cetyltrimethyl ammonium bromide (CTAB) buffer per mosquito (0.1 M Tris HCl pH 8.0, 1 M NaCl, 10 mM EDTA, 1% CTAB). Following incubation at 95°C for 30 min, the sample was left to cool and an equal volume of chloroform was added prior to centrifugation at 15,000 x g for 25 min at 4°C. The aqueous layer was transferred to a new tube and DNA was precipitated with an equal volume of isopropanol then washed twice with 70% ethanol prior to resuspension in TE buffer and quantification using a Nanodrop spectrophotometer. DNA was diluted to 50ng μ l⁻¹ and stored before 100 ng was added to a 10 μ l qPCR reaction using SYBR Green EXPRESS qPCR Supermix Universal (Life Technologies) as per manufacturers' instructions and qPCR was performed as per Section 2.2.8.

Wolbachia genomes per host genome were determined from a linear model fitted to the known plasmid standard curve values. This data was tested for normality and homogeneity of variances using the Shapiro and Bartlett tests respectively. Normal data was then assessed using a two-way analysis of variance (ANOVA) followed by Tukey Honestly Significant Differences (Tukey HSD) post-hoc pairwise testing. Data which deviated from the normal distribution was analysed via a Kruskal-Wallis test using a Bonferroni adjustment for multiple comparisons.

2.2.10 Cell line H₂O₂ assay preparation

H₂O₂ is the most stable form of ROS and thus acts as a correlate of overall ROS levels, it also has a highly specific biochemical assay which overcomes some of the issues in interpreting data inherent in other ROS probes, some of which have been shown to be highly non-specific (Kalyanaraman et al., 2012).

H₂O₂ generation by live cells was assessed using an Amplex Red Hydrogen Peroxide Kit (Invitrogen). Cells in the exponential growth phase (four days post passage in 75 cm flasks) were harvested in fresh Schneiders solution (+10% FBS, +1% penicillin-streptomycin) and viability was assessed by Trypan blue staining to ensure all lines had similar viability. 150 k cells were plated per well of a flat bottomed 96-well microplate and allowed to settle for one hour in the dark at 25°C. Media was then removed, cells were washed once with Phosphate-buffered saline (PBS) and 50 μ l Amplex Buffer in PBS was added followed by 50 μ l Amplex reaction solution (as per

manufacturers' guidelines) immediately prior to insertion into a FLUOstar microplate reader (BMG LabTech).

2.2.11 Adult mosquito H₂O₂ assay preparation

Pools of three mosquitoes were collected on ice in 2 ml tubes containing 1000 µl PBS with 2 mg ml⁻¹ 3-Amino-1,2,4-triazole (3-AT) and three borosilicate beads. Samples were homogenised at 50 Hz for 5 min in a TissueLyser LT (Qiagen) and centrifuged at 20,000 x g for 15 min at 4°C. The supernatant was transferred to a new tube and centrifuged again at 20,000 x g for 10 min at 4°C before 10 µl supernatant was added to a 96 well flat bottomed plate for the protein assay and 250 µl was added to a 10,000 k molecular weight cut-off (MWCO) Spinfilter Ultrafiltration centrifugal filter (VWR) and centrifuged at 14,000 x g for 20 min.

50 µl of filtrate was added to a separate flat bottomed 96-well plate for the H₂O₂ assay. Both plates were stored on ice until assays began.

2.2.12 Protein Assay

200 µl Bradford Reagent (Sigma-Aldrich) was added to samples in the protein assay plate, which was then shaken at 100 rpm for 30 s and incubated at 25°C in the dark for 10 min before being read at 595 nm using a FLUOstar microplate reader (BMG LabTech).

2.2.13 H₂O₂ Assay

The Amplex Red H₂O₂ Assay (Invitrogen) was used according to manufacturers' instructions with 50 µl Amplex reagent (containing 2x Amplex buffer) added to the 50 µl sample prepared above. The plate was then incubated in the FLUOstar microplate reader at 25°C where it was read kinetically at 545/590 nm excitation/emission over 40 cycles of 60 s with shaking at 100 rpm every two cycles to reduce clumping. The 30 min reading was used after confirmation that the assay kinetics were linear at this time point

Readings from protein and H₂O₂ samples were compared to standard curves of known concentration of albumin (Sigma Aldrich) and H₂O₂ (Invitrogen) in the respective assays. H₂O₂ was then normalised to protein content for adult assays and cell count for cell line assays (verified by post assay counts from randomly selected

wells within each line). This data was tested for normality and homogeneity of variances using the Shapiro and Bartlett tests respectively then assessed using a two-way ANOVA followed by Tukey HSD pairwise testing or by a Kruskal-Wallis test as per Section 2.2.9.

2.2.14 Primers

Control Gene Primers		
Gene	Forward Primer	Reverse Primer
S17	AP_S17_F AAGCCCCTGCGTAACAAGAT	AP_S17_R GTTATCTCTGCGCTCACGTTC

Table 2.10: Primers for the S17 housekeeping gene for both *Ae. albopictus* and *Ae. polynesiensis*.

Aalb qRT-PCR			
Gene	Forward Primer	Reverse Primer	Product
CAC	Alb_qCAC_F TGTTTCAGCTCGTCTTCGTCA	Alb_qCAC_R GGACTGGTGGTACTGGTGCT	71 bp
Rel1	Alb_qRel1_F4 GCTATCGGACACTGGACGTT	Alb_qRel1_R4 TGCGTTTCTTTCGTTTGGCT	132 bp
CSP	AP_qCSP_F AGAATGCGTAGCGGAGTGTC	AP_qCSP_R3 GACCGGTGAGAACATAACGAA	137 bp
Rel2	Alb_qRel2_F CCGCATCTTCATTCAGCTTT	Alb_qRel2_R TTTCGATACCAATCGGAGATG	81 bp
PIAS	Alb_qPIAS_F1 TCAAACCGGCAGATTACACA	Alb_qPIAS_R1 CGGGAAGGTCTTCTTGCTTT	57 bp
STAT	Alb_qSTAT_F8 ATCACCTGCCCTATCAACCG	Alb_qSTAT_R8 ACGACGACGCAAACATATCG	66 bp
TAK1	Alb_qTAK1_F1 GTTTCGGAAAATCCCAACTCAGG	Alb_qTAK1_R1 CTCCGCAACAAACCATGGAAG	62 bp
Duox	Alb_qDuox_F3 GGGAATAGCAAGGCTTCCGT	Alb_qDuox_R3 ACCAAGCGTTTGTGATTGGC	71 bp
DuoxA	Alb_qDuoxA_F3 TCAGCAATGGTGGGAACCTC	AP_qCSP_R3 ACAAAGTCAAAGCGCAGCAG	115 bp
OXR1	Alb_qOXR1_F CTACTCGTGGTCCCTGGTGT	Alb_qOXR1_R ATTGGGCTTTCAGTTTGTG	90 bp
Gpx	Alb_qGpx_F2 CCAACGAGGAGATCAAGCAC	Alb_qGpx_R2 TCAAACCTGGCTCCCTTCTG	52 bp
CuZnSOD	Alb_qCuZnSOD_F ATGTCAAGGGCACCATCTTC	Alb_qCuZnSOD_R GGCTTGAGTCCAGTCACCTC	82 bp

Table 2.11: Primers for qRT-PCR of immune pathway and ROS-related genes in *Ae. albopictus*.

Apol qRT-PCR			
Gene	Forward Primer	Reverse Primer	Product
CAC	AP_qCAC_F3 TTCCATTTCGGGTCAGAACTT	AP_qCAC_R4 TCCTGGAGCTGCTTTTGATT	116 bp
Rel1	None	None	NA
CSP	AP_qCSP_F3 CTTCGCCGGTGATGTAGTTC	AP_qCSP_R3 CGGACTCAAGCACGGATTAC	160 bp
Rel2	AP_qRel2_F GCAGTACCTGGAGCAGAACC	AP_qRel2_R CGAATGATGGCCAGAATCTT	69 bp
PIAS	AP_qPIAS_F TTTGTCCGTTGGGAAAGATG	AP_qPIAS_R ACAGTGACGCATCAAAGCAC	253 bp
STAT	AP_qSTAT_F2 AGAGCGTGATGGACGAGAAG	AP_qSTAT_R 2ATTGATCGTCCAAACCGAAA	90 bp

Table 2.12: Primers for qRT-PCR of immune pathway genes in *Ae. polynesiensis*. No primer pairs with a single product and suitable efficiency were found for Rel1.

Gene	Forward Primer	Reverse Primer
CAC	Cactus_dgen_F5 AGGTTTCAYGCMCCWTTCC	Cactus_dgen_R KCCRTYGTARTTCCACTGTT
Rel2	REL2_dgen_F2 TCATACACACGGCSAAGA	REL2_dgen_R3 AACCGRATCTTGATGTTATT
Duox	Duox_dgen_F AAGUGYGACGARAUGUACG	Duox_dgen_R TTGGTVUCRTCCTTCTTCTC
OXR1	OXR1_dgen_F AGYGTATTTTCVAAAGTWT	OXR1_dgen_R CTGCTTTTCGTGHGTTTTG
Gpx	Alb_GP_x_F CATGTGCTCATCATCGTCAA	Gpx_dgen_R GUGAAGUCCACUUGAU

Table 2.13: Degenerate primers designed with Primaclade (Gadberry et al., 2005) in order to amplify initial sequences that were too divergent for amplification with existing *Ae. aegypti* primers or for specific design of primers from multispecies alignments. All primers included produced correct products, NOX5 and NOXM degenerate primers not shown as all primers trialled were ineffective.

Gene	Forward Primer Reverse Primer	Apol product	Aalb product
CAC	AP_T7qCAC.F TAATACGACTCACTATAGGGGAGACAGCCGCACCTTCGATTCC AP_T7CAC.R TAATACGACTCACTATAGGGGTGTTTCCATCGATGTCACG	420 bp	383 bp
Rel1	Aa_Rel1A.F TAATACGACTCACTATAGGGATCTGCCCAACAACCTCAT Aa_Rel1A.R TAATACGACTCACTATAGGGTCTTCTTGACGCACTGGATG	451 bp	640 bp
CSP	AP_T7CSP.F1 TAATACGACTCACTATAGGGAGATCACGGACATTCCGGTGCGG AP_T7CSP.R1 TAATACGACTCACTATAGGGGAGACTCCACAAACTGTATGCTGCCGA	380 bp	383 bp
Rel2	AP_T7Rel2.F TAATACGACTCACTATAGGGAGATTGCAAACCTTTTCCACGAA AP_T7Rel2.R TAATACGACTCACTATAGGGAGAAGAATCGCCGAAGAGTTG	337 bp	385 bp
PIAS	Aa_iPIAS.F TAATACGACTCACTATAGGGATACACCCAGCAAGCCAGT Aa_iPIAS.R TAATACGACTCACTATAGGGAACCCCGCTATTGTATGCTG	428 bp	658 bp
STAT	Aa_iSTAT.F TAATACGACTCACTATAGGGCACCAACCTTCATCATCG AP_T7STAT.R TAATACGACTCACTATAGGGGAGACCGATAACTGCTTGGTGGTT	277 bp	277 bp
Duox	Alb_T7Duox.F TAATACGACTCACTATAGGGGAGACAAGGATCAGGATGGGAGAA Alb_T7Duox.R TAATACGACTCACTATAGGGAGAACCGTTCGACAAAACAACACA	NA	539 bp
DuoxA	Alb_T7DuoxA.F3 TAATACGACTCACTATAGGGAGATGCACCTTCCGAGACGAGGGT Alb_T7DuoxA.R1 TAATACGACTCACTATAGGGAGACATCGTCCTCATTGTCGTTG	NA	558 bp
OXR1	Alb_T7OXR1.F1 TAATACGACTCACTATAGGGAGAACTACCGTTCGATGGTTTTCG Alb_T7OXR1.R1 TAATACGACTCACTATAGGGGAGACTCGGGTGTA CT CGGTGATT	NA	558 bp
Jnk	Alb_T7JNK.F2 TAATACGACTCACTATAGGGAGAGATCCGCGGAGGGGTGCTGT Alb_T7JNK.R2 TAATACGACTCACTATAGGGAGAGTTCTACGGTGCTCGCGCT	NA	401 bp

Table 2.14: Primers to amplify cDNA templates for dsRNA synthesis. All sequences contain the T7 transcription start site followed by a sequence specific segment for primer binding. Primers starting Aa are *Ae. aegypti* primers from Erickson et al. (2009), all remaining primers were designed using Primer3 or Primer-BLAST from aligned Culicidae sequences or *Ae. albopictus* sequences once ascertained.

2.3 Results

2.3.1 Innate immune pathway gene sequences

The canonical immune pathway genes were sequenced from *Ae. albopictus* and *Ae. polynesiensis*, a closely related DENV vector from the South Pacific Islands, using degenerate primers based on available Culicidae genomes. The sequences represent only partial mRNAs due to the location of suitably conserved primer binding sites. However, an *Ae. albopictus* transcriptome was published soon after the sequences were obtained and on comparison showed only minor base pair changes, possibly as a result of strain differences or single nucleotide errors introduced during the cloning and sequencing process. Transcriptome sequences were thus used for the following comparative analyses due to their longer read-length.

Nucleotide and protein sequences were aligned with sequences from three existing mosquito genomes, *Ae. aegypti*, *An. gambiae* and *Cx. pipiens quinquefasciatus* (Table 2.15). The majority of genes were highly conserved at the amino acid level (Figure 2.3) and their divergence was consistent with the phylogeny of the species examined. CAC showed considerable variation even within the *Aedes* genus, displaying only 65% amino acid similarity between *Ae. albopictus* and *Ae. aegypti* (Figure 2.3).

	Aaeg	Aalb	Apol	Agam	Cpip
CAC	XM_001650216	140339	KT310349	XM_317542.4	XM_001846332.1
Rel1A	AY748242	145853	KT310347	XM_310177.3	XM_001844026.1
CSP	XM_001656977	082813	KT310350	XM_316513.4	XM_001862325.1
Rel2	AF498105	008087	KT310346	XM_308995.4	XM_001862241.1
PIAS	XM_001647772	135921	KT310348	XM_001688417.2	XM_001850599.1
STAT	EF175868	146249	KT310345	XM_311053.3	XM_001866572.1

Table 2.15: NCBI RefSeq IDs for the immune pathway homologues used in comparisons to *Ae. albopictus* and *Ae. polynesiensis* novel sequences. *Ae. albopictus* IDs are Aalb_OocyteEmbryoPharatarvaeAssembly contig references from the developmental transcriptome (<http://albopictusexpression.org/>, Poelchau et al. (2011)). *Ae. polynesiensis* partial mRNA sequences were deposited in GenBank and relevant IDs are shown.

Using the sequences obtained, dsRNA template (Table 2.14) and qRT-PCR primers (Table 2.11 and 2.12) were designed and validated to produce toolkits for functional manipulation and monitoring of all three innate immune pathways in *Ae. albopictus* and *Ae. polynesiensis*. Due to the sequence similarity between the two species,

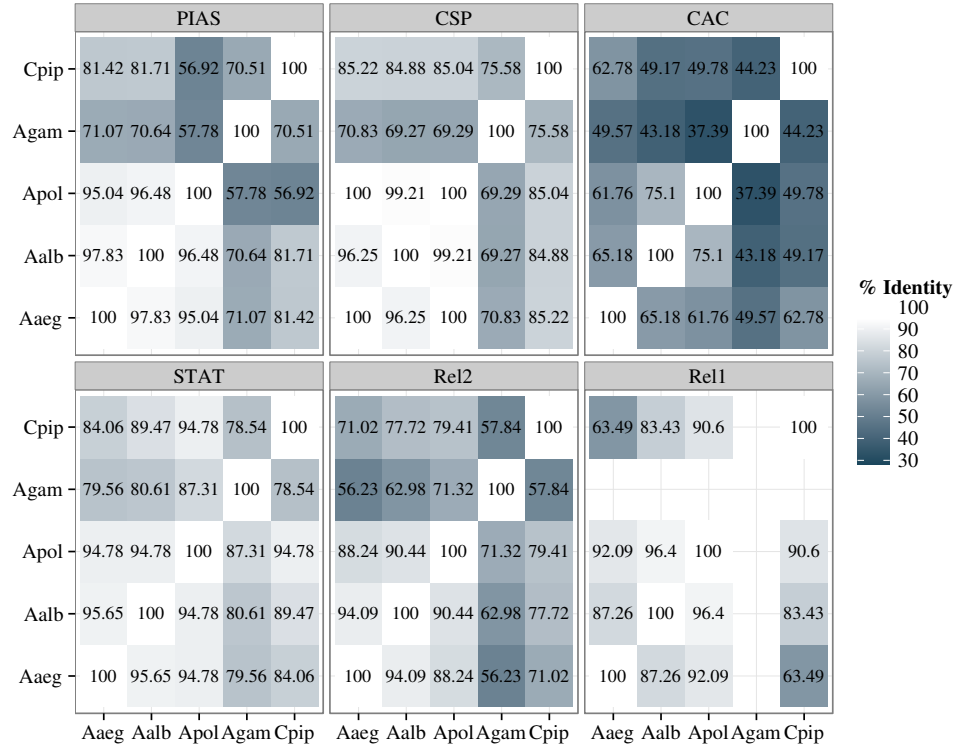
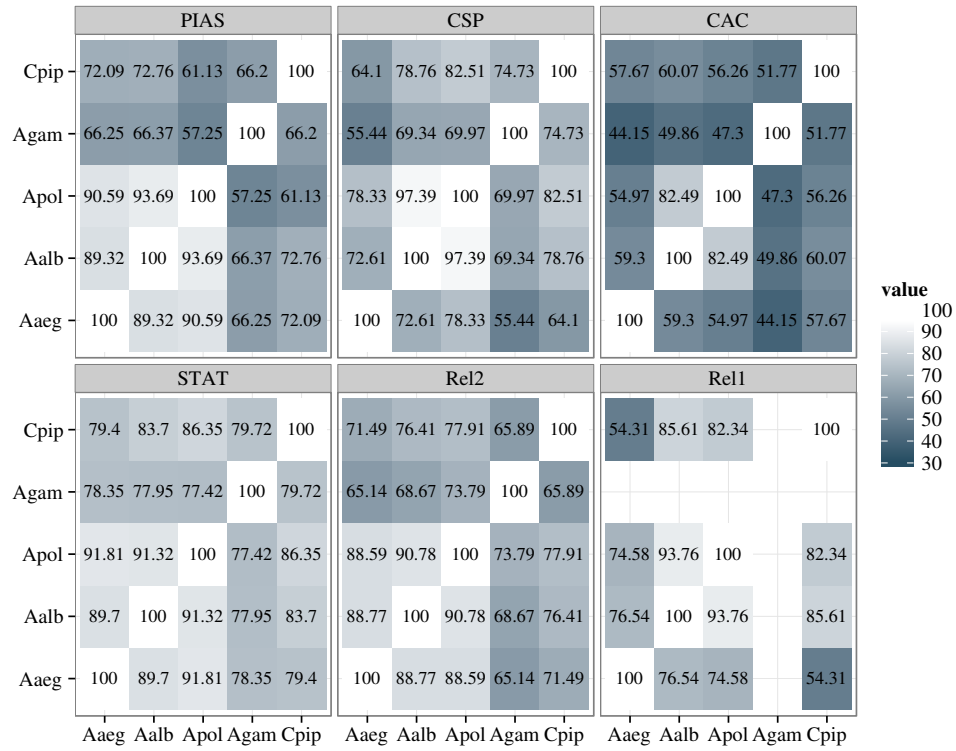


Figure 2.3: Comparison of nucleotide sequences (top) and amino acid sequences (bottom) from *Ae. aegypti* (Aaeg), *Ae. albopictus* (Aalb), *Ae. polynesiensis* (Apol), *An. gambiae* (Agam) and *Cx. quinquefaciatus* (Cpip). Percentage identity matrices were derived from Clustal Omega alignments (Sievers et al., 2011).

common dsRNA template primers were found but in some genes species-specific qRT-PCR primers were necessary to produce the desired efficiency of 1.8 - 2.1 and single peaks following melt curve analysis. These primers were then used to assess immune pathway gene expression in *Wolbachia*-infected *Ae. albopictus*, with a specific focus on the Toll pathway.

2.3.2 Immune gene expression in *Ae. albopictus*

Expression of CAC, Rel1, CSP, Rel2, PIAS and STAT mRNA in the *Wolbachia*-uninfected *Ae. albopictus* cell line Aa23-T was compared to the same cell line infected with *wAlb*, *wMel* and *wMelPop* (Figure 2.4). These transinfected lines differ significantly in density, respectively harbouring a mean of 2.50, 17.66 and 38.40 *Wolbachia* genomes per host genome (Figure 2.4. One-way ANOVA, $F_{(2,5)} = 71.00$, $p < 0.001$. Tukey HSD, $p < 0.01$ for all comparisons).

Gene expression was analysed using Bayesian MCMC methods in the MCMC.qPCR R package, returning a 95% credible interval. Only one significant difference was found (Figure 2.5), a 5.66-fold increase in STAT expression in Aa23-T.*wMel* (pMCMC = 0.014). STAT expression in Aa23-T.*wAlb* was also raised by a mean 4.25-fold but this was insignificant. The most densely infected line Aa23-T.*wMelPop* showed the lowest STAT increase of 2.66-fold and again this was not significant. There was no increase in expression of the Toll pathway TF Rel1 or decrease in expression of its negative regulator CAC, both of which might be expected during Toll activation.

Immune gene expression in Uju.*wAlb* and Uju.*wMel* was then compared to uninfected Uju.T and normalised to the housekeeping gene S17 (Figure 2.7). Uju.*wAlb* had a mean *Wolbachia* infection density of 1.93 *Wolbachia* genomes per host genome, while Uju.*wMel* had a substantially higher infection density of 9.62 (Figure 2.6). Some primers such as STAT and PIAS gave variable results and displayed poor efficiency in certain sets, which were therefore excluded from the analysis. Three independent repeats were performed for genes in the Toll pathway, which were of primary interest. No consistent significant difference in CAC or Rel1 was observed, although Rel1 was upregulated 2.17-fold in one Uju.*wMel* replicate of three (pMCMC = 0.006). Rel2 was upregulated 1.85 or 1.87 times across two Uju.*wMel* replicates (pMCMC = 0.01 and 0.034 respectively) and by 1.85-fold in one Uju.*wAlb* replicate (pMCMC = 0.024). TAK1 also showed inconsistent, slight upregulation in Uju.*wAlb* (1.85-fold, pMCMC = 0.032) and Uju.*wMel* (1.88-fold, pMCMC = 0.030).

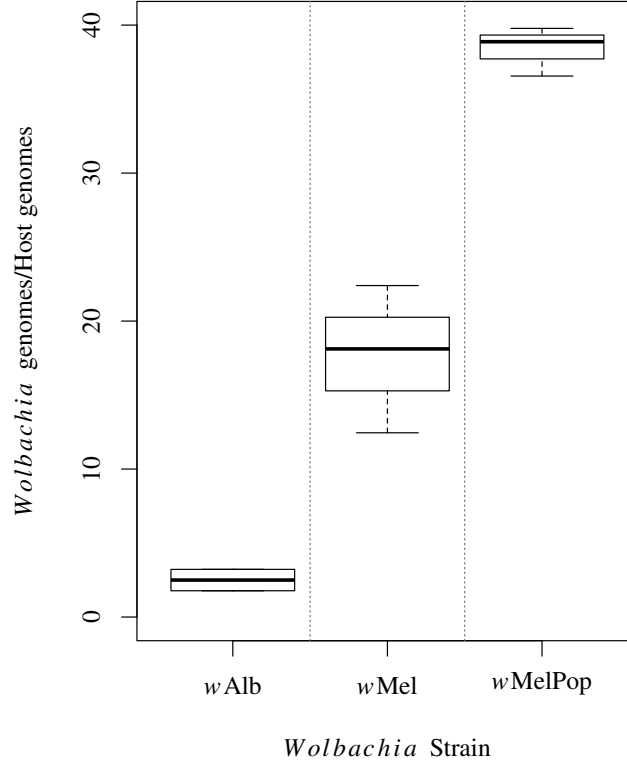


Figure 2.4: Ratio of *Wolbachia* genome copies to *Ae. albopictus* genome copies detected in Aa23-T.*wAlbB*, Aa23-T.*wMel* and Aa23-T.*wMelPop* cell lines. Genome copies were determined by absolute quantification using a standard curve of plasmid DNA containing the genes of interest. Results are from three biological replicates (two for Aa23-T.*wAlbB* due to loss of a sample).

2.3.3 H₂O₂ expression in *Ae. albopictus* adults and cell lines

Increased ROS levels were previously detected by flow cytometry and microscopy in naturally infected and antibiotic-cured *Ae. albopictus* cell lines (Brennan et al., 2008). Both assays utilised 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA), which is non-specific and will also detect some RNS (Halliwell and Whiteman, 2004; Winterbourn, 2008). It is thought to require a transition metal catalyst such as free iron, therefore the localisation of the probe with *Wolbachia*-containing vacuoles reported by Brennan et al. (2008) could be affected by cellular iron distribution and specifically the known sequestration of iron in insect vacuoles (Nichol et al., 2002). To avoid non-specificity and confounding factors, a H₂O₂-specific assay for reduced superoxide was chosen, following consideration of alternative assays (Halliwell and

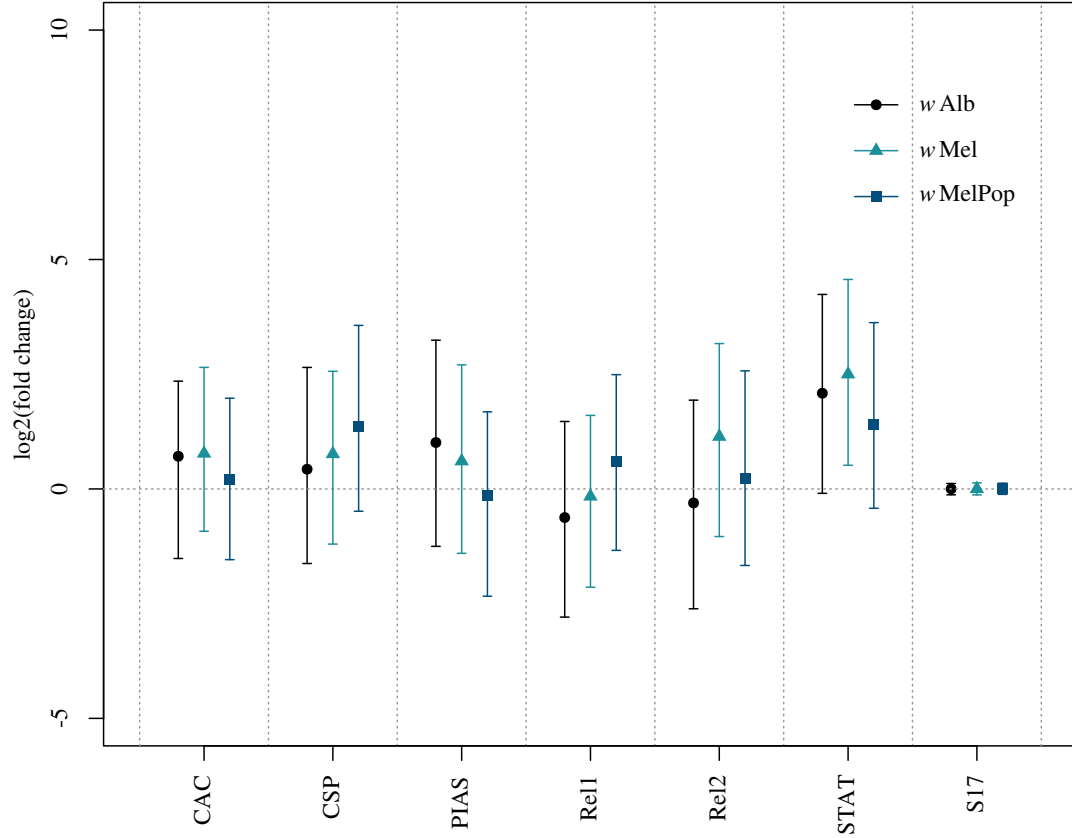


Figure 2.5: Immune pathway gene expression in Aa23-T.*wAlbB*, Aa23-T.*wMel* and Aa23-T.*wMelPop* cell lines compared to Aa23-T, normalised to expression of the housekeeping gene S17. Points represent the mean of three biological replicates and error bars denote 95% confidence intervals calculated using Bayesian MCMC methods in MCMC.qPCR R package.

Whiteman, 2004). This was also used by Pan et al. (2012) for ROS detection in *Ae. aegypti* adult samples.

All Uju mosquito and Aa23 cell lines were assayed for H_2O_2 to determine if *Wolbachia* infection increased ROS production and if any effect correlated with *Wolbachia* density (Figure 2.4).

Relative H_2O_2 generation in Aa23 cells across three independent experiments was significantly different between strains and analysed by Kruskal-Wallis testing as the data was non-normal (A: $\chi^2 = 43.80$, $df = 3$, $p\text{-value} = 1.67e-09$. B: $\chi^2 = 41.89$, $df = 3$, $p\text{-value} = 4.23e-09$. C: $\chi^2 = 26.04$, $df = 3$, $p\text{-value} = 9.36e-06$). Post-hoc

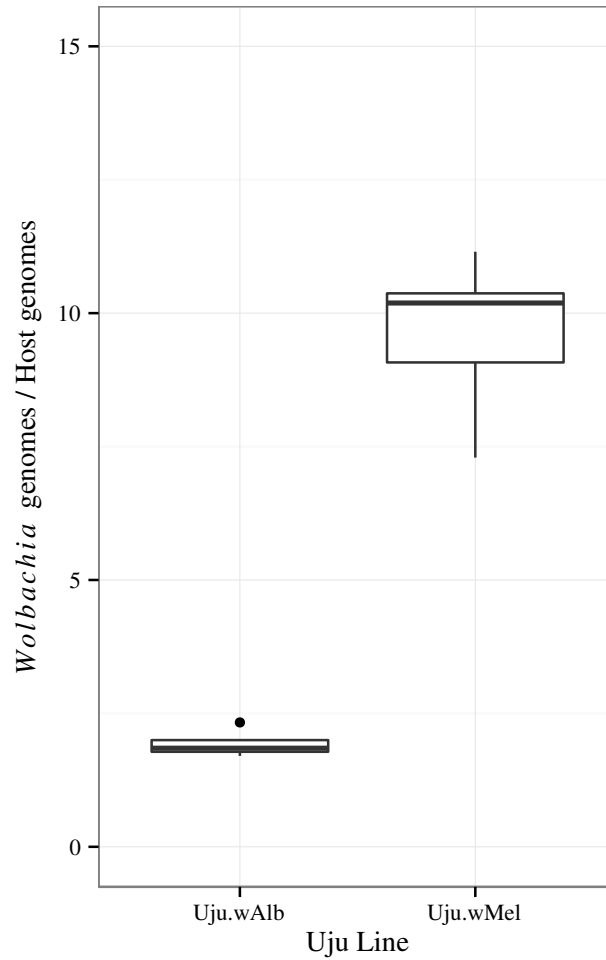
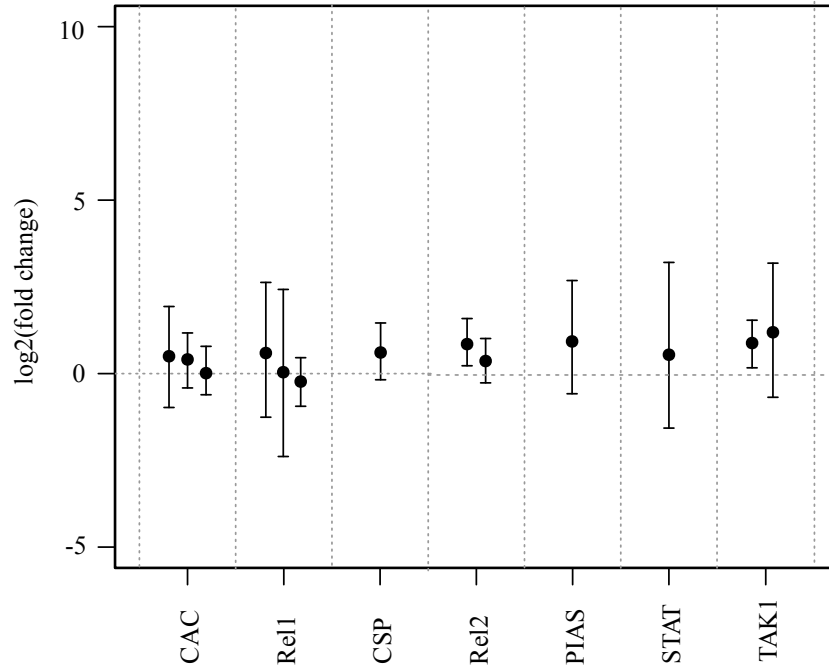


Figure 2.6: Ratio of *Wolbachia* genome copies to *Ae. albopictus* genome copies detected in Uju.wAlb and Uju.wMel adult lines. Genome copies were determined by absolute quantification using a standard curve of plasmid DNA containing the genes of interest. Results are from 4–5 biological replicates of pools containing three females.

bootstrap ratio testing showed significant differences ($p < 0.05$) between uninfected and infected lines as denoted on Figure 2.8, but these were inconsistent in direction across experiments, with each infected line producing more H_2O_2 than uninfected in two out of three experiments but producing less H_2O_2 in the third. No overall trend towards increased H_2O_2 in *Wolbachia*-infected cell lines was thus observed.

In crude adult extracts, there was no trend towards increased H_2O_2 levels in *Wolbachia*-infected lines. A typical result was a mean of 730 pmol H_2O_2 per μg protein in the uninfected Uju.T line compared to 646 and 689 pmol respectively in Uju.wAlb and Uju.wMel, resulting in no significant difference between the lines (Figure 2.9a).

(a) *Uju.wAlb*



(b) *Uju.wMel*

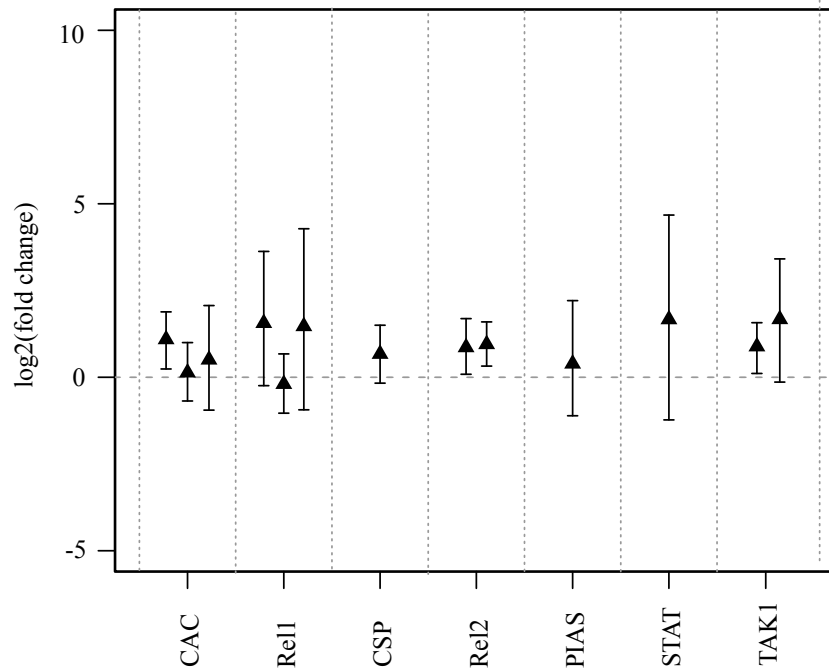


Figure 2.7: Immune gene expression in *Uju.wAlb* and *Uju.wMel* compared to *Uju.T*. Points represent mean of 3–5 biological replicates and error bars denote Bayesian 95% credible intervals. Each bar within a gene category represents an independent experiment.

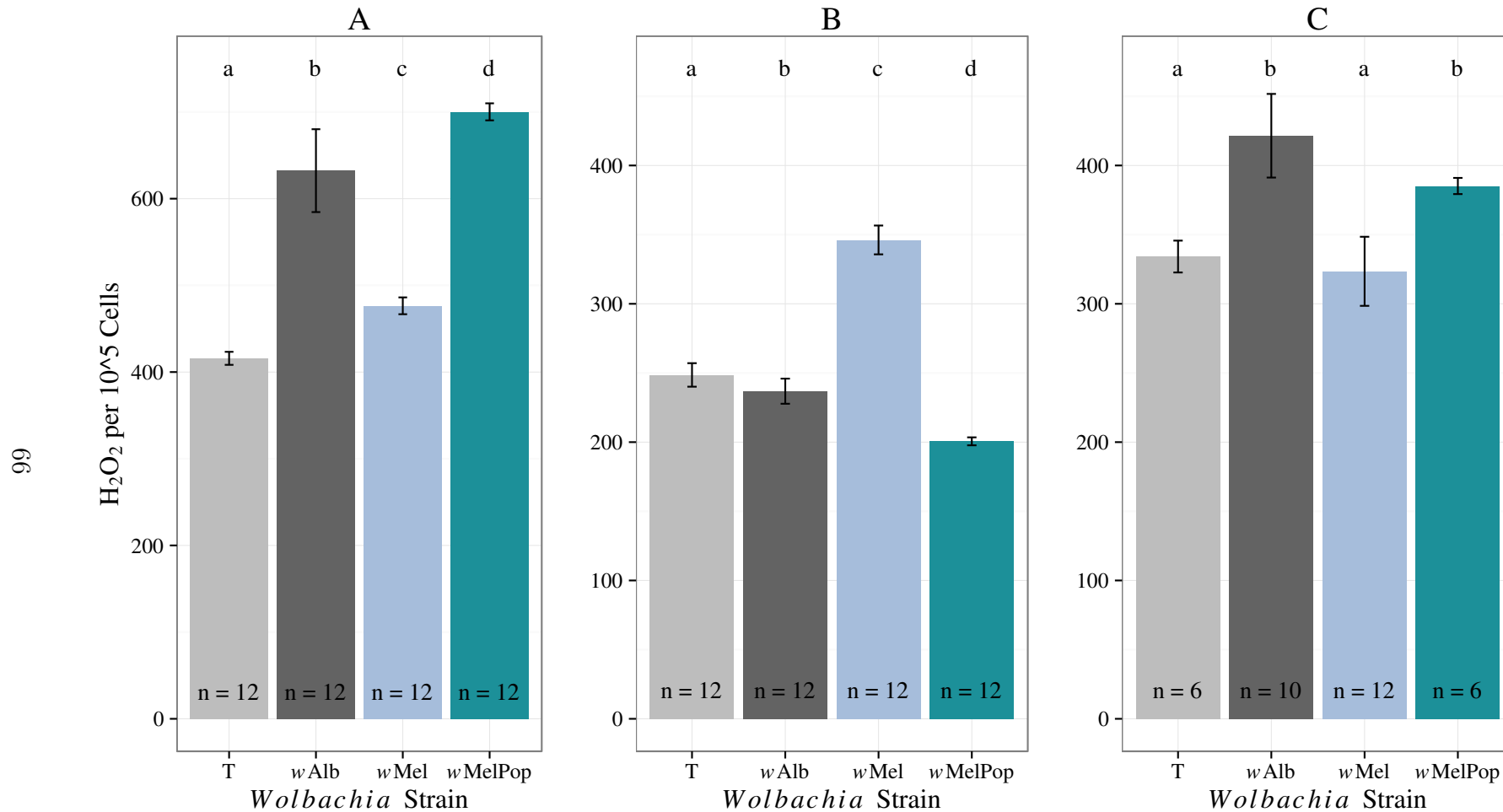


Figure 2.8: H_2O_2 detected in Aa23 cells, normalised to cell count. Graphs A, B and C represent independent experiments. Bars represent mean of n samples and error bars represent the standard deviation of the mean. Lower case letters represent significant pairwise differences at $p < 0.05$ derived from post-hoc bootstrap ratio testing.

ANOVA, $F_{(2,15)} = 0.592$, $p = 0.566$). Two further independent experiments had means in the same approximate range (min = 619, max = 1154) and showed no difference between lines (Figure 2.9b, ANOVA, $F_{(2,15)} = 1.06$, $p = 0.371$. Figure 2.9c, ANOVA, $F_{(2,12)} = 1.548$, $p = 0.252$).

2.3.4 ROS-Generating Enzyme and Antioxidant Expression

The lack of a detectable difference in H_2O_2 between strains could be because ROS production is similar or alternatively because there is a compensating increase in antioxidant enzyme expression to maintain redox homeostasis (Brennan et al., 2008). To differentiate between these hypotheses, expression of a selection of antioxidant and ROS-generating genes were assessed by qRT-PCR.

Pan et al. (2012) performed this analysis in *Ae. aegypti* and observed a 28-fold upregulation of ‘Duox2’ (Entrez Gene ID 5569376), which they described as an additional ROS-producing enzyme. However, this conflicts with existing evolutionary data on Duox orthologues (Kawahara et al., 2007). In *Ae. aegypti* and other sequenced members of the Culicidae, there are two NOX enzymes (NOXM and NOX5) and one Duox enzyme. A BLAST comparison and conserved domain analysis of the ‘Duox2’ protein sequence demonstrated that the gene contains neither a peroxidase nor other domains which characterise Duox enzymes (Figure 2.10). It is instead a DuoxA protein, sharing 68% amino acid identity with the *D. melanogaster moladietz* gene encoding Numb Interacting Protein (NIP), a maturation factor that is essential for the effective functioning of Duox and is thought to aid its transport to the plasma membrane (Grasberger and Refetoff, 2006; Xie et al., 2010).

Following degenerate PCR, Duox and DuoxA orthologues were sequenced from *Ae. albopictus* cDNA but NOXM and NOX5 were unobtainable despite repeated attempts. Degenerate PCR was also used to clone and sequence a range of antioxidant enzymes and regulatory transcription factors involved in redox homeostasis such as Jnk, OXR1 and TAK1 (Figure 2.2). All sequences were validated (or obtained in the case of NOX5 and NOXM) on release of the *Ae. albopictus* transcriptome (Poelchau et al., 2011).

In keeping with the lack of observed increase in ROS expression, there was no significant increase in expression of either Duox or DuoxA mRNA in *Wolbachia*-infected *Ae. albopictus*. There was also no significant change in any antioxidants

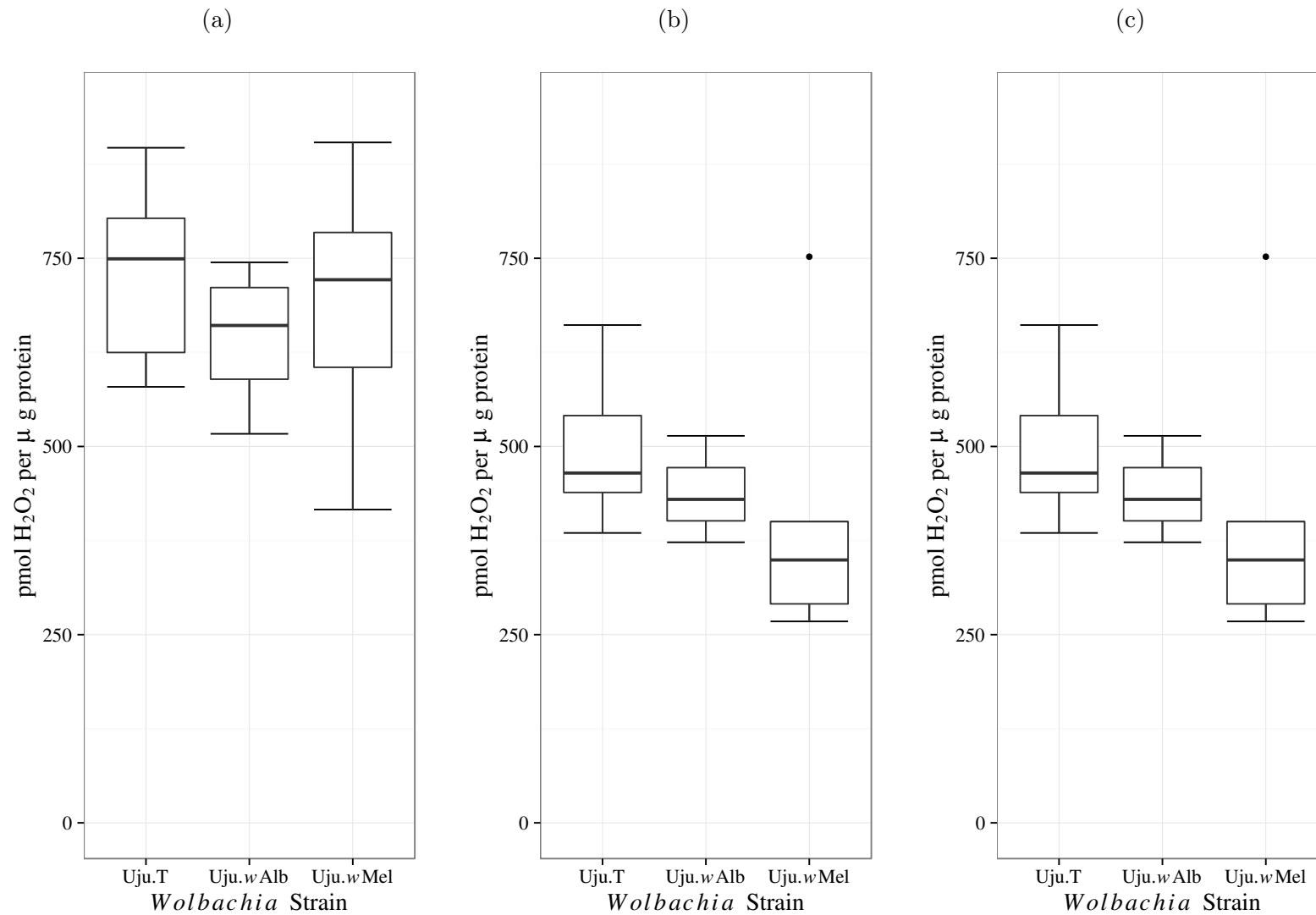


Figure 2.9: H_2O_2 detected in adult homogenates of *Ae. albopictus* lines Uju.T, Uju.wAlb and Uju.wMel, normalised to sample protein content. Data was obtained from six pools of three adult females per line in each of three independent experiments.

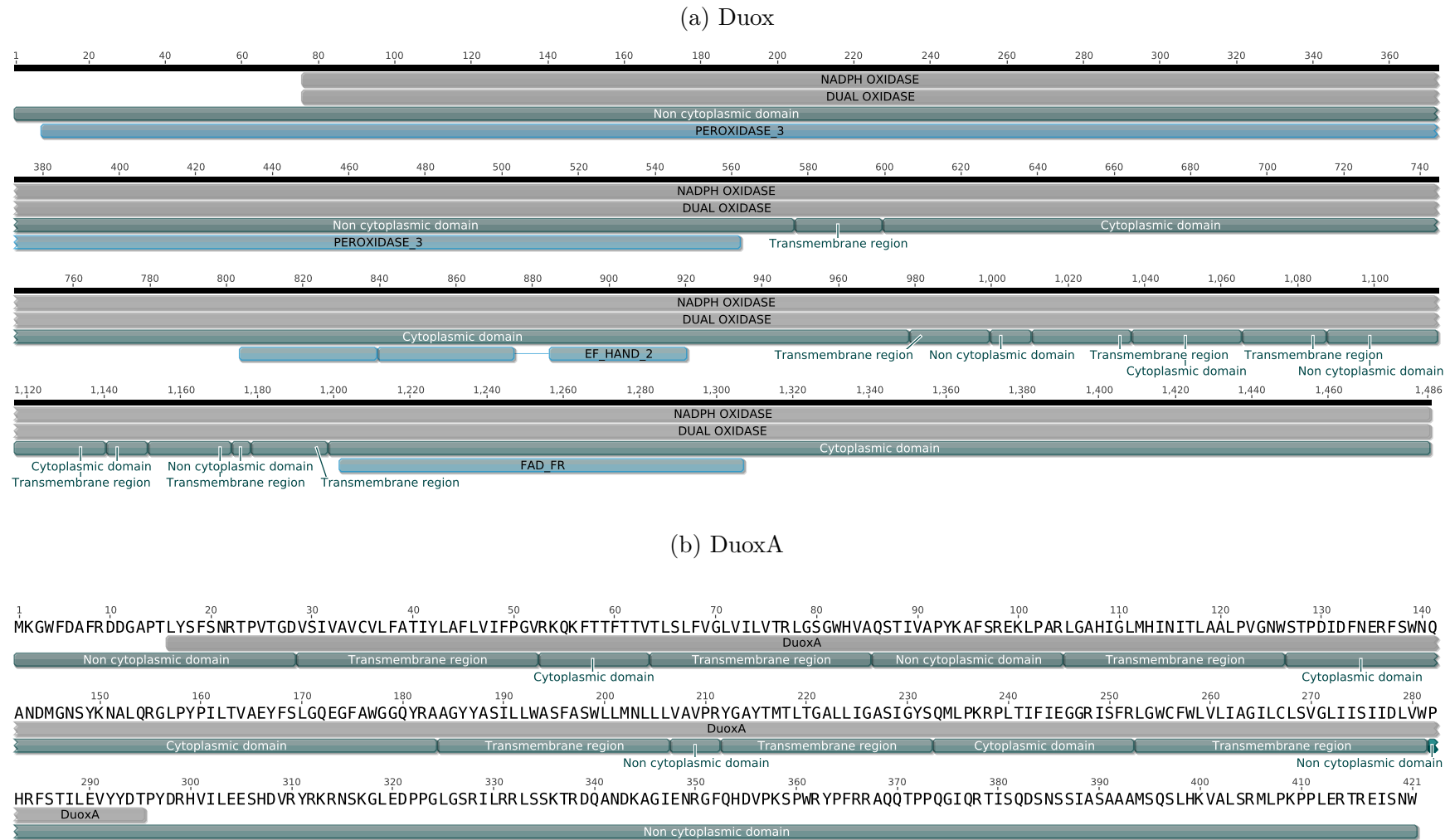


Figure 2.10: Protein domains highlighted during InteProScan analysis from Pfam (grey), PHOBIUS (teal) and Prosite profile (blue) databases. A) *Ae. aegypti* Duox1 (Protein RefSeq ID: XP_001658452.1) contains the characteristic NADPH Oxidase (NOX) domain, N-terminal peroxidase domain and EF-hand-containing calcium-binding domain. B) DuoxA (Protein RefSeq ID: XP_001658450.1), described as Duox2 in Pan et al. (2012), contains none of these domains and therefore does not generate ROS but is instead a Duox maturation factor. Alignment images are from Geneious (version 7.1.5, Biomatters, Available from <http://www.geneious.com> Kears et al., 2012).

Gene	Aaeg GenBank ID	Aalb Contig ID	Protein ID%	Nucleotide ID%
NOX5	XM_001660688_	075184	95	51
NOXM	XM_001654244	114843	89	80
Duox1	XM_001658402	144190	98	41
DuoxA	XM_001658400.1	102149	89	42
FoxO	EF088380	127112	81	41
Gpx	AF394234_	063197	86	67
Jnk	JQ291732.1	107370	97	43
OXR1	XM_001662152_	078559	98	81
MnSOD	AY745980	145590	85	78
CuZnSOD	DQ440061	143681	98	45
TAK1	XM_001658046	099738	88	77

Table 2.16: NCBI RefSeq IDs for a subset of *Ae. aegypti* genes associated with ROS production and antioxidant pathways. *Ae. albopictus* sequences were obtained by degenerate RT-PCR or from the developmental transcriptome (<http://albopictusexpression.org/>, Poelchau et al. (2011)), from which the relevant Aalb_OocyteEmbryoPharatarvaeAssembly contig references are supplied. Nucleotide and protein similarities were obtained by ClustalW pairwise alignment (Larkin et al., 2007).

assayed, only Uju.wAlb OXR1 was upregulated by mean 2.77-fold in one experiment of three.

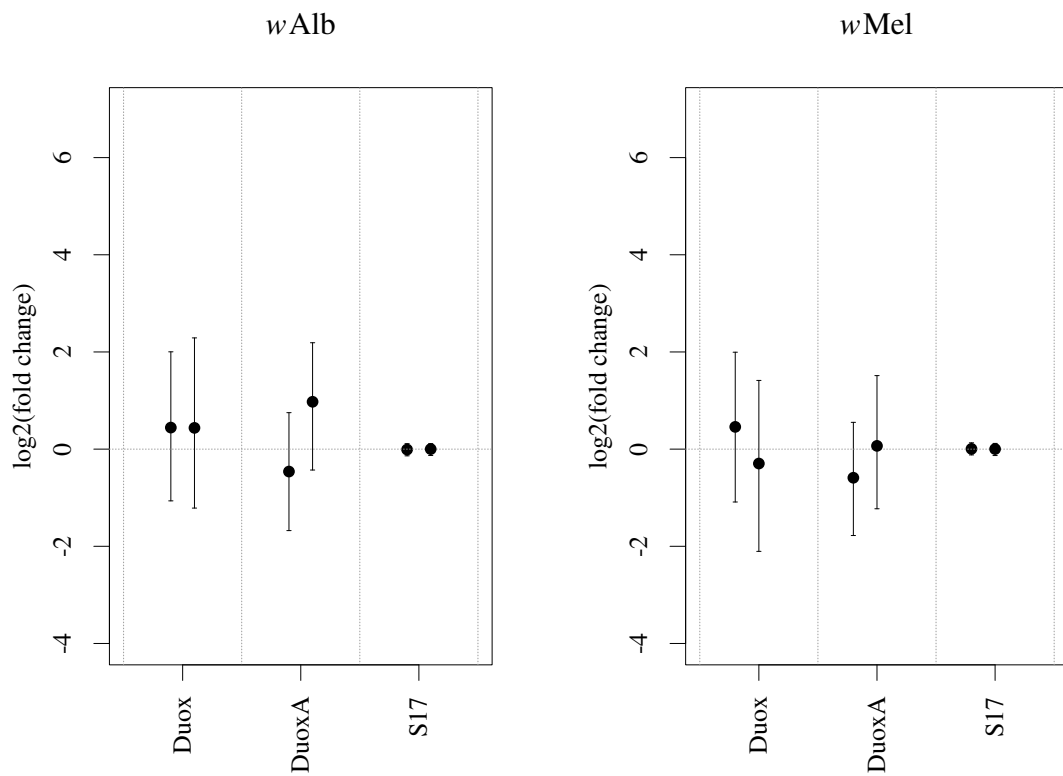


Figure 2.11: Expression of Duox and DuoxA in *Uju.wAlb* and *Uju.wMel* adult females, normalised to S17 expression and to uninfected *Uju.T*. Points represent mean of 3–5 pools of three females and error bars denote Bayesian 95% credible intervals. Each bar within a gene category represents an independent experiment.

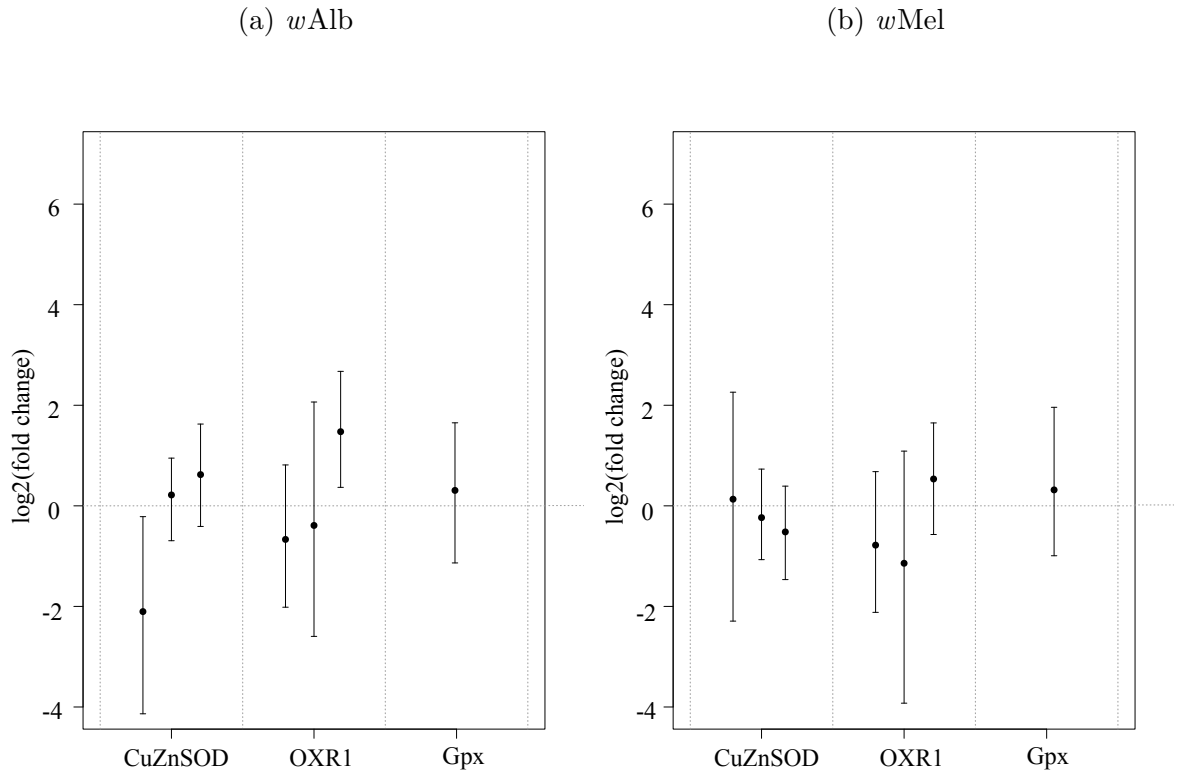


Figure 2.12: Expression of CuZnSOD, Gpx and OXR1 in Uju.*wAlb* and Uju.*wMel* adult females, normalised to S17 expression and to uninfected Uju.T. Points represent mean of 3–5 pools of three females and error bars denote Bayesian 95% credible intervals. Each bar within a gene category represents an independent experiment.

2.4 Discussion

No significant upregulation of ROS or any of the innate immune pathways was demonstrated in *Ae. albopictus*, although in cell lines there was some suggestion that STAT is upregulated which requires further investigation. Based on analogous studies, one would have expected to see upregulation on the order of xxxx in TFs and xxx in functional enzymes, which was not present.

Overall, these results suggest that the hypothesis of ROS-induced Toll pathway activation causing DENV refractoriness in *Ae. aegypti*, put forward by Pan et al. (2012), may not apply to *wMel*-infected *Ae. albopictus*. This conclusion based on the observation that *Uju.wMel* does not transmit DENV and CHIKV (Blagrove et al., 2013,?), with the caveat that tripartite interactions were not examined in this chapter. In turn, this indicates that immune upregulation and priming is not the predominant causal factor of *Wolbachia*-induced DENV inhibition, a conclusion which is strengthened by corresponding work in *D. melanogaster* (Rancès et al., 2012; Wong et al., 2011). Immune activation and ROS may still be important in *Ae. aegypti* as it is probable that the importance of immune pathways differs across different *Wolbachia*-host-virus interactions.

Such tripartite interactions were not investigated here and the possibility remains that although advance priming is not observed, immune pathways and ROS do play a role in *Ae. albopictus* post-DENV infection. Effects may also be confined to particular tissues and time points but data in this chapter is from whole 7 day old adult females only. These mosquitoes are old enough to bloodfeed but as DENV has an incubation period of over 10 days they may not be at the lifestage where DENV inhibition occurs, a point which is currently unclear. For instance, the blocking could occur in early replication of the virus following entry into the midgut through to late-stage inhibition in the salivary glands with tissue-specificity of *Wolbachia* and its associated molecular effects likely also playing a role. Evidence from *Ae. albopictus* and *Ae. aegypti* has focused primarily on the effect of age and tissue on *Wolbachia* density, showing that while *Wolbachia* is distributed throughout the body it is densest in the germline and reduces with age (Bian et al., 2010; Calvitti et al., 2015; Mousson et al., 2010). Transmission assays in *Uju.wMel* have shown that no infectious viral particles are present in excreted saliva, suggesting an earlier stage inhibition (Blagrove et al., 2012), but no experiments have yet been published tracking DENV infection in stable

Wolbachia-infected adults to elucidate the question and assays in cell culture (Section 1.5.4) fail to address questions about spatiotemporal expression in whole organisms.

Lack of immune activation in *Ae. albopictus* also leads to questions over its co-evolved immune adaptation and potential attenuation of immune responses to *Wolbachia*, including the potential role of ROS and innate immunity in controlling *Wolbachia* infection and proliferation. No functional assays examining this question were found in the literature but it is known that innate immunity controls Rickettsiales bacteria related to *Wolbachia*, such as tick-borne *Anaplasma phagocytophilum* or *Ehrlichia chaffeensis* (Baldrige et al., 2005; Liu et al., 2012). These bacteria are pathogenic so it is difficult to draw parallels with the complex but often commensal endosymbiotic interaction of *Wolbachia* and native or naive mosquito hosts. However, endosymbionts from other phyla have been shown to be regulated by AMPs, e.g. the weevil genus *Sytophilus*' primary endosymbiont (SPE) (Login et al., 2011). Some endosymbionts have also been shown to evade, suppress or resist immune responses (Haag et al., 2011).

Intriguingly, the *Wolbachia* genome contains many ankyrin repeats, a protein domain which is more common in eukaryotes (Iturbe-Ormaetxe et al., 2005) and found in NF- κ B proteins such as Rel1 and Rel2 and I- κ B proteins such as Cactus and Caspar. This raises the possibility that *Wolbachia*-derived proteins could directly interact or interfere with insect immune pathway proteins, as has been demonstrated in other bacteria and viruses (reviewed in Al-Khodori et al., 2010; Bitra et al., 2012).

The experimental evidence for gene expression presented in this chapter relates solely to the transcriptional level and necessarily chose a limited set of genes and samples. Further work at the protein level and functional assays using the sequences and primer toolkit provided in this chapter to knockdown relevant pathway genes will be required to gain a deeper understanding of *Wolbachia*-host immune interactions and determine the extent to which transcriptional regulation plays a role. The lack of concurrence with Brennan et al. (2008) regarding antioxidant upregulation in *Ae. albopictus* cell lines particularly merits some further investigation as the authors analysed proteins rather than mRNA transcripts. In all cases, the high variability in gene expression observed in this chapter both within and between experiments suggests that further experiments should include large sample sizes to increase confidence in low level changes.

The question of what causes DENV resistance in species which do not display immune activation remains open. Some alternative hypotheses have already been discounted, for example siRNA-mediated RNA-interference (RNAi) was shown not to be implicated in *D. melanogaster* *Wolbachia*-induced viral immunity (Hedges et al., 2012). It appears that any antiviral effect is *Wolbachia* density dependent, as inferred from the greater DENV resistance seen in *wMelPop* compared to *wMel*-infected *Ae. aegypti* and *Ae. albopictus* cell lines (Frentiu et al., 2010) and strengthened by later results showing that *wAlbB*, which does not induce DENV resistance in its low density natural infection of *Ae. albopictus*, can do so in a density dependent manner in *Ae. albopictus* cell lines (Lu et al., 2012). This is consistent with involvement of AMPs in resistance, as a linear positive correlation between *Wolbachia* density and Defensin D (DEFD) expression was observed by Lu et al. (2012). However, given the lack of evidence for increased AMP production (Blagrove et al., 2012) and immune pathway signalling in *Ae. albopictus* presented here, alternative hypotheses which are also compatible with density dependence must be pursued. These include resource competition for metabolites or lipids required by the virus, such as cholesterol and fatty acids, which will be investigated in Chapter 3.

Chapter 3

Wolbachia-induced modulation of *Ae. albopictus* lipid metabolism

3.1 Introduction

Substantial attention has been focused on the role of immune pathways in *Wolbachia*-induced arbovirus resistance in *Aedes* mosquitoes, but the lipidome of DENV-infected cells also undergoes significant changes affecting the viral lifecycle (Perera et al., 2012). Cholesterol plays an important role in entry of DENV and other arboviruses to mammalian cells (Gay et al., 2012; Poh et al., 2012; Shi and Luo, 2012; Zaitseva et al., 2010) and autophagy, encapsulation of cytoplasmic components in double membrane vesicle for degradation, causes triglyceride release from lipid droplets to provide energy for replication via β -oxidation (Heaton and Randall, 2010, 2011a; Judith et al., 2013; Rothwell et al., 2009). Once arboviruses reach a sufficient titre, it is likely that they also require particular lipid and cholesterol profiles for exit from the cell, as inferred from work in Semliki Forest Virus (SFV) (e.g. Marquardt et al., 1993). *Wolbachia*-induced modulation of lipid metabolism therefore requires investigation.

As per innate immunity and ROS homeostasis, lipid metabolism is not only implicated in DENV-mosquito interactions but is also critical to the *Wolbachia*-host relationship. Hosts provide both fatty acids and cholesterol to the bacteria, which lacks synthesis pathways for either metabolite in its streamlined genome (Wu et al., 2004). While insects are able to synthesise fatty acids and other lipid classes, they are sterol auxotrophs (Hobson, 1935). Cholesterol plays a key structural role in cell membranes and is essential for cell signalling and synthesis of steroid hormones vital for development so *Wolbachia*-host symbioses provide a likely arena for resource competition over dietary cholesterol. Current evidence points towards a reduction

in membrane cholesterol in *Wolbachia*-infected insects — total cholesterol in *Wolbachia*-infected *Ae. aegypti* was reduced by 15–25% (Caragata, 2012; Caragata et al., 2014), while free cholesterol in *wMel*-infected *Ae. albopictus* midguts was reduced by up to 51% (Blagrove, 2013). Increasing dietary cholesterol can reduce the viral refractoriness observed in *wMel*-infected *D. melanogaster* (Caragata et al., 2013), strongly suggesting that cholesterol modulation by *Wolbachia* plays a functional role in DENV infection, although the nature of that role in mosquitoes remains an open question.

Autophagy is another lipid metabolic pathway that is already known to be critical to *Wolbachia* density regulation, providing a mechanism for fatty acid release (reviewed in Singh et al., 2009) through breaking down lipid droplets but also posing a destructive risk to cytosol-dwelling pathogens and symbionts through a related pathway. Recent work by Voronin et al. (2012) found that activation of autophagy reduced bacterial density to a similar level as antibiotic treatment while suppression resulted in increased density. *Wolbachia* must therefore subvert or evade the autophagic response to maintain stable infections. This result is interesting in the light of autophagy’s role in DENV replication, whereby autophagic suppression significantly reduces DENV replication in human cells, an effect which is reversible on supplementation with free fatty acids (Heaton and Randall, 2010).

This result complements that of Perera et al. (2012), who demonstrated that inhibition of fatty acid synthase in mosquito cells at 4–12 h post DENV infection resulted in greatly reduced viral titres. Autophagy therefore appears to enhance viral replication by provision of fatty acids. However, this may not be the full story as autophagosome markers have been associated with viral replication complexes of both DENV and Japanese Encephalitis along with several non-arboviruses. Co-localisation with endosomes suggests a role for fusion of these intracellular compartments in viral uncoating while in other infections autophagosomes have been shown to form part of the membranous scaffold on which viruses replicate (reviewed in Shi and Luo 2012).

Interestingly, Teixeira et al. (2008) reported that *Wolbachia* protected *D. melanogaster* against a range of RNA viruses but not Insect Iridovirus VI (IIV-6), a DNA virus. As most DNA viruses replicate in the nucleus, autophagy is not expected to be as significant a requirement for their replication, although it is necessary for some (Kudchodkar and Levine, 2009). *Wolbachia* did not reduce replication or titre of some of the RNA virus types studied by Teixeira et al. (2008) but some viruses do not require autophagy or are damaged by it. For example, Sindbis virus, which was not tested

in the study, can be cleared by autophagy (Orvedahl and Levine, 2008; Orvedahl et al., 2010). Such viruses would be expected to maintain or increase their titre in an autophagy-suppressed system.

The influence of *Wolbachia* on broader host lipid metabolism remains unknown and the work of Perera et al. (2012) in highlighting the complex interactions between DENV and the mosquito lipidome have brought this question to the fore. Little data has been published on *Wolbachia* and lipid metabolism beyond its influence on cholesterol levels. Scheitz et al. (2013) investigated heritability and inter-population differences in *D. melanogaster* lipid profiles and found that *Wolbachia* abundance correlated with gain of odd-chained fatty acids in females and an increase in phosphatidylserines, a class of phospholipids which are responsible for signaling resulting in the clearing of apoptotic cells, suggesting that *Wolbachia* was inducing stress and apoptosis.

No such data is available for mosquitoes and there is uncertainty about the lipid-metabolic needs and capabilities of *Wolbachia* in addition to the multiple and complex roles of lipids in cell structure, metabolism and signalling. Therefore, specific *a priori* hypotheses regarding the effect of *Wolbachia* infection on the cell lipid profile would require significant speculation. An untargeted analysis from which functional hypotheses can be developed is therefore an appropriate investigative approach.

I performed cholesterol supplementation experiments to provide evidence for the hypothesis that cholesterol is a limiting factor in *Wolbachia* infection density. I also performed qRT-PCR on a range of genes related to lipid metabolism and autophagy to determine expression changes in *Wolbachia*-infected lines. In order to broadly identify candidate lipid classes and lipid pathways modulated in *Wolbachia*-host metabolic interactions, an untargeted lipidomic analysis of *Ae. albopictus* cell lines Aa23-T (uninfected), Aa23-T.wMel and Aa23-T.wMelPop was performed in collaboration with Dr Ulf Sommer (NERC Biomolecular Analysis Facility - Birmingham (NBAF-B), University of Birmingham).

3.2 Materials and Methods

3.2.1 *Ae. albopictus* gene expression

Aa23-T cell lines and Uju adult colonies were maintained as per Sections 2.2.1 and 2.2.2. Samples for *Wolbachia* density analysis and qRT-PCR were extracted, processed and

analysed as per Sections 2.2.3-2.2.8 and 2.2.9. Primers used in this analysis are listed in Table 3.1.

Gene	Forward Primer	Reverse Primer
<i>IAP</i>	Alb_qIAP_F1 ATCGCGAGAAGAGGAGCATC	Alb_qIAP_R1 TGCCATCATCATTGAGCCA
<i>atg8</i>	Alb_qATG8_F1 AGAGGATTCACCTAAGGCCAG	Alb_qATG8_R1 TGGTACAGCGAACCCATTGTTG
<i>FoxO</i>	Alb_qFoxO_F1 AAACTGGCCCATCAACGTCT	Alb_qFoxO_R1 TGACAACGACACCCCAAAGT
<i>FAS</i>	Alb_qFAS_F1 TCTGCGAGTATTGTGGCGTT	Alb_qFAS_R1 CACGATTGCAAAAATGCGCC
<i>ACAT</i>	Alb_qACAT_F CCACGTTGGATGCATAGGGA	Alb_qACAT_R CGGTGCGAAACGAAACTGACG
<i>DHR96</i>	Alb_qDHR96_F GCAATCTCTGCCGAACGAAG	Alb_qDHR96_R GCAAGGGAAAATACTCGGGA
<i>Npc1b</i>	Alb_qNpc1b_F CAACAGTCCTCTTCCCGGTC	Alb_qNpc1b_R TCGTCTAAGGAGTCGGGTGT

Table 3.1: Primers designed against *Ae. albopictus* transcripts (Poelchau et al., 2011) using Primer-BLAST (Ye et al., 2012).

3.2.2 *D. melanogaster* gene expression

D. melanogaster w1118 with *wMel* infection and a tetracycline-cured line were maintained at 25°C in a 12 h/12 h light/dark cycle on Carolina Biological Supply Company formula 4-24 (C4-24) instant food supplemented with yeast. For RNA extraction, *D. melanogaster* females were provided with apple agar plates for oviposition, which were then left overnight for hatching. Forty L1-L2 larvae were added per standard vial containing Carolina 4-24 food only to control cholesterol intake and adults were collected at 4–5 days old. 4–5 pools of three females were RNA extracted as per Section 2.2.3. cDNA synthesis, qRT-PCR and statistical analysis were performed as per Section 2.2.8

3.2.3 *D. melanogaster* cholesterol supplementation

C4-24 instant food consists of dried potato flakes, and in a 3.6 ml final volume contains approximately 2 ng gm⁻¹ of cholesterol, as determined by organic extraction and GC/MS (Horner et al., 2009). This is significantly lower than the optimal amount for fly growth and can therefore be considered a low cholesterol diet (Sang, 1956). Low cholesterol media was prepared fresh in each standard *Drosophila* tube with 2 g media and 5 ml dH₂O. Cholesterol supplemented tubes were made up using containing 50

Gene	Forward Primer	Reverse Primer
<i>ACAT</i>	Dm_qACAT_F CACAAACTGAAACCGCACAG	Dm_qACAT_R CGGACACGAAACAGAAGACCA
<i>DHR96</i>	Dm_qDHR96_F ACCAGGTGGCCCTACTCA	Dm_qDHR96_R CATGTTCTCTTTGGTATGGGGTA
<i>Npc1b</i>	Dm_qNpc1b_F CGGATTTTGTTCAGCAACT	Dm_qNpc1b_R GCCATTCTCAGTAAATCCTCGT
<i>RPL32</i>	Dm_RPL32_qF ATGCTAAGCTGTGCGACAAATG	Dm_RPL32_qR GTTGATCCGTAACCGATGT

Table 3.2: Primers for *D. melanogaster* cholesterol metabolism related genes from Bujold et al. (2010), RPL32 primers from Ponton et al. (2011).

or 200 μ g water soluble cholesterol (Sigma) in 200 μ l ethanol per g media, added at the same time as the dH₂O. Tubes were well mixed and allowed to soak for at least 1 min.

Forty L1-L2 larvae adults were plated in five tubes per treatment and three females per tube were collected at 4–5 days old for DNA extraction and *Wolbachia* density analysis as per Section 2.2.9.

3.2.4 Cholesterol depletion and supplementation in cell lines

1x10⁶ Aa23-T and Aa23-T.*w*Mel cells were seeded in 12-well plates with 1 ml Schneiders Medium (+10% FBS and 1% pen-strep) and media was replaced after 24 h with the same mixture (control), cholesterol supplemented and cholesterol depleted media. Methyl- β -cyclodextrin (M β CD; Sigma Aldrich Ltd; C4555) was used as a cholesterol depleting agent following Lin and Rikihisa (2003) and Hafer et al. (2009). Hafer et al. (2009) propagated mosquito cells in MEM-E media with M β CD added in an 8:1 molar ratio to serum cholesterol. Cholesterol levels in FBS are demonstrably variable as the authors measured 1.1 mg ml⁻¹ in their batch whereas Fisher Scientific technical documentation states an average of 300 μ g ml⁻¹. An 8:1 ratio of M β CD to cholesterol (FW 386.65 g mol⁻¹) implies the authors used 22.8 mM M β CD over four passages and did not report significant problems with cell growth, therefore 10 mM diluted in media from a 1 M stock in DMSO was used over over a 72 h incubation period. Cholesterol supplementation was undertaken using Synthescol animal-free cholesterol (Sigma Aldrich; S5442) at 200x dilution. 1% DMSO was added to both the cholesterol supplemented and control wells to account for any related toxicity.

After 72 h, media was removed, cells were resuspended in 300 μ l CTAB buffer and DNA was extracted then *Wolbachia* density qPCR was performed and analysed

as per Section 2.2.9.

3.2.5 Lipid extraction from Aa23-T cell lines

Aa23-T, Aa23-T.*w*Mel and Aa23-T.*w*MelPop cells were grown in 25 cm² flasks (as per Section 2.2.2) in 5 ml of media with passaging of 1 ml cell suspension to 5 ml of new media every five days. Eight flasks per line were independently passaged for at least five weeks before lipid and DNA extraction. Cells were quenched 72 h after the final passage, before confluence was reached. A centrifuge was pre-chilled to -9°C for at least 1 h and the metal fixed angle centrifuge rotor was frozen at -20°C for 1 h to ensure rapid quenching of cells. 1.6 ml quenching solution (60:40 HPLC grade methanol:H₂O (Sigma-Aldrich)) per flask was added to pre-weighed 2 ml tubes and left on dry ice for 15 min to cool to approximately -40°C. Cells were scraped into the flask media with minimum disruption and the cell suspension was transferred to a 15 ml tube for centrifugation at room temperature for 5 min at 1000 x g. 500 µl cell suspension was retained for DNA extraction. Supernatant was removed by pouring and cell pellets were resuspended in residual media by gently pipetting. 200 µl of this cell suspension was added to 1.6 ml quenching solution at -40°C and mixed immediately by inversion. Samples were centrifuged in the pre-chilled rotor at -9°C, 2500 xg for 5 min. A maximum of 10 samples were processed at a time to ensure all were kept below 0°C. Quenching solution was removed using a glass Pasteur pipette and the tube was weighed to estimate cell mass before being frozen at -80°C until extraction.

For lipid extraction, HPLC grade methanol (EMD Millipore), H₂O and chloroform (Sigma-Aldrich) was cooled on ice and 8 µl mg⁻¹ pellet mass methanol and 7.2 µl mg⁻¹ H₂O were added to each sample tube. Tubes were vortexed for 30 s and returned to ice. 8 µl mg⁻¹ chloroform was added to 1.9 ml glass vials and after pulse vortexing, the pellet and methanol/H₂O mixture were transferred using a glass Pasteur pipette. Samples were vortexed and incubated on ice for 10 min before centrifugation at 1500 x g for 15 min at 4°C. Biphasic samples were left at room temperature for 5 min before the upper layer containing polar metabolites was removed using a Hamilton syringe. 270 µl of non-polar lipid-containing lower layer was then transferred to a 1.8 ml glass tube and stored overnight at -80°C. Seven Aa23-T samples and six samples from each infected line were taken forward. 40 µl was removed to combine for a quality control

(QC) sample before all samples were dried under nitrogen, taken back up in 270 μ l chloroform and stored at -80°C until use.

3.2.6 FTICR-DIMS and data processing

Fourier Transform Ion Cyclotron Resonance Direct Infusion Mass Spectrometry (FTICR-DIMS) was performed by Dr Ulf Sommer (NBAF-B, University of Birmingham). Negative-ion mode DIMS analyses were conducted using a hybrid 7-Tesla linear ion trap FT-ICR mass spectrometer (LTQ FT Ultra, Thermo Fisher Scientific) equipped with an Advion Triversa chip-based direct injection nanoelectrospray ion source (Advion Biosciences). Samples were diluted 1:4 in 5 mM ammonium acetate (from a 1 M stock in methanol) in methanol/chloroform 3:1. Technical triplicates were run in controlled randomized order. Data were acquired from m/z 120-1200 in 12 wide SIM (selected ion monitoring) windows (Weber et al., 2011). Failed runs were repeated the same night.

After statistical analysis (see below), peaks of interest were subjected to further MS analysis using the same instrumentation, employing narrow SIM windows for the determination of isotope patterns, and MS2 / MS_n fragmentation using collision-induced dissociation (CID) and infrared multiphoton dissociation (IRMPD).

Data processing was performed using NBAF-B in-house scripts in MatLab. During replicate filtering, peaks were allowed if found in two out of three technical replicates and the blank filter required biological signals to be 2x the height of background signals. Sample filtering with 2 ppm error and a 75% filter resulted in a matrix containing 2044 peaks (m/z values). Normalization was performed using the probabilistic quotient normalization (PQN) method, and missing values were filled in the resulting data matrix using the k-nearest neighbors (kNN) algorithm. A generalized logarithmised version of this normalised matrix was generated based on QC samples only for use in multivariate statistical analysis.

3.2.7 LCMS and data processing

Reversed-phase high performance liquid chromatography Fourier transform ion cyclotron resonance mass spectrometry (RPLC-FT-ICR-MS) based analysis of the lipids was carried out on a Thermo Scientific Dionex Ultimate 3000 system connected to the same mass spectrometer as above. Samples were dried and each taken up in the original volume of solvent A (5mM ammonium acetate, 5% isopropanol in water) /

Time (min)	A (%)	B (%)	C (%)	Flow ($\mu\text{l}.\text{min}^{-1}$)
0.00	100	0	0	200
1.00	100	0	0	200
11.00	0	100	0	200
12.00	0	100	0	200
20.00	0	0	100	200
24.00	0	0	100	150
24.01	100	0	0	150
30.00	100	0	0	200

Table 3.3: Table for LCMS separation protocol. Eight μl of each sample were injected onto a Dionex Acclaim C30 (Thermo Scientific, 2.1 x 150 mm, 3 μm particles) and separated at 40 °C with a flow rate of 200 $\mu\text{l}.\text{min}^{-1}$ and a gradient from solvent A (5mM ammonium acetate, 5% isopropanol in water) over solvent B (5 mM ammonium acetate, 5% isopropanol, 5% water in acetonitrile) to solvent C (5 mM ammonium acetate, 5% water in isopropanol).

solvent B (5 mM ammonium acetate, 5% isopropanol, 5% water in acetonitrile) 1:1 and centrifuged for 10 min at 4 °C and 4000 rcf. Eight μl of each sample were injected onto a Dionex Acclaim C30 (Thermo Scientific, 2.1 x 150 mm, 3 μm particles) and separated as per Table 3.3. One LC-FT-ICR-MS analysis was performed per sample. Data was acquired in the negative ion mode from m/z 120-1200 at a nominal resolution of 50,000 in centroid mode. A QC sample was pooled from all biological samples and analysed repeatedly at the start, end, and equidistantly throughout the sequence. Two further QC sample runs were performed using data dependent acquisition (MSMS) of the most intense ions in the negative and positive ion modes, respectively.

RPLC-FT-ICR-MS lipidomics data were initially converted into netcdf (.cdf) format using Xcalibur 2.1 and processed using XCMS online (<https://xcmsonline.scripps.edu/>; Tautenhahn et al. 2012) to generate an intensity matrix with a list of peak retention times. The intensity matrix was imported into our SIMStitch pipeline immediately after the replicate filter, and hence included sample filtering, blank filtering, PQN normalization, kNN missing value imputation and the generalised logarithm transformation, as for DIMS processing above. The sample filter was set to 75% as no technical replicate filtering could be applied.

3.2.8 Identification of lipids

For both lipid datasets, peaks were annotated and putative empirical formulae calculated using the MI-Pack software (Weber and Viant, 2010), searching the KEGG and LipidMaps databases (Available at <http://www.genome.jp/kegg/> Fahy et al., 2009, 2007).

3.3 Results

3.3.1 Effect of dietary cholesterol manipulation on *Wolbachia* density

Evidence that cholesterol availability is a limiting factor for *Wolbachia* density was sought by dietary cholesterol supplementation in *wMel*-infected *D. melanogaster* w1118 using a baseline fly diet known to be very low in cholesterol (Bujold et al., 2010).

Mean *wMel* density in four pools of five female flies on a low cholesterol diet was 25.3 ± 2.26 *Wolbachia* genomes per host genome (M $\pm$ SD). Cholesterol supplementation had a significant overall effect on density (Figure 3.1a. One-way ANOVA, $F_{2,9} = 10.95$, $p = 0.003$), but this effect was negligible at medium cholesterol supplementation ($50 \mu\text{g ml}^{-1}$ (CHO-50): M = 26.9 ± 3.46 . Tukey HSD, $p = 0.782$). Higher supplementation of $200 \mu\text{g ml}^{-1}$ (CHO-200) led to a reduction in *wMel* density of 36% (M = 16.3 ± 4.37), which significantly differed from both other treatments (Tukey HSD. Control - $50 \mu\text{g ml}^{-1}$, $p = 0.013$. Control - $200 \mu\text{g ml}^{-1}$, $p = 0.005$). A second independent experiment (Figure 3.1b) showed a trend towards reduced *Wolbachia* density on cholesterol supplementation but this was insignificant (Kruskal-Wallis, $\chi^2 = 1.427$, $\text{df} = 2$, $p = 0.490$), due in part to high variability in the control samples (Control, 62.20 ± 18.43 . $50 \mu\text{g ml}^{-1}$, 48.72 ± 4.75 . $200 \mu\text{g ml}^{-1}$, 46.45 ± 3.61). Overall, there was no evidence for cholesterol as a *Wolbachia* density limiting factor at the concentrations tested. There was an initial indication that high levels of cholesterol could lead to reduced *Wolbachia* density, but this was unreproducible.

Cholesterol levels were also manipulated in Aa23-T.*wMel* cell lines using supplementation with synthetic cholesterol and depletion by methyl- β -cyclodextrin (M β CD), which led to significant alterations in *Wolbachia* density (Figure 3.2a. Kruskal-Wallis, $\chi^2 = 10.118$, $\text{df} = 2$, $p = 0.006$). Specifically, M β CD treatment led to a 66% reduction in *wMel* density compared to control samples (Control: 35.53 ± 3.95 *Wolbachia*

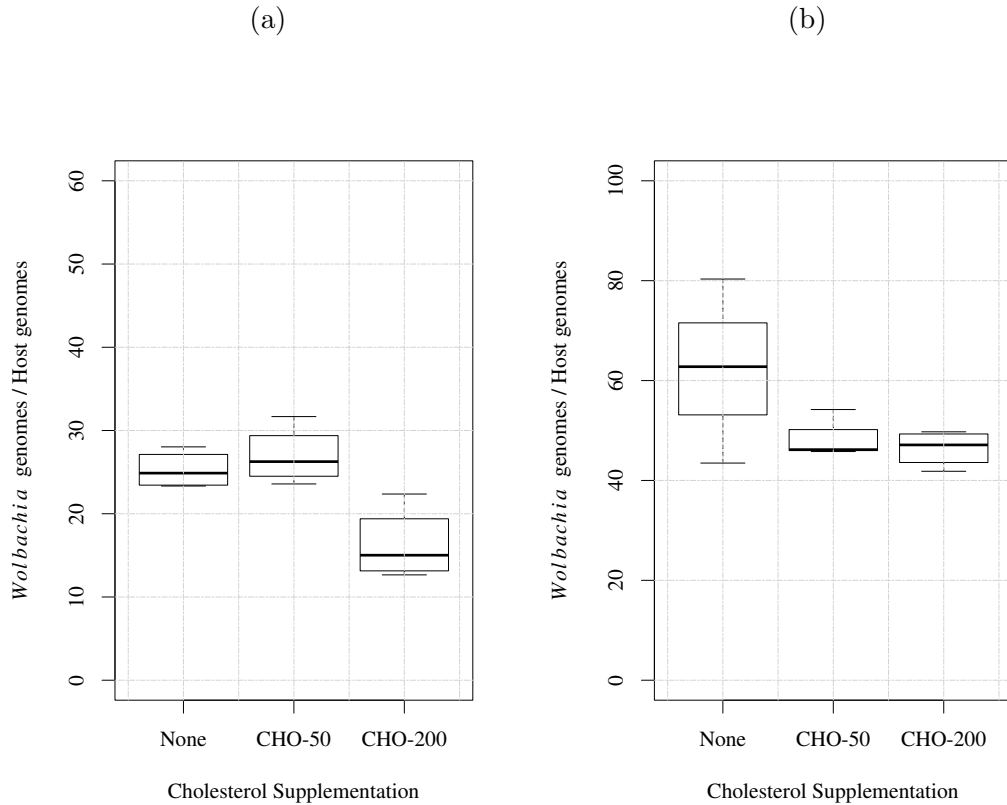


Figure 3.1: Box plot of *Wolbachia* density in *wMel*-infected *D. melanogaster* w1118 as determined by absolute qPCR of *Wolbachia* *Wsp* and the single-copy host gene *HTH*, normalised to a plasmid standard curve of known concentration. Graphs a and b represent two independent experiments. Plots represent 4–5 biological replicates from three treatments: None = control diet, CHO-50 = supplementation with 50 $\mu\text{g ml}^{-1}$ cholesterol, CHO-200 = supplementation with 200 $\mu\text{g ml}^{-1}$ cholesterol.

genomes per host genome. M β CD: 12.07 ± 7.73 . Wilcoxon rank sum test with Bonferroni p-value adjustment, $p = 0.024$). Cholesterol supplementation did not have any effect (33.79 ± 6.11 . Wilcoxon rank sum test with Bonferroni p-value adjustment, $p = 1$). A second independent experiment found much higher *Wolbachia* densities across all treatments, which may have been an artefact as these densities are unusually high, but in relative terms the results supported the initial experiment, with a 37% mean reduction in *Wolbachia* density on M β CD treatment and no significant difference on cholesterol supplementation (Figure 3.2b), indicating that cholesterol could be a *Wolbachia* limiting factor in this scenario.

Cell viability was not explicitly measured but there was no observed difference in growth rate between treatments over the course of the experiment. This is in line with

previous evidence that insect cells can continue to grow with low levels of membrane steroids (Carvalho et al., 2010; Silberkang et al., 1983).

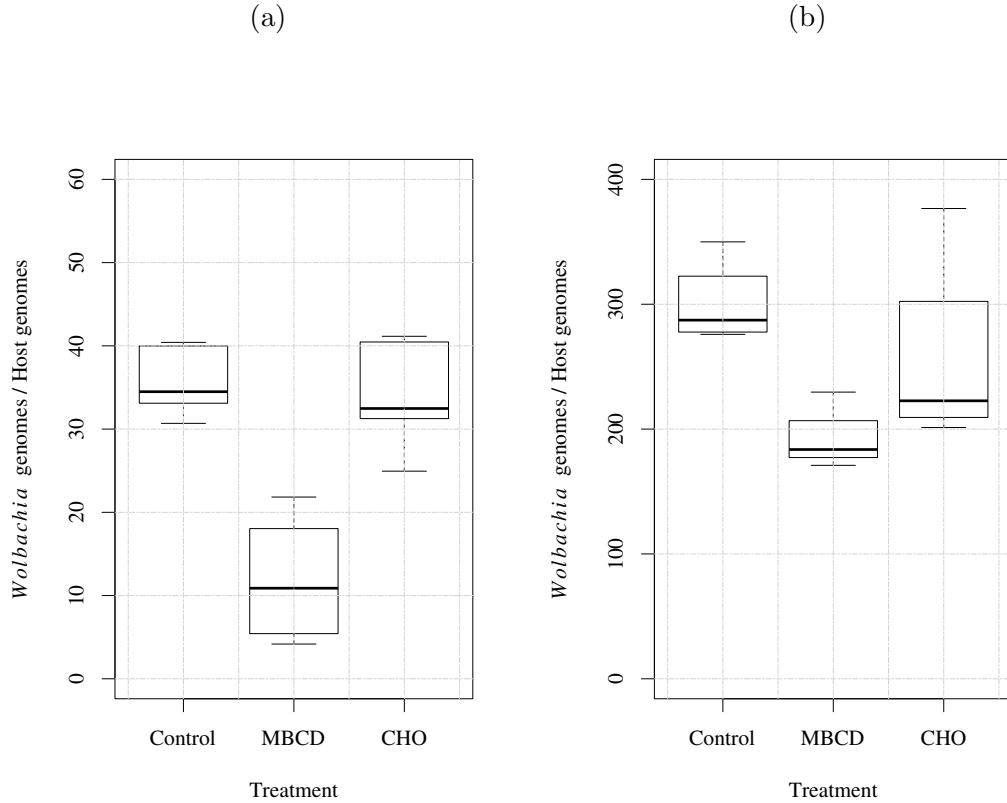


Figure 3.2: Box plot of *Wolbachia* density in Aa23-*T.w*Mel as determined by absolute qPCR of *Wolbachia Wsp* and the single-copy host gene *HTH*, normalised to a plasmid standard curve of known concentration. Graphs a and b represent two independent experiments. Plots represent 4–5 biological replicates from three treatments: Control = standard cell media, CHO = supplementation with synthetic cholesterol, M β CD = cholesterol depletion by M β CD.

3.3.2 Modulation of lipid metabolic gene expression

Cholesterol homeostasis is tightly regulated in insects by a series of genetic regulatory pathways. The nuclear receptor DHR96 has been shown to act as a master-regulator of cholesterol and triacylglyceride metabolism by binding cholesterol and regulating transcription of a broad range of downstream lipid metabolism genes (Bujold et al., 2010; Horner et al., 2009; Sieber and Thummel, 2012). It is therefore highly expressed on low cholesterol diets and reduced on cholesterol supplementation (Bujold et al., 2010). The Niemann-Pick C1-like 1 gene *D. melanogaster*-orthologue *Npc1b* is a

midgut protein essential for dietary cholesterol absorption (Voght et al., 2007) which is upregulated by cholesterol along with a sterol-o-acyltransferase termed *ACAT* (Bujold et al., 2010). These genes were thus used to determine if *Wolbachia*-infected hosts are modulating regulation of metabolic pathways to maintain cholesterol homeostasis in *D. melanogaster* and *Ae. albopictus*. Orthologues of all three genes were located through BLAST searches of the *Ae. albopictus* transcriptome (Poelchau et al., 2011) and expression was assessed by qRT-PCR.

D. melanogaster showed only negligible changes in mean *DHR96* expression while *Npc1b* consistently showed a reduction of 36–89% across three independent experiments, with two of those being statistically significant (pMCMC <0.001). Mean *ACAT* expression reduced 16–71% in *Wolbachia*-infected flies, but only the 71% reduction was statistically significant (pMCMC = 0.012) (Figure 3.3).

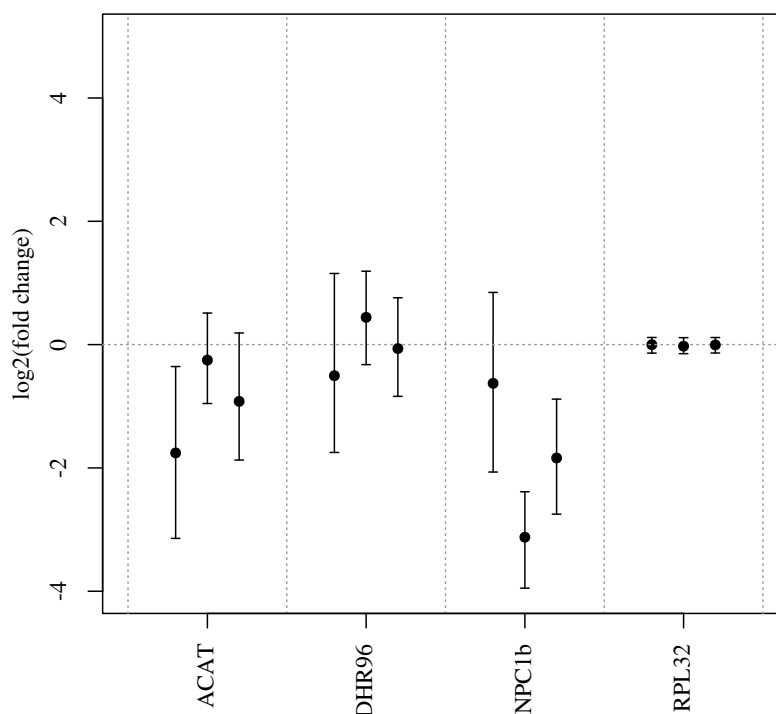


Figure 3.3: Cholesterol metabolic gene expression in *wMel*-infected compared to tetracycline-cured *D. melanogaster* w1118, normalised to expression of the housekeeping gene *RPL32*. Points represent the mean of 4–5 biological replicates and error bars denote 95% confidence intervals calculated using Bayesian MCMC methods in MCMC.qPCR R package. Separate bars within each gene represent independent experiments.

Relative gene expression in *Ae. albopictus* was inconsistent (Figure 3.4). *DHR96* was significantly upregulated by 1.81–2.23-fold in Uju.*w*Mel across two independent experiments (pMCMC <0.005), although not in a third. Uju.*w*Alb showed significant 1.73-fold upregulation of *DHR96* (pMCMC = 0.006) in one experiment but the other two showed a non-significant 1.15–1.32-fold increase. *ACAT* was upregulated 2.2-fold in one Uju.*w*Mel repeat but no significant difference was observed across any other lines or repeats. *Npc1b* expression was also inconsistent, with one repeat in Uju.*w*Mel giving a significant reduction of 58% but a second repeat recording a non-significant mean increase.

Expression of fatty acid synthase (*Fas*) was also included based on the role of fatty acids in *Wolbachia*-host metabolism and DENV replication. Only one Uju.*w*Alb sample showed significant 2.1-fold upregulation and differential expression in Uju.*w*Mel was inconsistent. Two experiments showed a mean increase of up to 1.76-fold, one of which was significant, but one experiment countered this with a downregulation of over 2-fold.

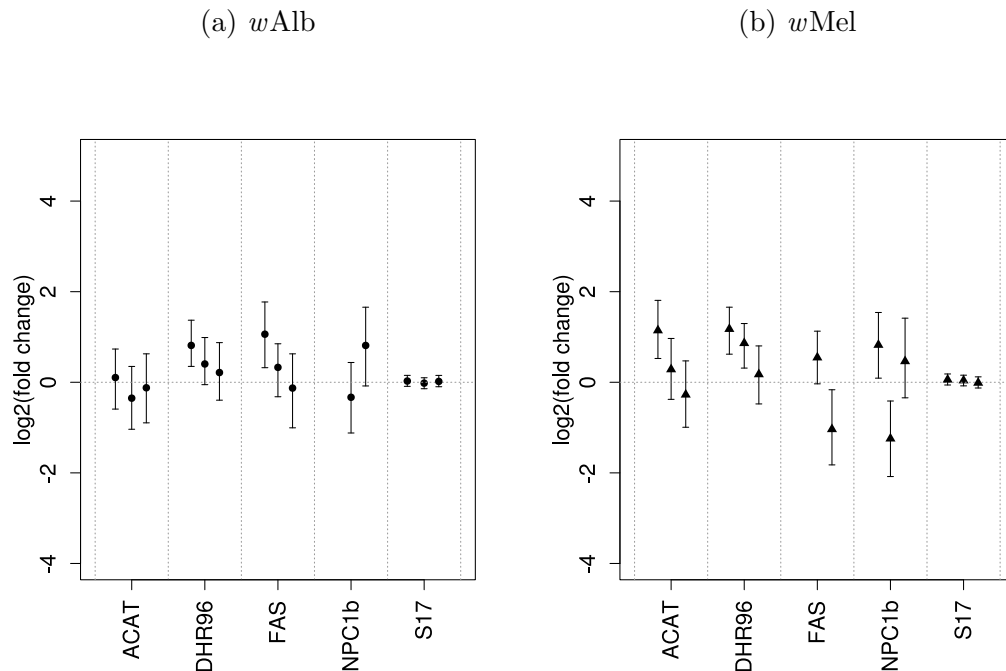


Figure 3.4: Lipid and cholesterol metabolism gene expression in Uju.*w*Alb and Uju.*w*Mel relative to uninfected Uju.T, normalised to the housekeeping gene S17. Points represent the mean of four biological replicates and error bars denote 95% confidence intervals calculated using Bayesian MCMC methods in MCMC.qPCR R package. Separate bars within each gene represent independent experiments.

Overall, there was a trend towards reduced expression of cholesterol-induced transcripts in *D. melanogaster*, particularly *Npc1b*, which offers some support towards the hypothesis of reduced free cholesterol due to *Wolbachia* resource competition. The picture in *Ae. albopictus* was more complicated with all genes except *DHR96* exhibiting changes in opposing directions across the 2–3 independent experiments. *DHR96* showed a consistent trend towards upregulation in both Uju.*wAlb* and Uju.*wMel* which could be a response to reduced cholesterol. However, this was not supported by corresponding changes in *ACAT* or *Npc1b* expression. There was no indication at the transcriptional level of any upregulation in fatty acid synthesis.

3.3.3 Expression of genes involved in autophagy

To corroborate the observation by Voronin et al. (2012) that *Wolbachia* modulate and are controlled by autophagy in *Ae. albopictus*, key genes in the autophagy regulatory pathway were chosen for analysis of expression by qRT-PCR. Autophagy can be induced by multiple triggers including oxidative stress and starvation. These two routes involve different signalling pathways, the former is associated with increased JNK activity leading to transcriptional regulation of genes such as *Puckered* and upregulation of *Atg* transcripts via the transcription factor FoxO (Wu et al., 2009). Starvation induced autophagy instead relies on insulin/PI3K signalling and appears to be independent of JNK but still ultimately upregulates *atg* transcripts.

ATG8 proteins are of specific interest as they were found to be upregulated in *Wolbachia*-infected hosts and observed localised to the *wBm*-containing vacuole in nematodes. Voronin et al. (2012) therefore suggest that *Wolbachia* could be modifying or recruiting this particular protein to assist with evasion of autophagy. *FoxO* and *atg8* were therefore chosen for qRT-PCR analysis. *Jnk* expression was tested in Chapter 2 and no significant changes in *Wolbachia*-infected lines were observed.

No significant increase in either autophagy related gene was found in Aa23-T.*wMel* or Aa23-T.*wMelPop* cell lines (Figure 3.6). In *Wolbachia*-infected adult mosquitoes, *atg8* was found to be significantly upregulated by 1.77–2.06-fold in Uju.*wMel* compared to Uju.T in two of three experiments (pMCMC <0.05), with the third showing an insignificant 24% decrease, but no differential regulation was observed in Uju.*wAlb*. FoxO showed no trend in expression in either Uju line (Figure 3.5).

Two other genes which mediate cross-talk between viral-resistance pathways were also assayed. Inhibitor of Apoptosis (IAP) prevents apoptotic cell death in *Aedes*

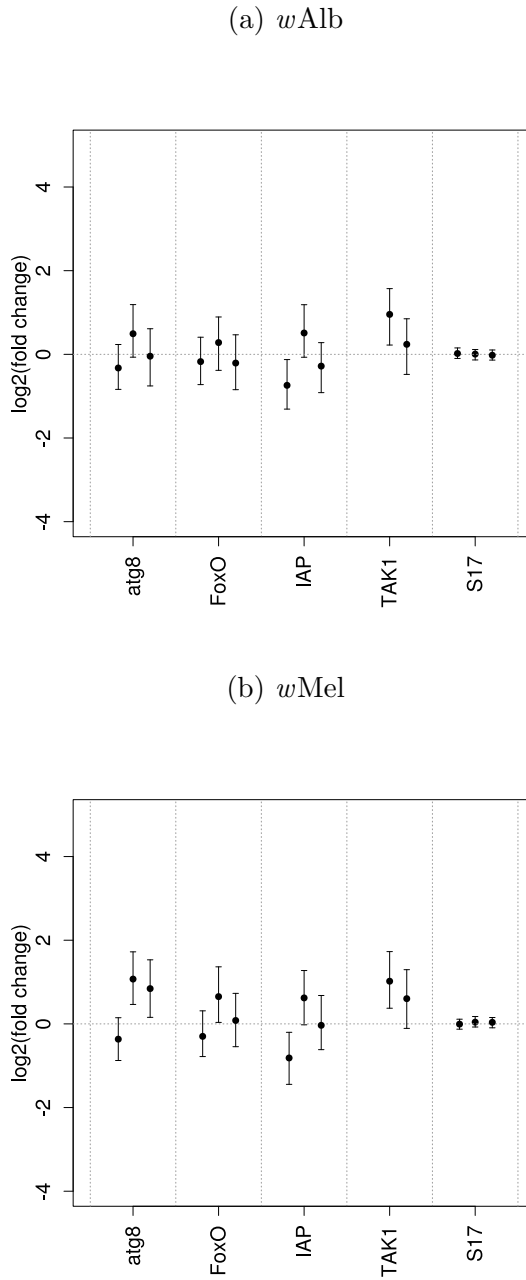


Figure 3.5: Autophagy related gene expression in *Uju.wAlb* and *Uju.wMel* relative to uninfected *Uju.T*, normalised to the housekeeping gene *S17*. Points represent the mean of four biological replicates and error bars denote 95% confidence intervals calculated using Bayesian MCMC methods in MCMC.qPCR R package.

mosquitoes (reviewed in Liu and Clem, 2011; Wang and Clem, 2011) and may play a direct role in viral immunity and symbiont persistence as well as reciprocally negatively regulating autophagy (reviewed in Eisenberg-Lerner et al., 2009).

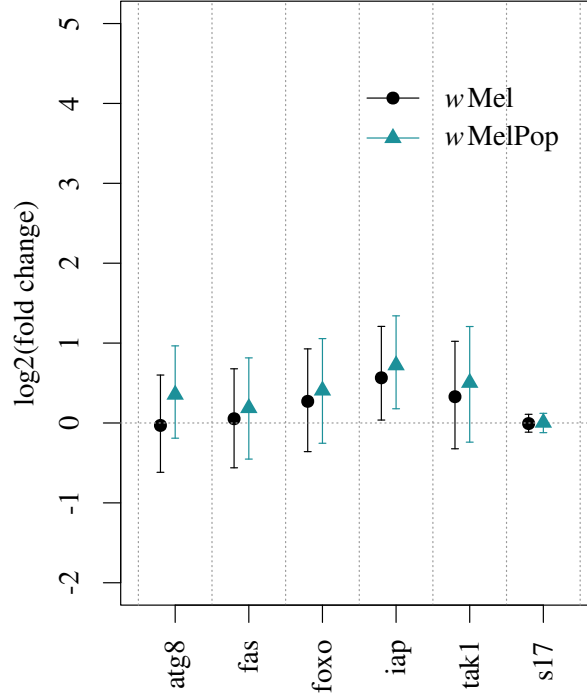


Figure 3.6: Autophagy related gene expression in Aa23T.*wMel* and Aa23T.*wMelPop* cell lines compared to Aa23T, normalised to expression of the housekeeping gene S17. Points represent the mean of four biological replicates and error bars denote 95% confidence intervals calculated using Bayesian MCMC methods in MCMC.qPCR R package.

Wolbachia has been shown to inhibit apoptosis in numerous symbioses (Fast et al., 2011; Landmann et al., 2011; Pannebakker et al., 2007) and there is direct evidence of IAP downregulation in *Wolbachia*-infected weevils (Vigneron et al., 2012) but this picture is not clear cut, for example *wMelPop* increases apoptosis in *D. melanogaster* ovaries (Zhukova and Kiseleva, 2012). IAP is cytoprotective during DENV infection in both *D. melanogaster* and *Ae. aegypti* (Chen et al., 2012) so has clear potential for modulation of *Wolbachia*-host-virus tripartite interactions. There was no overall trend in expression in either Uju.*wMel* or Uju.*wAlb*, although one experiment in each line showed significant downregulation of >40% (pMCMC <0.05).

In summary, there was some support for increased *atg8* expression suggesting increased autophagic activity in the densely infected Uju.*wMel* but not in Uju.*wAlb* or *Wolbachia*-infected Aa23-T cell lines. There was no clear evidence at the transcrip-

tional level for modulation of apoptotic pathways as a result of *Wolbachia* infection.

3.3.4 Lipidomics Analysis

With little clear difference in lipid metabolic pathways at the transcriptional level, an untargeted assay was performed to explore the overall lipid profile of *Wolbachia*-infected *Ae. albopictus* cells compared to uninfected Aa23-T.

Direct infusion mass spectrometry (DIMS) in combination with liquid chromatography mass spectrometry (LCMS) was performed using lipid extracts from Aa23-T, Aa23-T.*w*Mel and Aa23-T.*w*MelPop cells (in collaboration with Dr Ulf Sommer, NBAF-B, University of Birmingham ¹).

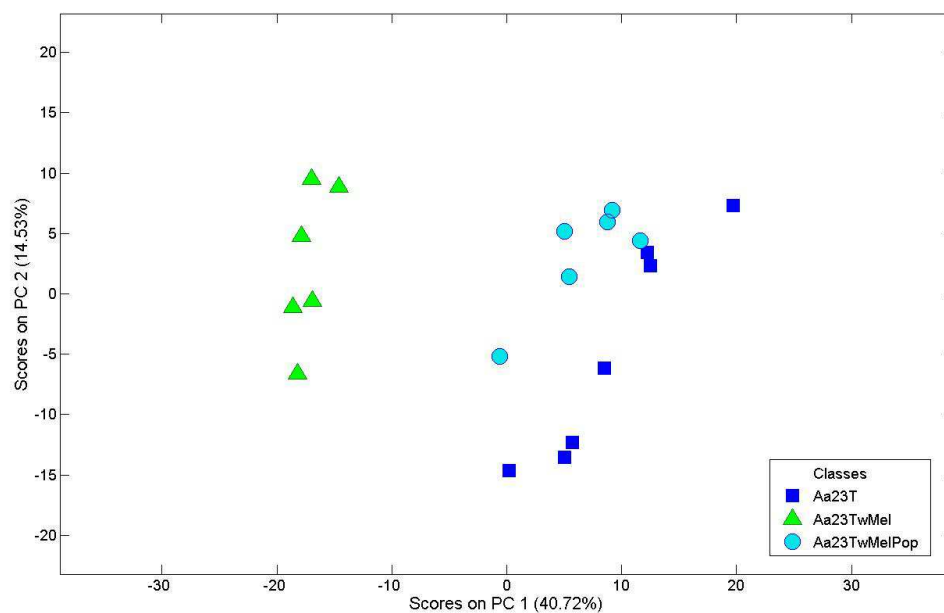
Negative-ion DIMS analysis of the lipid extracts resulted in 2044 peaks which gave three classes of distinct metabolic profiles, distinguished by principal component analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA). There was clear separation of Aa23-T and Aa23-T.*w*Mel via principal component 1 (PC1) and between Aa23-T and Aa23-T.*w*MelPop along the same axis to a lesser degree (Figure 3.7a). PLS-DA results showed that Aa23-T.*w*MelPop was separated from the other lines along latent variable LV1, while separation between Aa23-T and Aa23-T.*w*Mel was mostly along LV2 and LV1 to a lesser extent (Figure 3.7b).

The variability within groups was quite high and differences were most profound between Aa23-T and Aa23-T.*w*Mel, which was unexpected as this cell line typically has a lower density of *Wolbachia*. However, it is known that densities can fluctuate widely in cell lines (e.g. Khoo et al., 2013; Voronin et al., 2010) and qPCR of DNA samples taken from six separate culture flasks per line concurrently with lipid extraction revealed that at the time of sampling, mean *Wolbachia* density was 3.4-fold higher in Aa23-T.*w*Mel than Aa23-T.*w*MelPop.

LCMS was performed to strengthen the DIMS evidence. Analysis of 1345 peaks supported the pattern of separation along the principal components and latent variables observed from DIMS but showed clearer segregation of the *Wolbachia*-infected and uninfected cell lines (Figure 3.8). Further analysis was thus undertaken using the LCMS data set only.

¹Study design, sample preparation, data visualisation and biological interpretation performed by Jennifer Molloy. Mass spectrometry, statistical analysis and lipid identification performed by Dr Ulf Sommer, NBAF-B, University of Birmingham.

(a) DIMS PCA Plot



(b) DIMS PLS-DA Plot

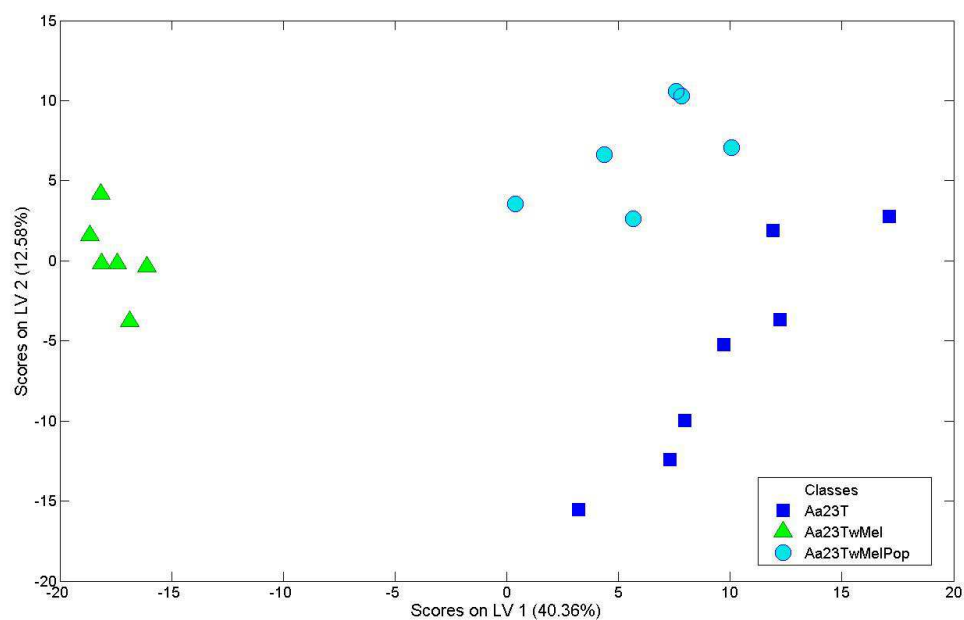
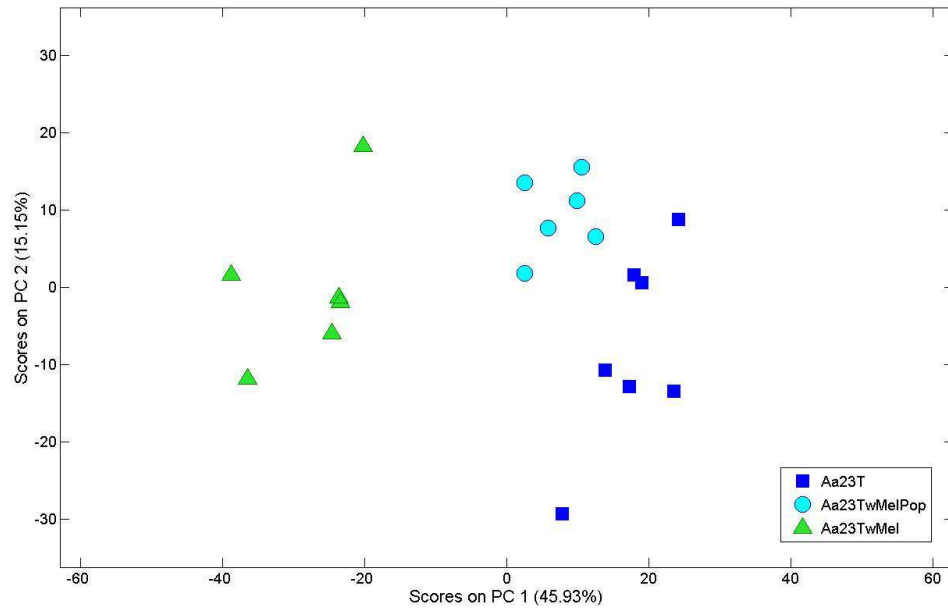


Figure 3.7: A) PCA plot showing segregation of total lipid profile derived from DIMS. B) PLS-DA of resulting data. Points represent 6–7 biological replicates from Aa23-T, Aa23-T.*w*Mel and Aa23-T.*w*MelPop cell lines and 2044 features from the negative-ion DIMS analysis were analysed. Quality Control (QC) samples are not shown.

(a) LCMS PCA Plot



(b) LCMS PLS-DA Plot

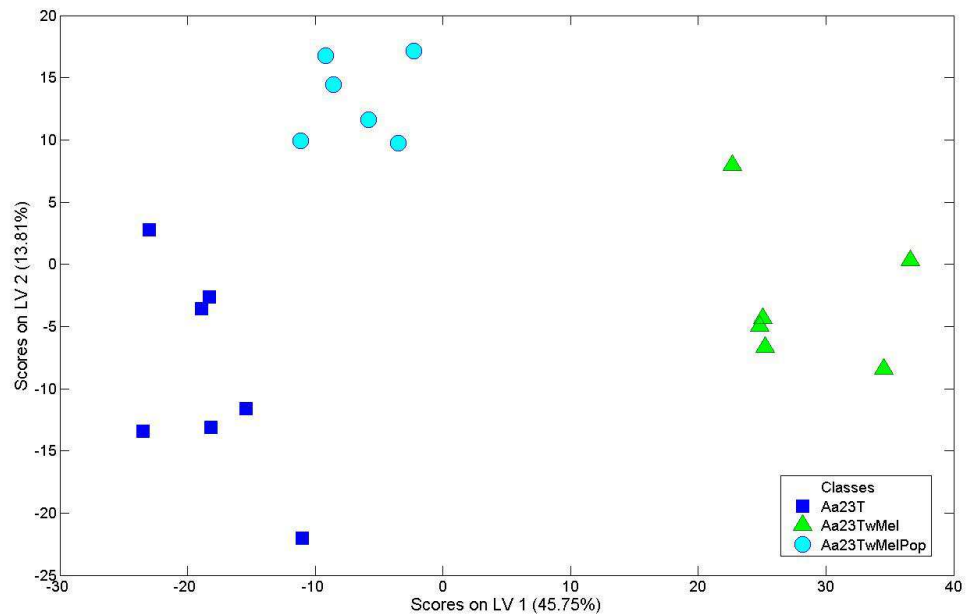


Figure 3.8: A) PCA plot showing segregation of total lipid profile derived from LCMS. B) PLS-DA of resulting data. Points represent 6–7 biological replicates from Aa23-T, Aa23-T.*w*Mel and Aa23-T.*w*MelPop cell lines and 1136 features from the LCMS analysis were analysed. Quality Control (QC) samples are not shown.

3.3.5 Differentially regulated lipids

The LCMS dataset contained 1113 lipids that were differentially regulated between cell lines (ANOVA, $p < 0.05$ after 5% Benjamini-Hochberg correction). Of these lipids, 851 were annotated using MiPack and LipidMaps allowing a clear picture of the lipid profile across several major classes (Table 3.4 and Figure 3.9).

<i>w</i> Mel	<i>w</i> MelPop	+	+	-	-	+	-	-	+	None
Sphingolipids										
Ceramide		3	43	0	11	5				
SM		1	11	0	8	5				
GlcCer		4	24	1	16	7				
LacCer		2	4	3	0	5				
CerP		1	1	0	0	1				
Phospholipids										
PC		17	60	5	21	58				
PE		10	14	0	3	12				
PI		46	10	11	7	54				
PG		15	12	5	4	22				
PS		26	47	2	7	63				
PA		23	13	4	6	26				
Fatty Acids										
DG		6	11	1	11	14				
TG		6	6	0	2	11				

Table 3.4: Count data for number of identified lipid species in each class, Columns show direction of significant change in abundance for Aa23-T.*w*Mel and Aa23-T.*w*MelPop respectively (ANOVA, $p < 0.05$). None = non-significant difference.

3.3.6 Sphingolipids

Ceramides and sphingomyelins were highly represented in significantly regulated sphingolipids (Figure 3.9). Variable importance is one measure derived from the PLS-DA latent variables that can indicate significant changes and the top ten list for comparisons between Aa23-T and Aa23-T.*w*Mel contains eight ceramides and one sphingomyelin species. While both sphingolipids play a major role in plasma membranes as structural materials they are also involved in signalling and a range of other functions within the cell. As little is understood about the biological role of specific sphingolipid species in mosquitoes, the class-level change (Figure 3.10) is of greatest interest to inform future hypotheses and functional experiments.

Ceramides are a hydrolysis product of sphingomyelin, although they can also be synthesised *de novo* from serine and palmitate. Of 63 identified ceramides, 58 were

significantly altered in *Wolbachia*-infected cell lines. Overall mean relative decrease across ceramides was 46% in Aa23-T.*w*Mel and 13% in Aa23-T.*w*MelPop, although some ceramide species were decreased by as much as 90% in Aa23-T.*w*Mel, for example Cer(d18:1/23:0).

Ceramide is often converted to more complex forms by the addition of phosphate or sugar groups. Ceramide 1-phosphate is one such form and three species were detected. CerP(d18:1/12:0) showed no significant difference while CerP(d18:1/26:1(17Z)) was reduced by 36% in Aa23-T.*w*Mel only and CerP(d18:1/24:1(15Z)) was increased by 1.51 and 2.43-fold in Aa23-T.*w*MelPop and Aa23-T.*w*Mel respectively.

Glucosylceramide (GlcCer) is the precursor of much more complex glycosphospholipids although its own role in insects is undefined. In *D. melanogaster* it is posited that GlcCer synthesis operates to reduce the ceramide levels and thus lower apoptosis risk. Other neutral glycosphingolipids detected contained different monosaccharide headgroups, but in the case of galactosylceramides (GalCer), it was not possible to positively distinguish between GalCer and GlcCer species with the same molecular mass. GalCer lipids are primarily found in brain and nervous tissues in mammals but again little information is available on potential roles in insects. Of eight GlcCer species unambiguously identified in Aa23-T cells, three showed no difference in abundance and five were reduced by 22–61% in Aa23-T.*w*Mel but unchanged or slightly increased in Aa23-T.*w*MelPop. Of the 47 ambiguous peaks detected, six were unchanged and all but three species were decreased by 23–78% in Aa23-T.*w*Mel while only 22 were reduced by up to 34% in Aa23-T.*w*MelPop, the rest were slightly increased in this less dense infection.

Lactosylceramides (LacCer) were also detected and 9 of 14 were significantly altered in *Wolbachia*-infected cells. LacCer species with fatty acid chains of C₂₀ to C₂₄ were decreased in both Aa23-T.*w*Mel and Aa23-T.*w*MelPop. For example, LacCer(d18:1/22:0) was reduced by 81% in Aa23-T.*w*Mel and 51% in Aa23-T.*w*MelPop. However, species with shorter fatty acid chains were increased in Aa23-T.*w*Mel by 1.52–1.90-fold and showed limited variability in Aa23-T.*w*MelPop.

The clear trend across ceramides and derivative classes is for depletion in the presence of dense *Wolbachia* infections (Figure 3.10). This is reflected in levels of sphingomyelin, which consists of a ceramide with a phosphorylcholine headgroup. 25 species were identified of which 21 were significantly differentially regulated in *Wolbachia*-infected lines. All but one of these were downregulated in the denser *w*Mel-

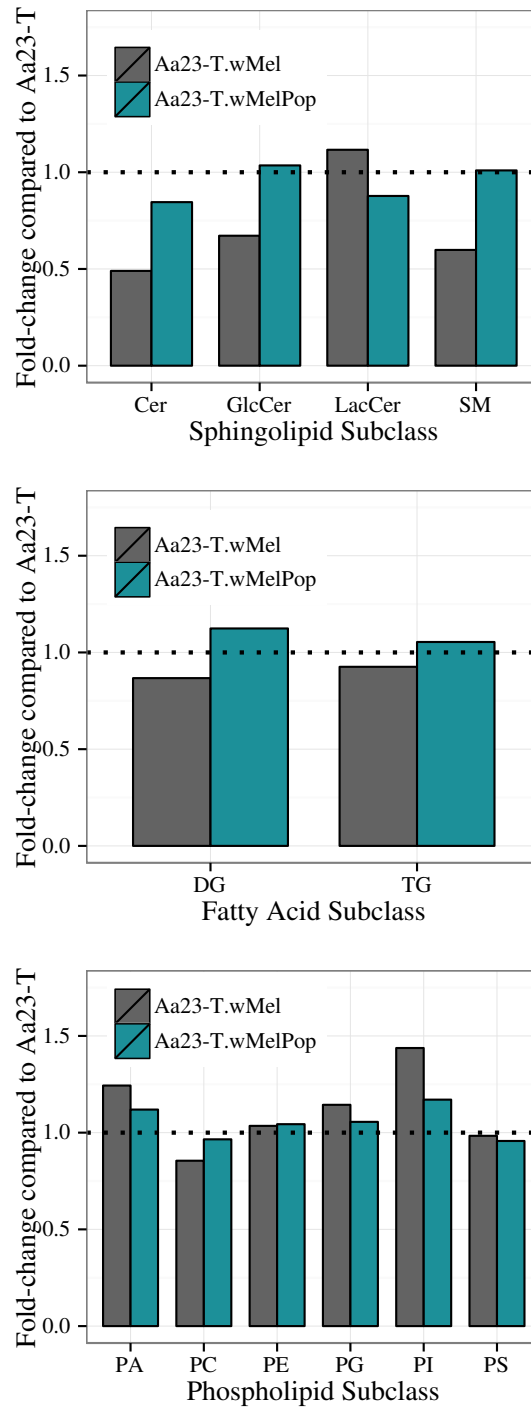


Figure 3.9: Mean fold-change relative to Aa23-T across all lipid species identified in each lipid class in Aa23-T.*wMel* and Aa23-T.*wMelPop*. Horizontal line depicts no change.

infected line, with 11 also downregulated in Aa23-T.*wMelPop* (Table 3.4). The mean decrease across sphingomyelin species in Aa23-T.*wMel* was 40% (Figure 3.9). In Aa23-T.*wMelPop* there was no mean change across the whole group but for example,

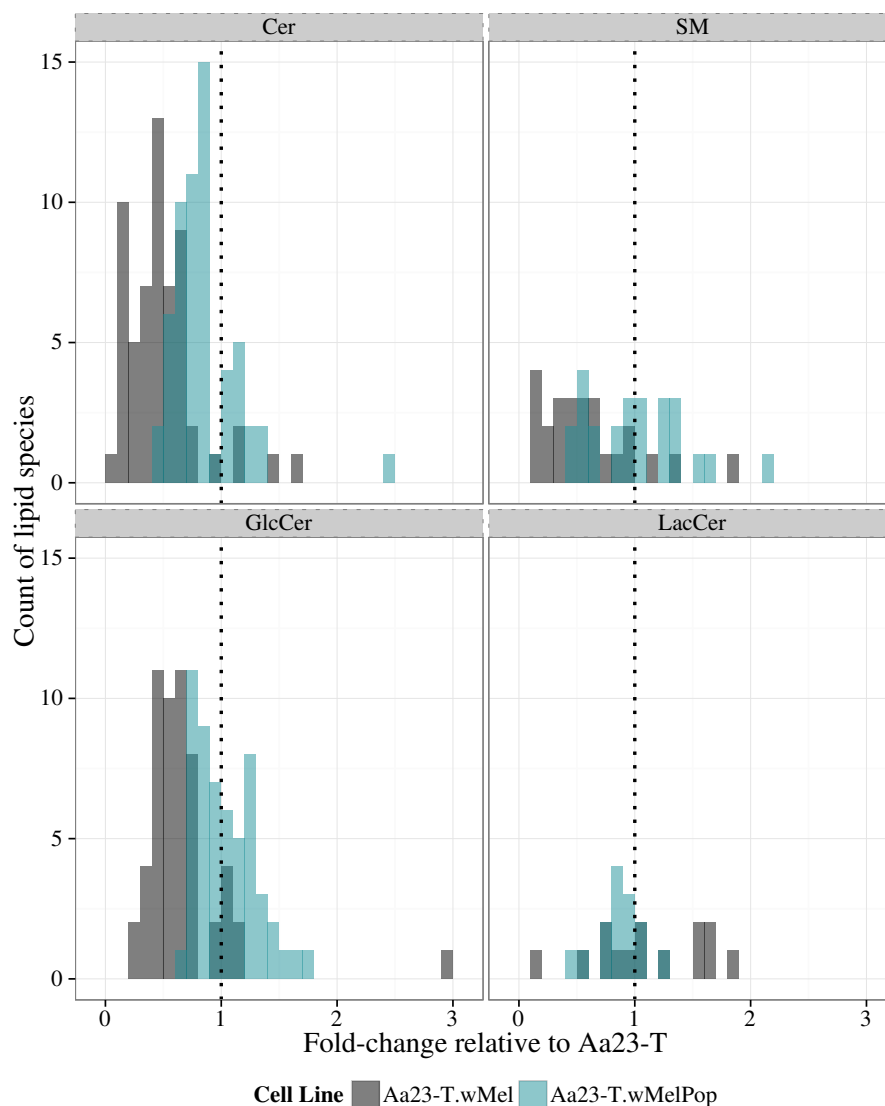


Figure 3.10: Histograms of fold-change in sphingolipid classes, representing number of species exhibiting fold-change in each bin (bin size = 0.1).

the five most reduced species in Aa23-T.wMel (79–80% decrease) were all reduced by 39–50% in Aa23-T.wMelPop. There does not appear to be any correlation with chain size or saturation.

Several precursors that would have provided further detail on modulation of sphingolipid pathways were not detected in either DIMS or LCMS, such as sphingosine or palmitic acid, so only a partial view of the sphingolipid metabolic network was obtained (Figure 3.11). For example, 15 of the 17 ceramides which showed a reduction only in the Aa23-T.wMel line or showed no significant overall change were dihydroceramides, a intermediate in the *de novo* synthesis pathway. There is insuf-

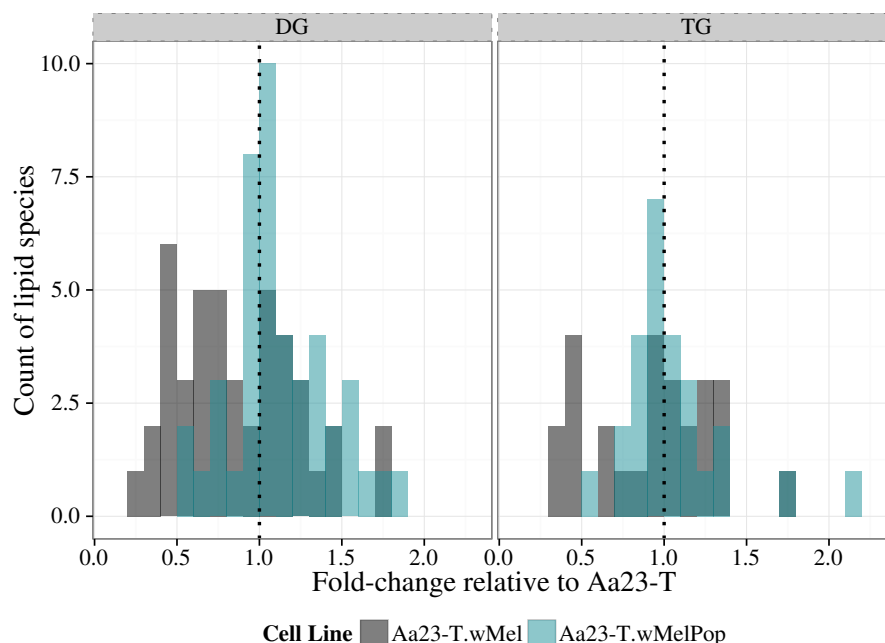


Figure 3.12: Histograms of fold-change in fatty acid classes, representing number of species exhibiting fold-change in each bin (bin size = 0.1).

Triacylglycerols (TGs) also showed little mean fold-change between lines across the class (Figure 3.12. Aa23-T.*wMel* = 0.93, Aa23-T.*wMelPop* = 1.05), but 8 out of 12 short chain TGs (12:0) were decreased in Aa23-T.*wMel* (mean fold-change = 0.76). The significance of chain length in either class of lipids in this context is currently unknown.

Scheitz et al. (2013) correlated increased odd-chain fatty acids with *Wolbachia* infection but it is not possible to repeat this analysis without absolute concentration data. In addition, their interpretation was based on *D. melanogaster*'s lack of capacity to synthesise odd-chain fatty acids, which would therefore be supplied by symbionts or gut microflora. This does not hold in cell cultures as the media is supplemented with mammalian-derived serum (FBS) which will contain odd-chained FAs and therefore confound or mask a potential effect of *Wolbachia* infection.

3.3.8 Phospholipids

Phospholipids carry out a wide range of functions in the cell, most critically they are able to form lipid bilayers and are therefore required in the plasma membrane, but many also participate in signalling. Most showed little cross-class change in

Wolbachia-infected cells, although phosphatidylinositol (PI) lipids were upregulated (Figure 3.13).

PIs induce the PI-3-kinase (PI3K) pathway which plays an essential role in cell survival and growth. 128 distinct species were detected of which 73 were differentially regulated. The across class mean increase was 1.44-fold in Aa23-T.*w*Mel and 1.17-fold in Aa23-T.*w*MelPop but 43 out of 73 species were upregulated to some extent in both *Wolbachia* infections and 26 of these were upregulated by >2-fold in Aa23-T.*w*Mel.

Phosphatidylserine (PS) species are important for recognising and removing apoptotic cells. Collectively, they were not differentially regulated by *Wolbachia* infection (Mean fold-change in Aa23-T.*w*Mel = 0.98, Aa23-T.*w*MelPop = 0.96). 147 species were detected although many of these were ambiguously identified within the class. None featured in the top 50 lipids by vector importance ranking for either *Wolbachia* infection.

Glycerophosphoglycerols (PG) were slightly elevated across the class (Mean fold-change: Aa23-T.*w*Mel = 1.14, Aa23-T.*w*MelPop = 1.05). As was typical across all lipid classes, changes were usually in the same direction in both Aa23-T.*w*Mel and *w*MelPop, with larger effect sizes in the more densely infected Aa23-T.*w*Mel (Table 3.4). Likewise, glycerophosphate species (PA) were slightly elevated (Mean fold-change: Aa23-T.*w*Mel = 1.24, Aa23-T.*w*MelPop = 1.12), with the majority of species showing no change or upregulation in both *Wolbachia*-infected cell lines.

Phosphatidylethanolamines (PEs) and Phosphatidylcholines (PCs) are the primary components of cellular membranes in *D. melanogaster* and have previously been found in high abundance in mosquitoes. PCs are cylindrically shaped and spontaneously forms lipid bilayers while PEs are cone shaped and thought to help control membrane curvature and increase membrane fluidity. 162 PC and LysoPC species were detected, 104 of these were significantly differentially regulated in *Wolbachia*-infected lines and the vast majority of these were downregulated in Aa23-T.*w*Mel, by up to 84%. There were 17 species showing upregulation across both infected lines but no distinctions could be drawn between characteristics of upregulated versus downregulated species due to the ambiguous identification. PCs provide the choline headgroup on sphingomyelin, which was also reduced in *Wolbachia*-infected cells, thus adding support to the trend for sphingolipid depletion.

Only 40 PE species were detected, of which 12 were unaffected, 16 were downregulated by 14–82% and 9 were upregulated 1.22–2.99-fold in Aa23-T.*w*Mel. Changes

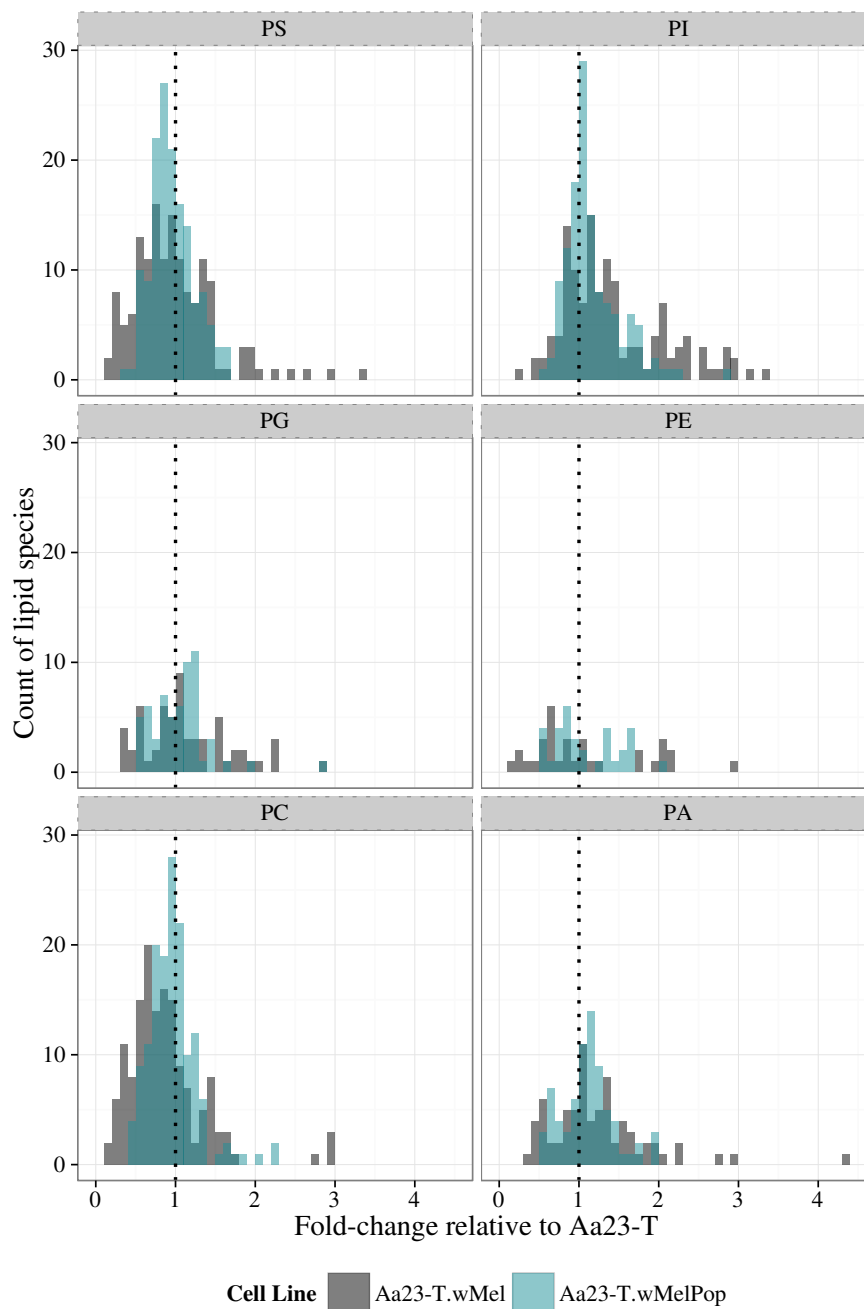


Figure 3.13: Histograms of fold-change in phospholipid classes, representing number of species exhibiting fold-change in each bin (bin size = 0.1).

in Aa23-T.wMelPop were mostly in the same direction but with a smaller effect size. No trends in terms of chain length, saturation or other characteristics were detected, although most PEs were ambiguously identified, including some which share a molecular mass with a PC species. Overall, there was no difference in expression when

averaged across the class.

3.3.9 Other lipids

Other lipids with altered expression outside of these main classes were detected but none were highly ranked in terms of variable importance and there are limited clues in the literature as to the likely consequence of their altered expression. No further investigation was carried out at this time.

3.4 Discussion

We used mass spectrometry to profile the lipidome of *Wolbachia*-infected *Ae. albopictus* cells and observed a distinct segregation between uninfected and infected cells, which was heightened at high *Wolbachia* density. *Wolbachia* infection resulted in the downregulation of several lipids that affect membrane structure, fluidity, curvature and protein complex assembly in lipid rafts. Some of these identified lipids also function as signalling molecules and control important pathways such as trafficking, autophagy and apoptosis. All of these roles suggest likely interactions with cytosolic bacteria and viruses such as *Wolbachia* and DENV. Such organisms might evolve the ability to alter the cell lipidome to ensure their own preservation and replication and this is indicated in DENV-infected *Ae. albopictus* cells, where infection substantially altered the cellular lipid profile to favour viral propagation (Perera et al., 2012). Here I discuss the above results in the context of *Wolbachia*-host interactions only and then suggest potential implications for DENV infection based on existing evidence from each bipartite relationship. It is also possible that tripartite interactions with differing or emergent properties contribute to the refractory phenotype observed, but unpicking these will require experimental designs including both infection factors.

3.4.1 *Wolbachia*-host interactions and lipid metabolism

Wolbachia-host resource competition over cholesterol has previously been hypothesised because both organisms are cholesterol auxotrophs and rely on an exogenous dietary supply. In support of this, the limited range of cholesterol derivatives detected in the DIMS/LCMS analyses showed a reduction in Aa23-T.*w*Mel cells and evidence from cholesterol supplementation experiments indicated that depletion by M β CD can cause a reduction in *Wolbachia* density. However, increased dietary cholesterol at

the levels supplied in Aa23-T *wMel* cells and *wMel*-infected *D. melanogaster* had no effect. This suggests that at very low levels cholesterol can be a limiting factor in *Wolbachia* density but that a threshold is quickly reached that is sufficient for normal *Wolbachia* densities to persist. To investigate further, cells could be adapted to cholesterol-free media and supplementation provided over a broader range. One limitation of the results presented here is that cellular cholesterol was not confirmed in the treatments via biochemical means and this assay should be incorporated into future experiments. Although this thesis is focused on genetic control applications, resource competition has interesting implications for the ecological and evolutionary biology of symbiosis and cholesterol could provide a useful system for investigations in this area.

Cholesterol is vital to both *Wolbachia* and the host due to its role in membrane structure, where it imparts rigidity and plays a key role in the formation of lipid rafts and the recruitment of membrane proteins (reviewed in Van Meer et al., 2008). In cholesterol depleted *D. melanogaster*, sphingolipids are increased and hypothesised to play a similar structural role (Carvalho et al., 2010) but in *Wolbachia*-infected *Ae. albopictus* there was specific depletion of bioactive sphingolipids such as ceramide and sphingomyelin in both Aa23-T.*wMel* and Aa23-T.*wMelPop*. *Wolbachia*, like most bacteria, do not have the gene pathways required to produce sphingolipids but unlike many other obligate intracellular bacteria, they also have very limited capacity for any other lipid metabolism such as sphingolipid degradation. The *Wolbachia* genome retains genes for fatty acid elongation and limited transformations of glycerolipids and glycerophospholipids (Wu et al., 2004). This means that changes in lipid composition are unlikely to be due to the direct action of *Wolbachia* metabolic enzymes but more likely involve a host response to *Wolbachia* infection or manipulation of host lipid pathways by *Wolbachia*.

SM, cholesterol and Cer depleted membranes are more fluid than enriched membranes (reviewed in Van Meer et al., 2008) and this could be advantageous for *Wolbachia* as cellular membranes are reconfigured to provide the double membrane-bound, golgi-derived intracellular vesicles in which it dwells (Cho et al., 2011). These lipids are also involved in forming lipid rafts in plasma membranes which most evidence suggests are useful for bacterial invasion and persistence (reviewed in Lafont and Van Der Goot, 2005), suggesting that another explanation for the depletion should be sought.

An alternative possibility is that sphingolipid depletion limits autophagy and apoptosis even where *Wolbachia* is causing cellular stress. Accumulated ceramides are able to activate both autophagic and apoptotic pathways by interfering with the mTOR pathway (reviewed in Jiang and Ogretmen, 2014; Young et al., 2013) and have been shown to target autophagosomes to mitochondria (Sentelle et al., 2012). This is interesting because Voronin et al. (2012) have already demonstrated that autophagy plays an important role in maintenance and control of *Wolbachia* infections. In this chapter I examined expression of the autophagy-related gene *Atg8* which Voronin et al. (2012) saw was upregulated in *Wolbachia*-infected *Brugia malayi* and the inhibitor of apoptosis gene *IAP*, hypothesising an increase in both in *Wolbachia*-infected lines. *Atg8* expression in Uju.*wMel* was increased, suggesting raised autophagic pathway activity, and *IAP* was upregulated in Aa23.*wMel* and Aa23.*wMelPop* cell lines, but there was no evidence for modulation of other genes in either adult or cell lines and in neither case were both genes upregulated together, suggesting that any effect is decoupled, nuanced or more complex than a straight link between *Wolbachia* infection and activation of autophagic and apoptotic pathways. Autophagy can be triggered by oxidative stress or starvation so to investigate signs of upregulation upstream the transcription factor *FoxO*, which is linked to activation of the former latter pathway, was assayed to obtain evidence of upregulation with no difference observed in any line or strain.

One weakness of using a transcript level approach to quantifying gene expression is that several key regulators of autophagic and apoptotic pathways such as FoxO are also controlled by phosphorylation and the relative influence of transcript abundance is therefore lessened. qRT-PCR was a useful starting point for examination of these pathways, but is not an appropriate tool for gaining nuanced evidence of activity – protein level assays and prevalence of post-translational modification combined with biochemical or enzyme activity assays would be required to give a clearer picture of *Wolbachia*-modulated activity.

Phospholipids and FAs showed less dramatic class-wide differences. There were minor overall depletions in both DG and TG in the densely infected Aa23-T.*wMel* line. It is unclear how this relates to flux in FA synthesis and degradation because straight chain FAs and monoacylglycerols were not detected, which can be remedied by optimisation of extraction procedures and mass spectrometry setup for this lipid class. Competition for FAs from *Wolbachia* might be expected to lead to increased

FAS expression to compensate and meet the host metabolic needs, but alterations in FAS expression at the mRNA level were not observed.

Phospholipid classes showed no overall change although within each class there were differentially regulated species. Due to a paucity of information on the role of specific phospholipid species in insects, the functional significance of these findings is difficult to predict but the class level changes indicate clear avenues for future work. The clearest modulation in phospholipids was a 1.44-fold increase in PIs in Aa23-T.*w*Mel and an overall downregulation of PCs.

PIs play key roles in receptor-mediated signal transduction, membrane dynamics and cell remodelling via actin and other cytoskeletal components (reviewed in Di Paolo and De Camilli, 2006). Several vacuolar pathogens are known to modulate PI levels either directly through production of PI metabolic enzymes or through manipulation of host pathways (reviewed in Hilbi, 2006; Weber et al., 2009). *Wolbachia* does not encode PI modifying enzymes but it could manipulate or subvert host pathways. PI increase in bacterial infection has precedent, for example, *Shigella flexneri* upregulates various PI species (Pendaries et al., 2006) post-entry, which is thought to provide protection from apoptosis. PIs thus provide another likely candidate lipid class for involvement in maintenance and regulation of *Wolbachia* infections. PCs are primarily structural molecules and also play a role in the aforementioned lipid raft domains. They are expected to form a major part of intracellular bacterial membranes, but the composition of the *Wolbachia* membrane is currently unknown. Many bacterial infections appear to increase rather than deplete PCs and several pathogenic bacteria are able to synthesise their own using host-provided choline (Geiger et al., 2013).

While other lipids were modulated, and each class included at least a few species with significant differences, the above class changes are most significant and immediately suitable for the design of downstream functional assays, which was the intention behind undertaking an untargeted analysis. In combination, the changes observed suggest a substantial reprogramming of the host lipid metabolic network (examples described for other bacteria in Vromman and Subtil, 2014) but the flux within the system and the mechanism of this modulation is yet to be elucidated.

3.4.2 Implications for DENV refractoriness

These results are striking because the same lipid classes that are depleted in *Wolbachia* infections were selectively enriched in DENV-infected *Ae. albopictus* cells (Perera

et al., 2012).

SM and Cer were upregulated in both the whole cell and replication complex membranes during DENV infection, including enrichment of specific Cer species in the 16K pellet. Perera et al. (2012) suggest that this may be a cellular response or might reflect a direct need for Cer in the viral lifecycle, particularly in forming negative curvature of lipid bilayers as observed in some DENV-induced membrane structures. Unsaturated PC was the most increased phospholipid in DENV-infected C6/36 *Ae. albopictus* cells, which Perera et al. (2012) suggest is again due to its role in membrane curvature and maintaining fluid membranes, which may aid remodelling by viruses.

In addition to generating favourable membrane properties, Cer enrichment in DENV infection also appears to upregulate autophagy for fatty acid provision and this is a requirement in both mammalian and mosquito cells (Heaton and Randall, 2010; Perera et al., 2012). Cer also increases apoptosis in some circumstances, but DENV antagonises apoptosis pathways and is a persistent infection so it seems unlikely that Cer is being used for this purpose. As *Wolbachia* is targeted by autophagy and is therefore under significant selection pressure to subvert or downregulate the process, these two organisms oppose each other in terms of host cell lipid regulation, which could play a role in the refractory phenotype of interest.

3.4.3 Conclusion

The results presented in this chapter have provided new directions for research into *Wolbachia*-induced modulation of host lipid metabolism and implications for DENV infection and transmission, in addition to providing some additional support for cholesterol depletion and modulation of autophagy by *Wolbachia*. The lipidomic analysis exemplifies the usefulness of untargeted assays to uncover interactions from which specific hypotheses can now be generated and pursued in greater detail using the same technologies in combination with targeted molecular and biochemical assays.

There are some experimental design considerations which will provide greater understanding of the viral refractory phenotype that is currently being introgressed into wild-type populations, which is one of the most important avenues of investigation in order to implement and monitor this genetic control approach more effectively.

One consideration is that all results in this chapter were obtained from the Aa23-T heterogeneous cell line derived from larvae, or from whole body analyses of adult

insects. However, the primary lipid storage organ in adults is the fat body and the physiology of different tissues will affect all three primary experimental factors: lipid composition, *Wolbachia* density and viral titre. Repeating the lipidomic study in dissected adult insects would therefore be of interest and for similar reasons, a subcellular investigation may also be revealing. For example, Perera et al. (2012) found different lipid profiles in both the ER and 16k replication complex. Regardless of the system chosen for further investigation, combining DENV infection with lipid supplementation and lipidomic profiling should be a priority.

In summary, the role of sphingolipids and a small range of other lipids has been highlighted as a priority area for future research into *Wolbachia*-based *Ae. albopictus* population replacement for DENV control, which should add to an emerging picture of the molecular mechanisms underpinning the *Wolbachia*-host relationship in this species.

Chapter 4

Engineering female-specific late-acting lethal traits for genetic control of *Ae. aegypti*

4.1 Introduction

Female-lethal, self-limiting population suppression is a variation on current mosquito genetic control techniques using tetracycline-repressible (tet-repressible) expression of dominant lethal effector genes to kill all (bisex) offspring of transgenic insects. Sex-specific alternatively spliced genes can be used to produce lethal levels of effector proteins in female offspring only, which is useful in mosquitoes because it removes the need to separate females prior to release (Figure 4.1). Pre-release sexing is time-consuming and expensive (Section 1.6), but important because it reduces potential distraction of transgenic males from mating with wild-type (WT) females (McInnis et al., 1994; Rendón et al., 2004; Rendon et al., 2000) and also the biting and disease transmission risk posed by released females.

Early and late timing of lethality bring different benefits. Early female removal maximises cost savings in mass-rearing facilities, but in the field a late-acting phenotype has been shown to more effectively and more quickly suppress populations where density-dependent mortality is operating at the larval-stage (Section 1.6.2, Phuc et al. (2007)). Unfortunately, as both bisex and sex-specific lethal constructs currently rely on the same tetracycline-repressible gene expression system, it is not possible to achieve both phenotypes within the same transgenic line, although orthogonal systems to allow this are under development (Section 1.6.1.1 and Chapter 5).

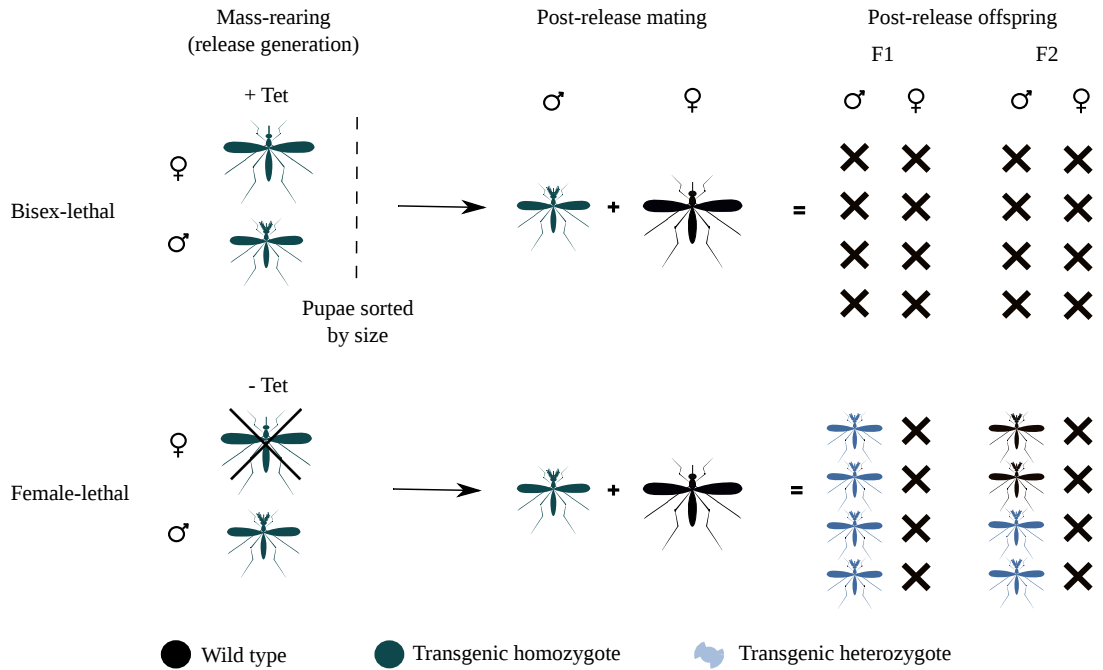


Figure 4.1: Schematic of main differences in mass-rearing, release and lethal allele spread between bisex-lethal and female-lethal self-limiting population suppression. Primary alterations are the ability to rear the female-lethal release generation in the absence of tetracycline, thereby inducing female death and retaining healthy males for release. This circumvents the need for mechanical sorting by size. Releases are carried out in the same manner but another significant difference arises from the survival of heterozygous F₁ males who will continue to mate with WT females and transmit lethal transgenes. Levels will diminish thorough further generations without sustained release, with models suggesting persistence of the transgene for around nine generations in populations of diamondback moth (*Plutella xylostella* L.) and olive fly (*Bactrocera oleae*) reared in the absence of tetracycline (Harvey-Samuel et al., 2014).

Existing female-lethal lines developed in *Ae. albopictus* and *Ae. aegypti* use sex-specific splicing of the *actin-4* gene expressed primarily in flight muscle (Labbé et al., 2012 and Oxitec Ltd, unpublished data). Females express a functional version of the bacterial protein tTAV, a transcriptional activator, which is toxic to cells via transcriptional ‘squenching’ (Section 1.4.3). When highly expressed in flight muscles via a positive feedback loop, tTAV produces flightless females which are unable to escape predation, locate mates, find blood meals to produce eggs and generate viable offspring. These mosquitoes are thus functionally sterile and the trait is effectively adult-stage lethal. However, a fully lethal phenotype activated before eclosion in late larvae or pupae is preferable for ease of monitoring and to prevent regulatory issues

associated with release of transgenic adult females. In addition, it would arguably provide less scope for the evolution of resistance.

Such improvements require a sex-specific alternatively spliced gene with a broader expression profile to provide more flexible regulation of lethal effectors. In other insect species, *doublesex* (*dsx*) and *transformer* (*tra*), which form part of the sex determination pathway, have performed this role in female-lethal constructs. Examples include the Lepidopteran species pink bollworm (*Pectinophora gossypiella*) and diamondback moth (*Plutella xylostella*) (Jin et al., 2013) as well as the Dipterans medfly (*C. capitata*) (Fu et al., 2007) and olive fly (*Bactrocera oleae*) (Ant et al., 2012). *Ae. aegypti* appears to lack a *tra* gene but its *dsx* homologue was characterised by Salvemini et al. (2011) and a mini-gene based on this sequence was engineered by Oxitec Ltd. This transgene has since being used to explore an alcohol-induced early sexing system (Collado, 2013) and successfully generated early-acting, larval-stage female lethality using a tTAV positive feedback loop (Figure 4.2A, Oxitec Ltd., unpublished data).

In this chapter, I build on previous work to replace the tTAV feedback loop with specific toxic effectors Nuclear inhibitor of Protein phosphatase 1 (Nipp1) and Reaper (RprKR). I also engineer pupal-stage lethality by selecting putative pupal-specific promoters using an *Ae. aegypti* developmental transcriptome dataset (Akbari et al., 2013a) and characterising their regulation of downstream markers and effectors in a two-component tet-repressible genetic system (Figure 4.2B).

4.1.1 Toxic Effectors

Toxic effectors including Nipp1, ReaperKR and Michelob_X have been used successfully in mosquito bisex dominant lethal lines (Alphey, 2007). The advantages of using known effectors controlled by a tetO-tTAV feedback loop rather than tTAV performing the role of both regulator and toxic effector are two-fold from both a biological and regulatory perspective. Firstly, the mechanism is more precisely understood than tTAV-induced transcriptional squelching, enabling more informed consideration and monitoring of resistance mechanisms. A second and related point is the flexibility enabled by use of different effectors that act independently via distinct mechanisms. Alternatives are then available in the case of resistance arising to one effector or, when it is desirable to transform or introgress constructs into new *Ae. aegypti* strains, in the case of an incompatible genetic background.

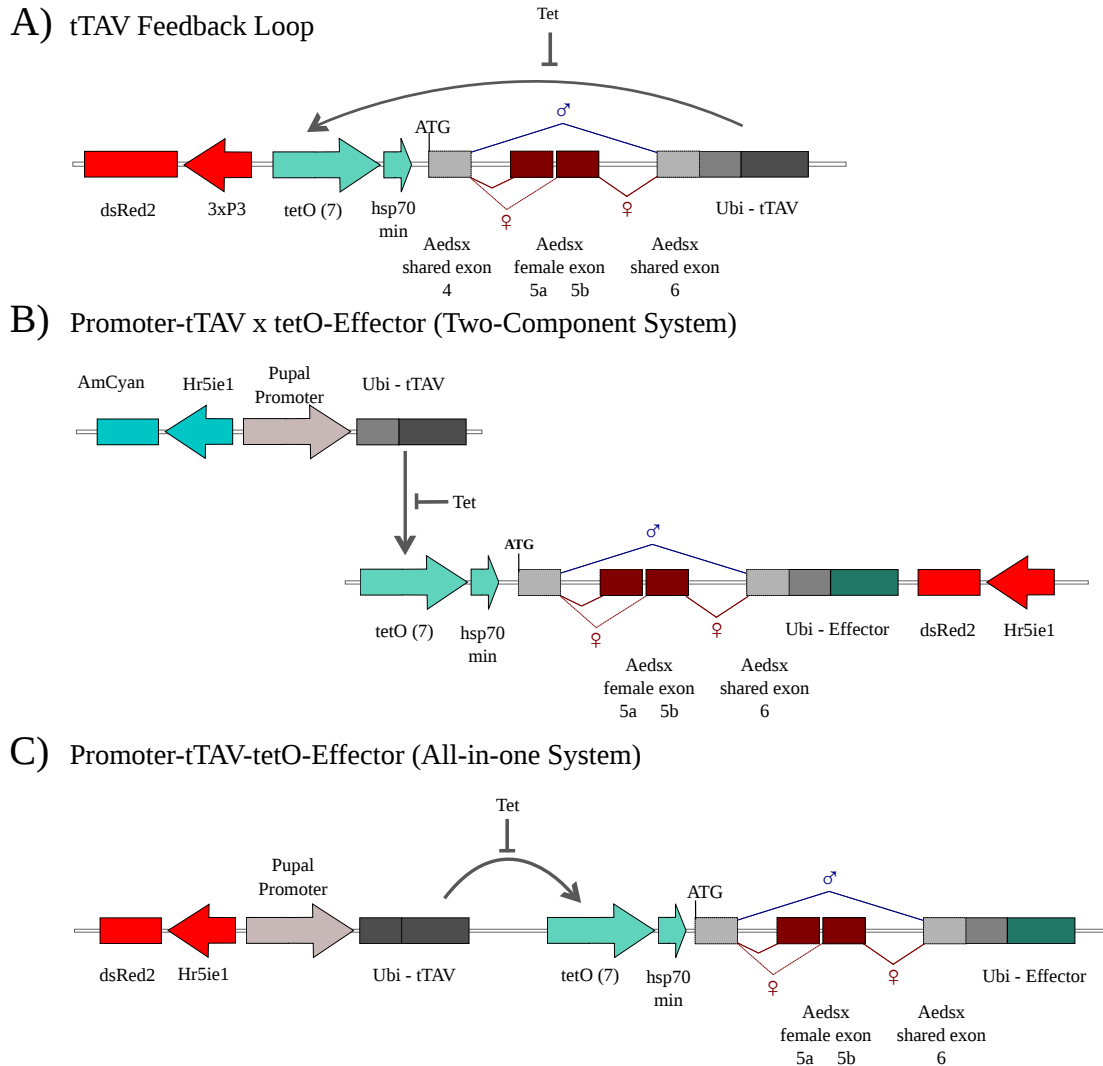


Figure 4.2: Schematic diagrams of lethal gene expression systems where binding of tTAV to the tetO operator is inhibited by the antibiotic tetracycline. A) tTAV positive feedback loop. tTAV is expressed under a hsp70 minimal promoter, controlled by regulatory elements around the genomic insertion site. The protein then binds the tetO operator and promotes its own expression, potentially leading to high levels of tTAV causing cell death via transcriptional squelching. B) Two component system. One construct contains tTAV under the control of a specific promoter e.g. pupal-stage promoter. In double-heterozygote offspring from crosses of single transgenic insects, tTAV binds to a tetO operator in the second construct, promoting transcription of an effector gene. C) All-in-one-system. tTAV under the control of a specific promoter binds to a tetO operator on the same construct and promotes transcription of an effector protein.

An advantage of a synthetically engineered system is that the range of potential effector genes is very wide, increasing the potential for effector switching in cases of resistance. Effectors are usually endogenous or orthologous proteins involved in cell

death, although the use of toxins such as those found in venomous arachnids and sea anenomes has been explored (Haghighat-Khah, 2014; St John, 2012). The Nipp1 and ReaperKR effectors used in this chapter are both *Ae. aegypti* proteins whose pro-apoptotic role is conserved across their Dipteran orthologues.

Nipp1 encodes a nuclear scaffold protein which acts as an inhibitor of serine/threonine protein phosphatase type 1 enzymes (PP1s). PP1s have a highly conserved catalytic subunit (PP1c) involved in a wide variety of essential cell functions including the cell cycle, metabolic reactions and RNA processing. Subcellular location and catalytic specificity are determined by a range of regulatory subunits, including Nipp1, which binds PP1s in the nucleus and has a strong inhibitory effect on catalysis by PP1c (Van Eynde et al., 2004). Nipp1 is indispensable to early embryonic development and also cell proliferation. Overexpression causes apoptosis, tissue death and potentially lethality. This effect can be rescued by simultaneous overexpression of PP1c, suggesting that PP1c inhibition is the cause of cell death rather than disruption of other Nipp1 functions, such as its role in spliceosome formation (Parker et al., 2002).

Reaper (Rpr) is a pro-apoptotic protein; an antagonist of IAP proteins which bind to apoptosis mediating capsases and prevent cell death. Reaper, HID and Grim, collectively termed the RHG proteins after their shared IAP binding motif, both directly bind IAP proteins and also stimulate the ubiquitination and subsequent degradation of IAP (Olson et al., 2003). Conversely, IAP ubiquitin-ligase activity can lead to degradation of Rpr because Rpr lysine sites are so closely associated to IAP via the RHG domain interaction that they can be ubiquitinated. This two-way control of protein abundance thus regulates apoptosis but such complexity is undesirable in a genetically engineered system because it increases the level of Rpr expression required to favour apoptosis. For this reason, the construct tested in this chapter uses a lysine deficient Rpr generated by Olson et al. (2003) known as ReaperKR (RprKR), which is less susceptible to IAP-mediated ubiquitination and degradation. RprKR is hence more likely to induce apoptosis at lower levels of expression than endogenous Rpr because the active protein persists for longer in the cell.

4.1.2 Pupal promoters

Synthetic biology approaches to designing engineered lethal systems allow significant control over the spatio-temporal expression of effectors by re-purposing promoter regions from other genes that display the desired expression profile. The original

bisexual-lethal *Ae. aegypti* strain generated by Oxitec Ltd (OX513A) used a ubiquitous promoter to express tTAV. Its final late-acting expression profile was therefore achieved via positional effects owing to regulatory elements surrounding the random genomic insertion site of the *piggyBac* transposon-mediated construct (Figure 4.2A). *piggyBac* is a mobile genetic element that transposes vector cargo sequences into chromosomal TTAA sites, which are widely distributed throughout the genome. This reliance on positional regulatory effects means that phenotypes are very difficult to reliably reproduce in new lines. While recognising that positional effects will still play a strong role, regulatory elements that are more likely to give the desired expression pattern in any given genomic environment need to be identified.

Expression in female-lethal strains using *actin-4* has two desirable and endogenous properties: splicing that is female-specific and that occurs only in a late-developing tissue that is sensitive to the action of tTAV. However, *dsx* is spliced throughout development and in numerous tissues by an unknown regulator. A specific promoter driving tTAV expression in the desired spatio-temporal pattern is therefore required. In model insects such as *D. melanogaster*, promoter databases have been compiled alongside large datasets on gene expression, making selection much simpler than in species such as *Ae. aegypti*, for which far less data exists. However, the recent publication of a large developmental transcriptome dataset (Akbari et al., 2013a) and the wider variety of RNA-Seq and microarray data being generated in this species mean that it is now becoming easier to identify putative regulators for expression patterns of interest, for instance in the characterisation of five germline-specific *Ae. aegypti* promoters for the development of genetic drive systems by Papathanos et al. (2013).

I select and test three pupal promoter candidates from this recent dataset to complement the *Ae. aegypti* osiris gene pupal promoter characterised by Conway (2014). This research will thus contribute to the genetic toolkit necessary to deliver reliable and reproducible genetic control systems for this important vector species.

4.2 Materials and Methods

4.2.1 Transgenic constructs tetO-*dsx*-Nipp1 and tetO-*dsx*-RprKR

tetO-*dsx*-Nipp1 and tetO-*dsx*-RprKR (Figure 4.2B) were designed and constructed by Dr Tarig Dafa'alla (Oxitec Ltd.) starting from OX4699 PB[tetO7-mutAed Δ ubiquitin-TAV-SV40-3xP3-DsR] (unpublished construct). The 3xP3 driven DsRed2 marker was replaced by a Hr5ie1-DsRed2-SV40 segment and tTAV was replaced by the effector Nipp1 amplified from OX3547 (Fu et al., 2007) or RprKR amplified from OX4776 (unpublished construct).

4.2.2 Selection of pupal promoter regions

Genes putatively expressed almost exclusively in pupal-stage *Ae. aegypti* were selected from developmental stage RNA-Seq transcriptome data (Akbari et al., 2013a). Genes with the highest ratio of pupal to non-pupal expression were shortlisted and the gDNA, mRNA, cDNA and 2 kb upstream promoter sequences were obtained from NCBI databases. A genomic features file was generated to examine the location of the genes and surrounding features in the *Ae. aegypti* genome (UCBC Genome Browser). This data retrieval was performed semi-automatedly using a custom BioPython script. Genes were selected as described in Section 4.3.1 on the basis of having relatively little non-pupal expression and a 'high', 'mid' and 'low' absolute expression level. Other criteria considered included being located at least 5-10 kb from other genes and outside of gene clusters which may exhibit complex co-regulation.

4.2.3 Transgenic constructs low-, mid- and high-tTAV

Constructs were generated in collaboration with Sarah Scaife (Oxitec Ltd). 2 kb regions upstream of the chosen genes low-, mid- and high-Pro were amplified from *Ae. aegypti* gDNA using primers designed by Sarah Scaife (Table 4.3) and Q5 or Phusion polymerase (New England Biolabs, Ipswich, MA, US; Table 4.2). Touchdown PCR cycling was performed as per Table 4.1. Appropriately sized PCR products were purified using the Qiagen QIAquick PCR purification kit or gel extracted using the Qiagen MinElute kit per manufacturer's instructions (Qiagen, Venlo, Netherlands). Purified products were then digested using AscI-AvrII and ligated into con-

struct OX4531 (PB[Hr5ie1-AmCyan-SV40-Hex1.2pro-ubi-ttAV-SV40]) to replace the Hex1.2pro hexamerin promoter with the pupal promoter of interest.

(a)		(b)			
Reagent	Volume μ l	Step	Temp °	Time	Cycles
cDNA	0.5	1	95	4 min	1
PCR BioTaq	0.3	2	95	30 s	
5x BioTaq Buffer	6	3	60	30 S	9
10 mM Primer F	0.6	4	72	60 S	
10 mM Primer R	0.6	5	95	30 S	
dH ₂ O	22	6	55	30 s	35
Total	30	7	72	60 s	
		8	72	5 min	1

Table 4.1: a) PCR reagent recipe and b) thermocycling conditions for PCR.

Plasmids were transformed into XL10-Gold ultracompetent cells as per manufacturer’s instructions (Aligent Technologies, Santa Clara, CA) and colonies were screened using diagnostic primers (Table 4.4) and PCR BioTaq (Bioline Reagents, London, UK; Table 4.2). Two colonies per construct displaying the correct sized PCR product were cultured overnight in 3 ml LB-Amp media and mini-prepped using the Fermentas GeneJET Plasmid miniprep kit (Thermo Fisher Scientific, Waltham, MA). Mini-preps underwent diagnostic restriction digests (Table 4.5) and were sequenced by GATC Biotech AG (Constance, Germany) to confirm promoter inserts were correct before being cultured overnight in 250 ml LB-Amp media and maxi-prepped using the QIAfilter Endofree Plasmid Maxi Kits (Qiagen, Venlo, Netherlands) per the manufacturer’s instructions.

4.2.4 Microinjections

An injection mixture containing 200 μ g. μ l⁻¹ low-, mid- and high-tTAV maxi-prepped plasmid and 250 μ g. μ l⁻¹ helper mRNA encoding *piggyBac* transposase or 500 μ g. μ l⁻¹ tetO-*dsx*-Nipp1/RprKR plasmid DNA and 700 μ g. μ l⁻¹ helper mRNA was suspended in injection buffer (5 mM KCl and 0.1 mM NaH₂PO₄, pH 6.8) (Morris et al., 1989). Semi-desiccated *Ae. aegypti* Asian WT eggs were injected under halocarbon oil (Sigma Aldrich, MO, USA) during the preblastoderm stage using a Femtojet II microinjector (Eppendorf AG, Hamburg, Germany) with alumniosilicate extruded capillary needles. Eggs were then left vertically in deionised water overnight to enable oil to drain before

	Volume μ l		
Reagent	Dream Taq	Q5/Phusion	PCR BioTaq
Enzyme	0.1	0.75	0.1
10 x Buffer	2	NA	NA
5 x Buffer	NA	10	2
10 mM dNTPs	8	1	NA
10 mM Primer F	8	2	0.2
10 mM Primer R	8	2	0.2
10 x BSA	0	1	NA
dH ₂ O	16.7	31.25	7.5
DNA	2	2	NA
Total	20	50	10

Table 4.2: PCR reagents and volumes per reaction used in construction of low-, mid- and high-tTAV.

Construct	Gene	Amplification Primers
low-tTAV	low-Pro	11044AscF (Forward) 5'-GGTGTGGGCGCGCCCGAGGATTATTCCAGACGGCTAGG-3' 11044AvR (Reverse) 5'-GGTGTGCCTAGGTTTATCGCCTGGCTAACCAAGATG-3' 2516 bp product
mid-tTAV	mid-Pro	4788AscF (Forward) 5'-GGTGTGGGCGCGCCGGGCTCCATAGGGTTACAGCAG-3' 4788AvR (Reverse) 5'-GGTGTGCCTAGGTTTGGCTGGCTGCGAACACAC-3' 2510 bp product
high-tTAV	high-Pro	7085AscF (Forward) 5'-GGTGTGGGCGCGCCGGAGCTGGGCCACACCCTG-3' 7085AvrR (Reverse) 5'-GGTGTGCCTAGGGTTGCTGCTGTTGAAATCGTTTCCG-3' 2526 bp product

Table 4.3: Primers used to amplify promoter regions of low-, mid- and high-Pro genes. All primers were designed by Sarah Scaife (Oxitec Ltd.)

slides were transferred to pots containing damp cotton wool. After 4 days at approx. 100% relative humidity, eggs were hatched under low pressure in deoxygenated water.

Injection survivors were fed ground Tetramin[®] fish food (Tetra GmbH, Melle, Germany) until pupation, whereupon they were sex separated and pooled. Two G₀ males were mated to 10 WT females or 3-8 G₀ females were mated 1:1 with WT males in small pots. Cotton wool soaked in 10% sucrose solution supplemented with 14 μ g.ml⁻¹ penicillin and 14 μ g.ml⁻¹ streptomycin was provided *ad libitum* throughout.

Crosses were blood fed two days post-addition of WT adults, eggs were collected after an additional four days and hatched after maturing for at least a further four

Construct	Gene	Colony PCR Primers
low-tTAV	low-Pro	AmCyanddiagF (Forward) 5'-TCACCTACGAGGACGGCGG-3' 11044DiagR (Reverse) 5'-TTCTACCCAACCTGAACCAGTGACCC-3' 930 bp product
mid-tTAV	mid-Pro	AmCyanddiagF (Forward) as low-Pro 4788DiagR (Reverse) 5'-CTGCGGACCCCTTATTCCCG-3' 930 bp product
high-tTAV	high-Pro	AmCyanddiagF (Forward) as low-Pro 7085diagR (Reverse) 5'-GAGCGTGTATGTTCCCTTGCGTTCC-3' 934 bp product

Table 4.4: Primers used for colony PCR of putative low-, mid- and high-tTAV transformed colonies. All primers were designed by Sarah Scaife (Oxitec Ltd.)

Construct	Diagnostic digest	Sequencing Primers
high-tTAV	XmaI 8.4 kb, 3.1 kb, 364 bp	7085diagR (Reverse) (as colony PCR) 7085diagR2 (Reverse) 5'-GTGTGTCCTACTTTGCTTCGGTACG-3' 7085diagR3 (Reverse) 5'-GATTCAAGAAGCATGTGATAACGATCAGT-3'
low-tTAV	XmaI 8.4 kb, 3.1 kb, 364 bp	11044DiagR (Reverse) (as colony PCR) 11044DiagR2 (Reverse) 5'-GTGACACTGACACAGATGGAGAACTTCC-3' 11044DiagR3 (Reverse) 5'-GTCTGCCCAGACCGATTGTAAGG-3'
mid-tTAV	NheI-XbaI 8.9 kb, 2.4 kb, 694 bp	4788DiagR (Reverse) (as colony PCR) 4788DiagR2 (Reverse) 5'-TTCCATTCCAGACAAATAGTATGGATACC-3' 4788DiagR3 (Reverse) 5'-GGGCTTATGTTGCACAGAGCCG-3'

Table 4.5: Restriction enzymes and additional primers used in digest and sequencing of low-, mid- and high-tTAV. All reverse primers were paired with AmCyanddiagF and were designed by Sarah Scaife (Oxitec Ltd.)

days. G₁ larvae were screened for fluorescence on a Leica MZ95 microscope (Filters: DsRed2: excitation HQ545/30x; emission HQ620/60m; Chroma Technology (Rockingham, VT)). Photos were taken using a Canon PowerShot S5IS fitted to the eyepiece with a MM99 adaptor (Martin Microscopes).

Primer	Direction	Sequence
DiagIEF	Forward	5'-GATAATGTCGGGCAACGTATCCG-3'
Diag6-IE1	Forward	5'-CTATTATCGATTTGCAGTTCGG-3'
AmCydiagF	Forward	5'-TCACCTACGAGGACGGCGG-3'
DiagttAV2	Reverse	5'-GAGCTGGTTCTCCAGTGTCTCG-3'
Seq3-ttAV2	Reverse	5'-CGTTGGAGCTGTTGAACGAAG-3'

Table 4.6: Primers used for sequencing constructs low-, mid- and high-tTAV. All primers designed by Sarah Scaife or Tarig Dafa'alla (Oxitec Ltd)

Transgenic larvae were separated by sex and phenotype before being outcrossed to WT adults on eclosion. Where possible, single transgenic males were outcrossed to 10 WT females and single females to two WT males.

Where further generations appeared to contain multiple insertions, out-crossing of single males was repeated to allow for selection of single insertion transgenic line, which were then maintained in a heterozygous state by outcrossing of pooled transgenic males to WT females during each subsequent generation.

4.2.5 General Mosquito Rearing

Subsequent mosquito rearing took place in 15 x 15 x 15 cm BugDorm cages (MegaView Science, Taiwan). Mosquitoes were provided with sugar water containing 10% sucrose solution supplemented with 14 $\mu\text{g}.\text{ml}^{-1}$ penicillin and 14 $\mu\text{g}.\text{ml}^{-1}$ streptomycin to prevent bacterial growth. Insectaries were maintained at 60% humidity and 27 °C with a 12 h/12 h light/dark cycle. Horse blood was provided periodically on Parafilm® covered metal panels heated by warm beanbags and eggs were collected and stored on filter paper before being hatched under low pressure. Larvae were routinely reared in plastic trays on a diet of ground Tetramin® fish food (Tetra GmbH, Melle, Germany).

4.2.6 Splicing PCR and transcript sequencing

Double transgenic male and female pupae of tetO-*dsx*-Nipp1-T and tetO-*dsx*-RprKR-T lines crossed to pro-tTAV were collected. RNA was extracted and DNase treated using the Noragen Total RNA Purification Kit (Noragen Biotek Corporation, Ontario, Canada) according to the manufacturer's instructions. RNA was quantified using a GeneFlow NanoPhotometer (GeneFlow Ltd., Lichfield, UK) and cDNA was synthesised from 500 ng RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions.

A 1:10 dilution of cDNA was amplified to detect expression of *Nipp1* or *RprKR* and splicing pattern of *dsx-Nipp1/RprKR* in all samples using the *Ae. aegypti* house-keeping gene muscle actin (AeMuAc) as a positive control and gDNA and a no-RT sample as controls to confirm absence of gDNA contamination.

Splicing PCRs were performed as per Table 4.1 using the primers in Table 4.7. A second PCR was performed for OX5010-B male and female samples in a total volume of 50 µl and spliceform bands were gel extracted using the Qiagen QIAQuick Gel Extraction Kit (Qiagen, Hilden, Germany), cloned into pJET vectors (GeneJET PCR Cloning Kit, Fermentas, Vilnius, Lithuania) and then transformed into XL-10 cells (Stratagene, La Jolla, CA, USA). Positive recombinants were identified using PCR with pJET primers (Table 5.4), purified via miniprep (GeneJET Plasmid Miniprep Kit, Fermentas) and sequenced by GATC Biotech (Konstanz, Germany) using the same pJET primers. Sequences were aligned to the construct and visualised using Geneious (version 7.1.5, Biomatters, Available from <http://www.geneious.com>).

Primer Name	Direction	Primer Sequence
<i>Aed</i> <i>dsx</i> -F (630)	Forward	5'-GAGATGATGCCCCCTGATGTACG-3'
<i>Nipp1</i> (167)	Reverse	5'-CCCAACATGGGCTTGCGACC-3'
<i>RprKR</i> (1117)	Reverse	5'-TTACTGGCTCGGACGGCG-3'

Table 4.7: Primers spanning the *dsx-Nipp1* and *dsx-RprKR* cassette to identify spliceforms.

4.2.7 Testing *dsx-Nipp1* or *dsx-RprKR* effector lines

Single insertion effector lines were crossed at G₄ or G₅ to pro-tTAV G₄ males. pro-tTAV (OX4811-N) is a construct designed and injected by Conway (2014) expressing tTAV under the control of a pupal-stage promoter from the Osiris gene AAEL004298. 100 tetO-*dsx-Nipp1* or tetO-*dsx-RprKR* females from each line were crossed with 25 pro-tTAV males in 15 x 15 cm cages and eggs collected post blood feeding.

Day	mg per larva
1	0.08
2	0
3	0.16
4	0.32
5	0.64
6+	0.32

Table 4.8: Tetramin[®] feeding regime for *Ae. aegypti* larvae

Eggs from *dsx*-effector x pro-tTAV crosses were hatched in deionised water under vacuum for at least 2 h. L1 larvae were aliquoted into three pots of 200 in 300 ml deionised water with or without the addition of 30 $\mu\text{g}.\text{ml}^{-1}$ chlortetracycline (Sigma Aldrich, St. Louis, MO, USA). Larvae were fed a standard feeding regime of ground Tetramin[®] fish food (Table 4.8). Due to differences in growth rates across experiments, feeding on day 3 was delayed until day 4 if food remained in the water to avoid over feeding and water fouling. Offspring were screened for fluorescence and sex at the pupal-stage. WT and double-heterozygote males and females were counted and separated into pots while single transgenics were only counted. Pupae were allowed to eclose in a 30 x 30 x 30 cm BugDorm cage (MegaView Science Company Ltd., Talchung, Taiwan), with one cage containing all replicates of one line and treatment combination. Unclosed or half-closed dead pupae were counted once all pupae in a cage had either eclosed or died.

4.2.8 Testing pupal promoter lines

All G₂ females from putative single insertion promoter lines low-, mid- and high-tTAV were crossed to 25 G₄ tetO-*dsx*-Nipp1-T males. Offspring were reared and assayed for female specific lethality as per Section 4.2.7.

4.2.9 Expression profile of pupal promoter driven transgenes

100 G₃ females from single-insertion pupal promoter lines low-, mid- and high-tTAV were crossed with 25 males from the marker line OX3987-D (AttB-Hr5ie1-DsRed2-teto21-hsp-adh-AmCyan). This has a whole body Hr5ie1 driven DsRed marker and weakly expresses AmCyan in the eyes due to the location of the site-specific integration docking site attB. AmCyan is also under the control of a tetO minimal promoter so expression should be driven in tissues where tTAV is present in the absence of tetracycline.

Offspring were reared in trays containing 2 L of deionised water with or without 30 $\mu\text{g}.\text{pl}^{-1}$ chlortetracycline (Sigma Aldrich, St. Louis, MO, USA) and fed ground Tetramin[®] fish food *ad libitum*. Pupae were removed from the tray at a single time point on the first day of pupation and then at 2 h intervals further pupae were removed, screened, sexed and allowed to develop. Pupae were then screened and photographs of representative examples were taken for each of the timepoints: fourth

instar larvae (L4), 12-14 h, 24-26 h, 36-38 h, 48-50 h, one day adults. Microscope and camera details can be found in Section 4.2.4.

4.2.10 Expression profile of pupal promoter driven endogenous genes

A developmental time series for expression of low-, mid- and high-Pro was generated by rearing WT larvae in 2 L deionised water. Larvae were collected at instar L1-4. Pupae were picked on the first day of pupation and again at 1 h intervals then left to develop for 6, 12, 18, 24, 36 and 48 h. RNA was extracted and cDNA synthesised as per Section 4.2.6. PCR was performed with primers designed against the relevant endogenous genes using PrimerBLAST (Table 4.9), a 30 μ l BioTaq reaction as per Table 4.2 and the PCR thermocycling conditions in Table 4.1.

Primer	Direction	Sequence
AAEL004788 F	Forward	5'-GTCGGCCAGAAGAACGAGAA-3'
AAEL004788 R	Reverse	5'-GGTGCTTGTTGCCCTTGTTT-3'
AAEL011044 F2	Forward	5'-CAGCTCCCCAAGGACGAAG-3'
AAEL011044 R2	Reverse	5'-GAATCGAGCCATTGCCCCC-3'
AAEL007085 F3	Forward	5'-ACACCGGAGATCACAAGAGC-3'
AAEL007085 R3	Reverse	5'-CAGTCCTCCTTCGTGGATGG-3'

Table 4.9: Primers used in PCR for expression of endogenous genes driven by selected pupal promoters.

4.2.11 Statistical Analysis

All statistical analysis was undertaken using R. Survival to pupation and subsequent eclosion were analysed separately to demonstrate pupal-stage lethality where present. Pupation was analysed via count data using a Poisson distribution log-linear GLM, because the starting frequency of each phenotype in the initial sample was unknown (Crawley, 2014). Eclosion data was analysed using a binomial distribution GLM because due to the pupal count data, the exact proportion of mosquitoes eclosing or not could be calculated and analysed as a proportion. A saturated model containing the three factors; tet-treatment, transgenic status and sex plus all interactions, was tested against the null model using a χ^2 ANOVA and Akaike information criterion (AIC), with interactions and factors removed in a step-wise manner.

Where a saturated GLM was fitted, further tests were performed to confirm that the off-tetracycline, female transgenic result was the only specific significant three-way

interaction. A cell means model was fitted using the R package multcomp. This model was based on a new factor derived as the interaction between type, treatment and sex. Pairwise contrasts were then produced for all factors. All factors in the model have two levels so these contrasts were reduced to a single test, corresponding to the single degree of freedom of the three-way interaction.

4.3 Results

4.3.1 Pupal Promoter Candidates

Three candidate genes with pupal-specific high, medium and low expression were selected from a shortlist of 25 genes showing highest pupal expression in comparison to other life stages in the *Ae. aegypti* developmental transcriptome dataset from Akbari et al. (2013a): AAEL011044 (low-expression, low-Pro), AAEL004788 (medium-expression, mid-Pro) and AAEL007085 (high-expression, high-Pro) (Figure 4.3). The shortlist was produced from Akbari’s analysis of relative transcript abundance dataset, which is expressed as fragments per kilobase of exon per million fragments mapped (FPKM). This measure normalises for transcript length and number of fragments sequenced.

A range of expression strengths was selected because even when tTAV is expressed at the right time in effector-susceptible tissues, promoters may be too weak to cause an effect or too strong. In the latter case, levels of tTAV could be high enough that tetO binding is not fully suppressed by tetracycline, causing lethal or sub-lethal effects regardless of rearing conditions. The three selected genes were all comparatively isolated, with nearest neighbours at least 7 kb away and in some cases >100 kb up or downstream. Importantly, they were not clustered or arranged in a way that indicates complex cis-regulation. While Akbari et al. (2013a) provided developmental staging data, there is limited information on tissue expression patterns in the vast majority of *Ae. aegypti* genes so orthologues were examined in *D. melanogaster* where much larger transcriptome datasets are available.

Mid-Pro is a putative osiris gene. These are a family of highly conserved proteins in holometabolous and hemimetabolous insects that display remarkable syntenic conservation and are thought to have arisen from gene duplication and neo-functionalisation (Shah et al., 2012). In *D. melanogaster* the majority form a cluster in the Triplo-lethal (Tpl) locus, but mid-Pro is an orthologue of the *Drosophila* Osiris 21 (*Osi21*,

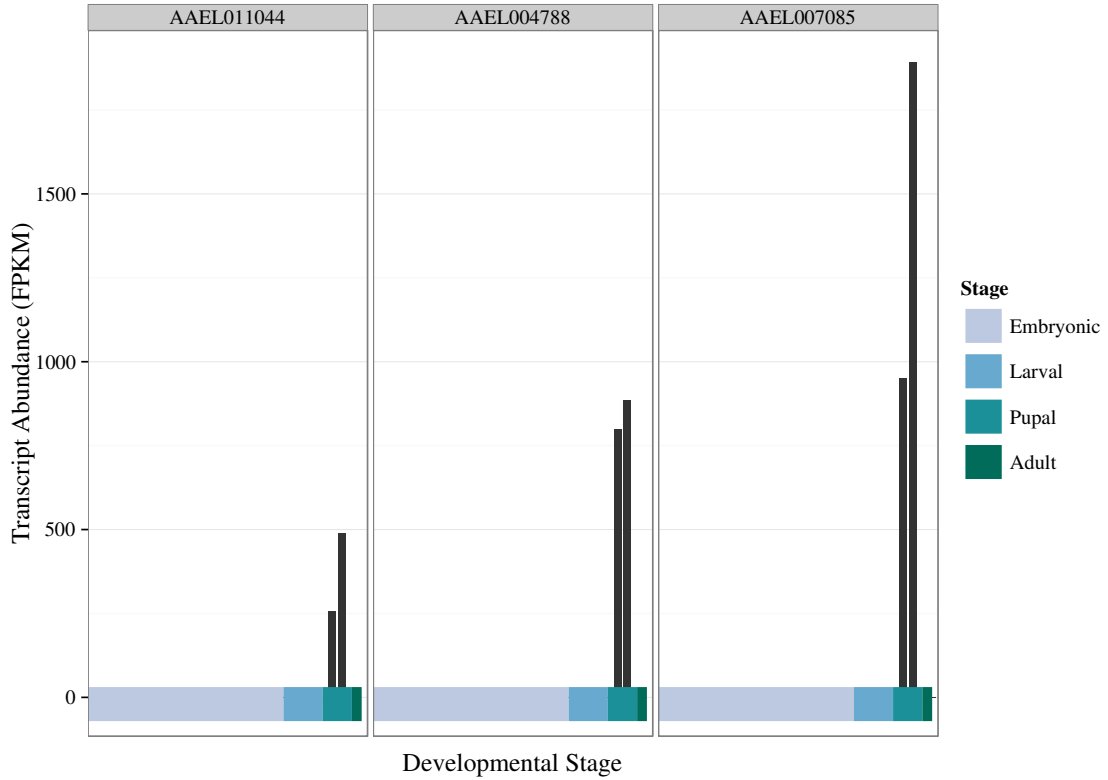


Figure 4.3: Normalised RNA-Seq transcript abundance expressed as fragments per kb of exon per million fragments mapped (FPKM) for the selected low-, mid- and high-expression pupal-specific genes at each life stage. Data for all embryonic and larval life stages described in Akbari et al. (2013a) is displayed in chronological order. Pupal and adult stage data is displayed with male expression on the left and female on the right. Adult data represents carcass expression only, data from gonads and blood-fed females is not displayed.

CG14925), which is outside of the *Tpl* locus and does not display syntenic conservation. It may have evolved a divergent function to other family members, thus escaping the selection that is maintaining co-localisation and potentially shared regulation. There have been recent clues as to what this function might be, for example Lee et al. (2013a) demonstrate involvement in controlling membrane homeostasis in endosomes and lysosomes, but otherwise little is known about the protein. The *Osi21* expression pattern appear to be conserved between *Ae. aegypti* and *Drosophila*, in which it also demonstrates very high pupal-specific expression from 2-3 days post white prepupae (modENCODE data from <http://www.flybase.org>, (Graveley et al., 2011)), suggesting that the gene is expressed for a maximum of around 48 h.

Tightly time-bounded expression makes it less likely that tissue expression patterns are detected because the point of sampling is less likely to intersect with peak expression, but for *D. melanogaster* the standard modENCODE tissue expression

dataset (modENCODE data from <http://www.flybase.org>, Roy et al. (2010)) picks up moderately high expression of *Osi21* in pupal fat body. Osiris genes generally are expressed in all non-neuronal tissues derived from the ectoderm, including epidermis, hind and foregut, the trachea (Shah et al., 2012) and the developing wing (Ren et al., 2005). The promoter region from another member of the osiris family (AAEL004298, orthologue of *D. melanogaster Osi11*, CG15596 Shah et al. (2012)) was selected and successfully trialled as a pupal-stage promoter driving tTAV expression by Conway (2014). The resulting construct OX4811-N (pro-tTAV) will be used in this chapter. Lethality in bisex-lethal systems using pro-tTAV was observed to be very late acting (Conway, 2014), so my screen aimed to find a promoter inducing death before eclosion.

A massive level of transcription and chitin-synthesis is required in the pharate adult, initiated by a peak in ecdysone hormone production in pupae 6-12 hours after larval-pupal ecdysis (molting) (Margam et al., 2006). An over-representation of cuticle gene expression in pupae is therefore to be expected and this was borne out in the shortlist, with many genes identified as proteins involved in cuticle synthesis either through previous annotation or the presence of a chitin binding domain. The necessary coordination of multiple genes required to regulate and provide raw material for this rapid cuticle proliferation is reflected in the tight genomic clustering of cuticle protein genes, which are often arranged as tandem arrays expressed at similar times and at similar levels, indicating shared regulation. The linked arrangement of multiple very similar genes may be a result of selection pressure to increase transcript levels and speed up the transition from pupae to adult eclosion, which is completed in only 48 hours. Transcription of a single gene will eventually reach a maximum but duplication would continue to allow increased transcript production and therefore be positively selected under this hypothesis (Togawa et al., 2008). Unfortunately, the genomic arrangement just described makes them unsuitable as candidates for promoter extraction.

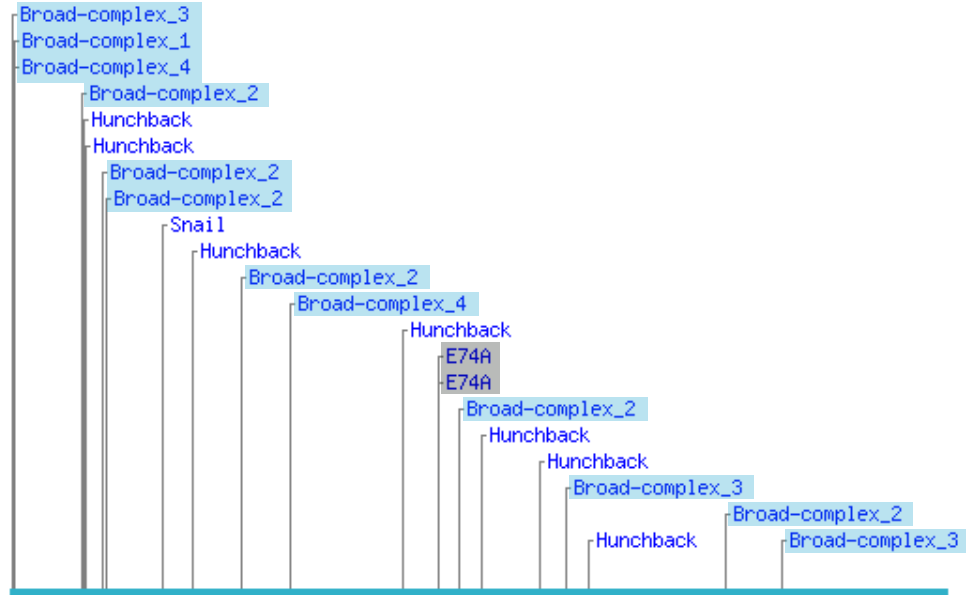
On the other hand, there are comparatively isolated putative cuticle proteins like high-Pro, which are more likely to be under straight-forward promoter control. The closest four *An. gambiae* genes CPR17/18/19/20 with 62-74% amino acid similarity are also all expressed in a pupal-specific manner with expression peaking in the late pupal-stage, making them likely to be contributing primarily to the pre-ecdysial

exocuticle in the pharate adult (Togawa et al., 2008). Low-Pro is also isolated, contains a chitin-binding domain and shows weak homology to cuticle proteins such as CPR69 in *An. gambiae* species, but there are no other clues to its function or expression. All genes were cross-checked against the *Ae. aegypti* developmental gene expression study by Harker et al. (2013) but none were included on the custom microarray used (archived at ArrayExpress <http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-46/>) so no support was obtained for the RNA-Seq transcriptome data.

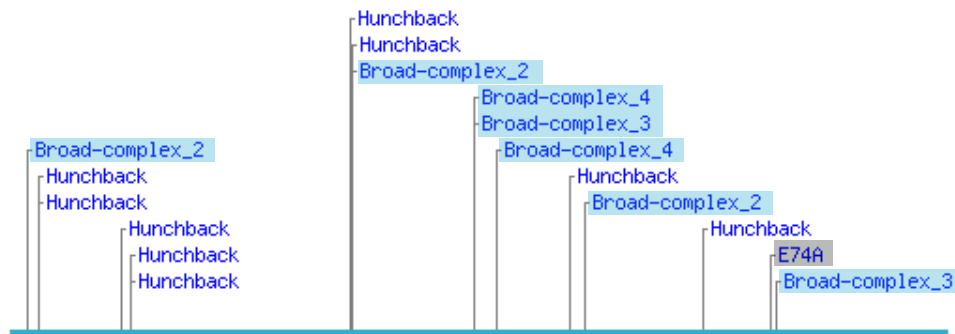
A further consideration when selecting regulatory elements for transgenic constructs is any potential environmental modulation of expression. Promoter regions containing transcription factor binding sites (TFBSs) or other regulatory elements that are affected either directly or indirectly by pollutants, insecticide application and many other stimuli that could potentially be encountered by F₁ offspring in the wild are of concern because they might reduce the efficacy of the lethal construct. *Ae. aegypti* microarray reporters demonstrating modulation of expression included small but significant changes during *Wolbachia* infection for all genes, although *Wolbachia* is not a naturally occurring symbiont. Both cuticle proteins were upregulated in strains selected for insecticide resistance (low-Pro in Tetreau et al. (2012); high-Pro in Poupardin et al. (2012)). This demonstrates that environmental factors could potentially influence introgressed transgene expression under the control of these promoters in genetic backgrounds that have been exposed to insecticides. Any effect would be dependent on the exact regulatory mechanisms, not all of which will involve the promoter region.

To establish the likely transcription factors involved in regulating expression from these promoters, the sequence 2 kb upstream of each gene's annotated transcription start site was checked against *D. melanogaster* TFBSs using ConSite (Figure 4.4, <http://consite.genereg.net/>, Sandelin et al. (2004)). All sequences contained putative binding sites for Hunchback, which is typically involved in embryonic development but has a very simple binding sequence of repetitive T bases, so it is unclear whether this is biologically relevant. More interestingly, Broad complex and E74A binding sites were present in all promoters and both of these transcription factors are ecdysone-responsive. They also both perform a conserved function in regulating pupal development of several holometabolous insects (Fletcher and Thummel, 1995a,b; Uhlirova et al., 2003; Zhou and Riddiford, 2002). In *Ae. aegypti* Broad complex genes

low-Pro (AAEL011044)



mid-Pro (AAEL004788)



high-Pro (AAEL007085)

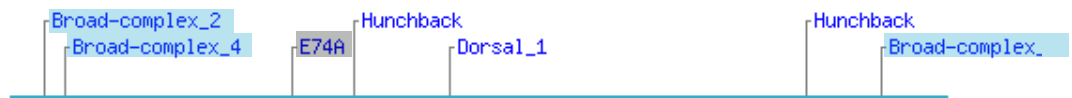


Figure 4.4: Transcription factor binding sites (TFBSs) found in the 2 kb upstream flanking region of the low-, mid- and high-Pro pupal genes selected from the *Ae. aegypti* developmental transcriptome Akbari et al. (2013a). All promoter regions contained Broad complex (light blue) and E74A binding sites (grey). These transcription factors are responsive to hormone 20-hydroxyecdysone (20E), a steroid hormone which initiates pupal development, suggesting that they are ideal candidates for pupal-stage promoters. Images generated by ConSite (<http://consite.genereg.net/>, Sandelin et al. (2004)).

are also involved in vitellogenesis, another 20E activated process, but E74A isoforms were not detected in vitellogenic tissues (Chen et al., 2004), suggesting that this TFBS profile could be characteristic for pupal-stage promoters in *Ae. aegypti* (Figure 4.4).

4.3.2 Production of Transgenic Lines

Constructs tetO-*dsx*-Nipp1 and tetO-*dsx*-RprKR with effector genes driven by a tetO operator and hsp70 minimal promoter (Figure 4.2) were generated by Dr Tarig Dafa'alla (Oxitec Ltd.). Constructs containing pupal promoters driving expression of tTAV were generated in collaboration with Dr Sarah Scaife (Oxitec Ltd.). These are OX5050 (high-tTAV, containing high-Pro), OX5051 (low-tTAV, containing low-Pro) and OX5053 (mid-tTAV, containing mid-Pro). Data on construct microinjections, lines established and transformation rates for all five constructs are provided in Table 4.10.

Construct	Name	Injected	G ₀ adults	Positive G ₁	% TR	Initial G ₁ Lines	Tested Lines
OX5012	tetO- <i>dsx</i> -Nipp1	2852	64	8	12.5	11	10
OX5013	tetO- <i>dsx</i> -RprKR	2670	95	16	16.8	19	2
OX5050	high-tTAV	1714	49	16	32.7	16	5
OX5051	low-tTAV	993	34	13	38.2	13	4
OX5053	mid-tTAV	960	21	3	14.3	4	3

Table 4.10: Information on transgenic lines established by microinjection of five constructs: tetO-*dsx*-Nipp1, tetO-*dsx*-RprKR, low-, mid- and high-tTAV. G₀ represents all injection survivors reaching adult lifestage. Positive G₁ figure represents pools containing transgenic larvae, giving the minimum number of independent insertion events from which the transformation rate (% TR) was calculated as a percentage of injection survivors. Initial G₁ lines represent the estimated number of independent lines once larvae had been separated from pools by fluorescent phenotype. Tested lines represent the number of single-insertion lines with no sex-linkage or transgenic lethality which were verified by PCR and then included in subsequent assays for female lethality off-tetracycline. Only one line per original pool was taken forward for testing so it is likely that these represent independent insertions, although no flanking PCR was performed.

Rates of survival were low compared to values typically achieved in *Ae. aegypti* microinjections, ranging from 2.2-3.5% compared to 20.5% in Kokoza and Raikhel (2011), 23.3% in Nimmo et al. (2006) and a variable average of 10% at Oxitec Ltd. However, minimum transformation rates calculated as the number of positive pools

as a percentage of the surviving injection survivors, were high at 12.5-38.2% compared to previously reported rates of 4-11% (Kokoza and Raikhel, 2011; Lobo et al., 2002; Nimmo et al., 2006). All lines displayed the expected Hr5ie1 promoter-driven fluorescent phenotype, with varying levels of intensity (Figure 4.5). Lines were established as single insertions through repeated single-male crossing to WT females where necessary and all sex-linked lines or lines displaying sex-ratio distortion and reduced transgenic survival were discarded. Lines taken forward for testing were verified by PCR to confirm lack of contamination with other constructs, which was a possibility due to the same marker being expressed in the two effector lines (DsRed2) and three pupal promoter lines (AmCyan) respectively. No contamination was discovered but some lines were found to be expressing Nipp1 when thought to contain the tetO-*dsx*-RprKR construct, indicating a very early error during injections or line separation and accounting for the small number of tetO-*dsx*-RprKR lines tested.

4.3.3 Sex-specific splicing of tetO-*dsx*-Nipp1 and tetO-*dsx*-RprKR effector constructs

Different spliceforms were expected in males and females in both effector constructs (Figure 4.6). Only the female spliceform containing *dsx* exon-4-5b-6 will produce an in-frame sequence encoding the effector protein.

RNA was extracted from double-heterozygote male and female pupae from crosses between tetO-*dsx*-Nipp1-T and tetO-*dsx*-RprKR-T lines with the pro-tTAV pupal promoter line in order to assess *dsx* splicing. PCR in all lines produced the expected *Ae. aegypti* muscle actin cDNA band at 881 bp and no genomic contamination was detected in any samples. tetO-*dsx*-Nipp1 males had a single amplicon at the expected size of 1603 bp while the female *dsx* exon-4-5b-6 spliceform was observed at 2072 bp and a fainter band at 2530 bp representing the *dsx* exon-4-5a-5b-6 variant, which does not encode functional Nipp1. In tetO-*dsx*-RprKR males, a single band of 656 bp was observed as expected. In females, a strong band at 1125 bp represented the RprKR-encoding female spliceform *dsx* exon-4-5b-6 and a fainter band at 1583 bp corresponds to the *dsx* exon-4-5b-5a-6 spliceform. However, a third faint band was also detected around 1600 - 1800 bp which does not represent an expected splicing pattern in the construct.

The band appeared inconsistently in repeated PCRs so it was not possible to sequence it, suggesting it was at low frequency. Some incomplete or unusual splicing

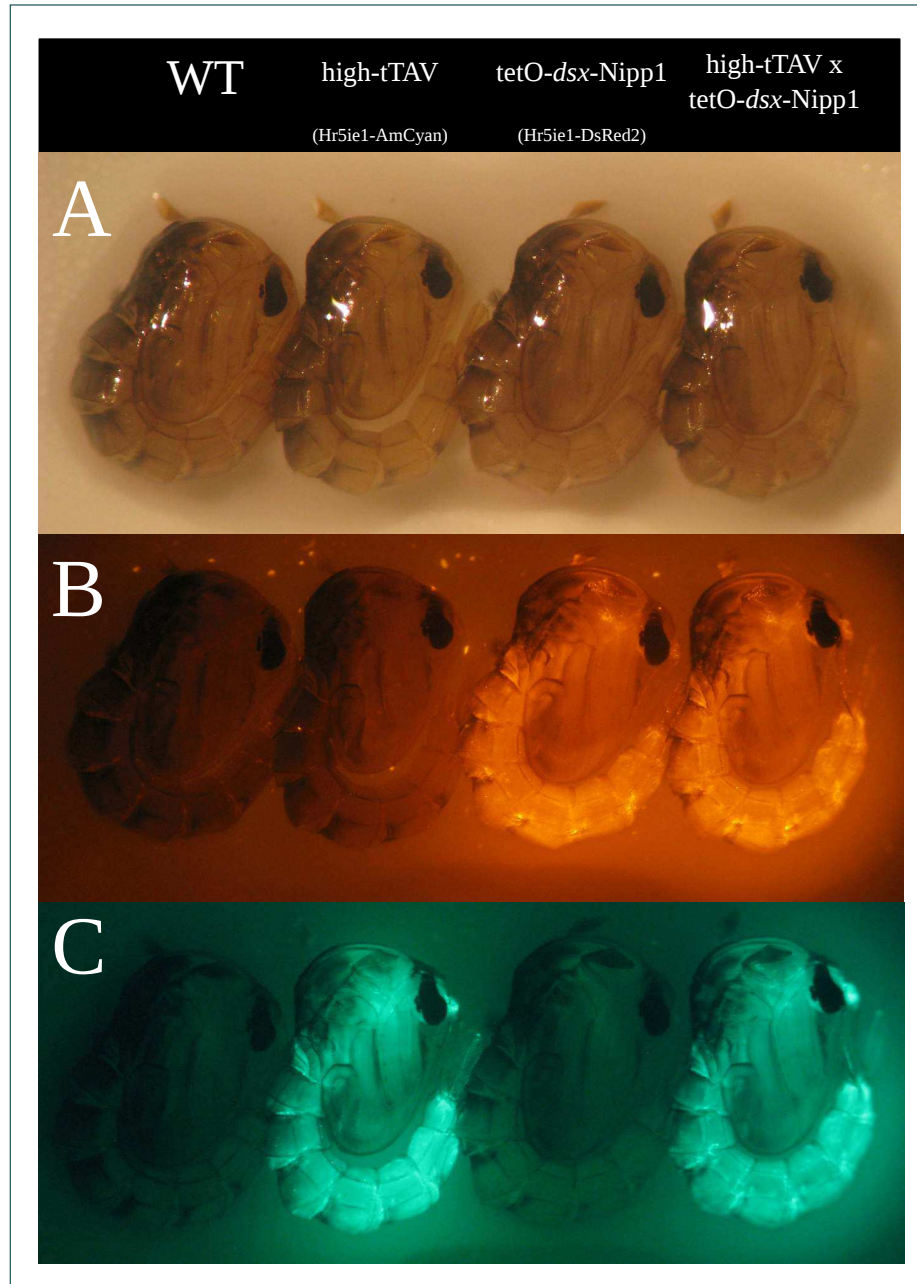


Figure 4.5: Female pupae from a cross between high-tTAV-D and tetO-*dsx*-Nipp1-F heterozygotes reared on-tetracycline demonstrating whole body expression of AmCyan and DsRed2 respectively, under the control of the ubiquitous Hr5ie1 promoter. These lines display a typical expression pattern with diffuse fluorescence spread through all tissues, although other lines differed in intensity of the marker. A) White light. B) Red filter: excitation HQ545/30x; emission HQ620/60m. C) Blue filter: excitation D436/20x; emission D480/40m.

may be expected to occur at low frequency and the use of 35-40 cycles in PCR reactions which would have produced visible bands from very small starting amounts

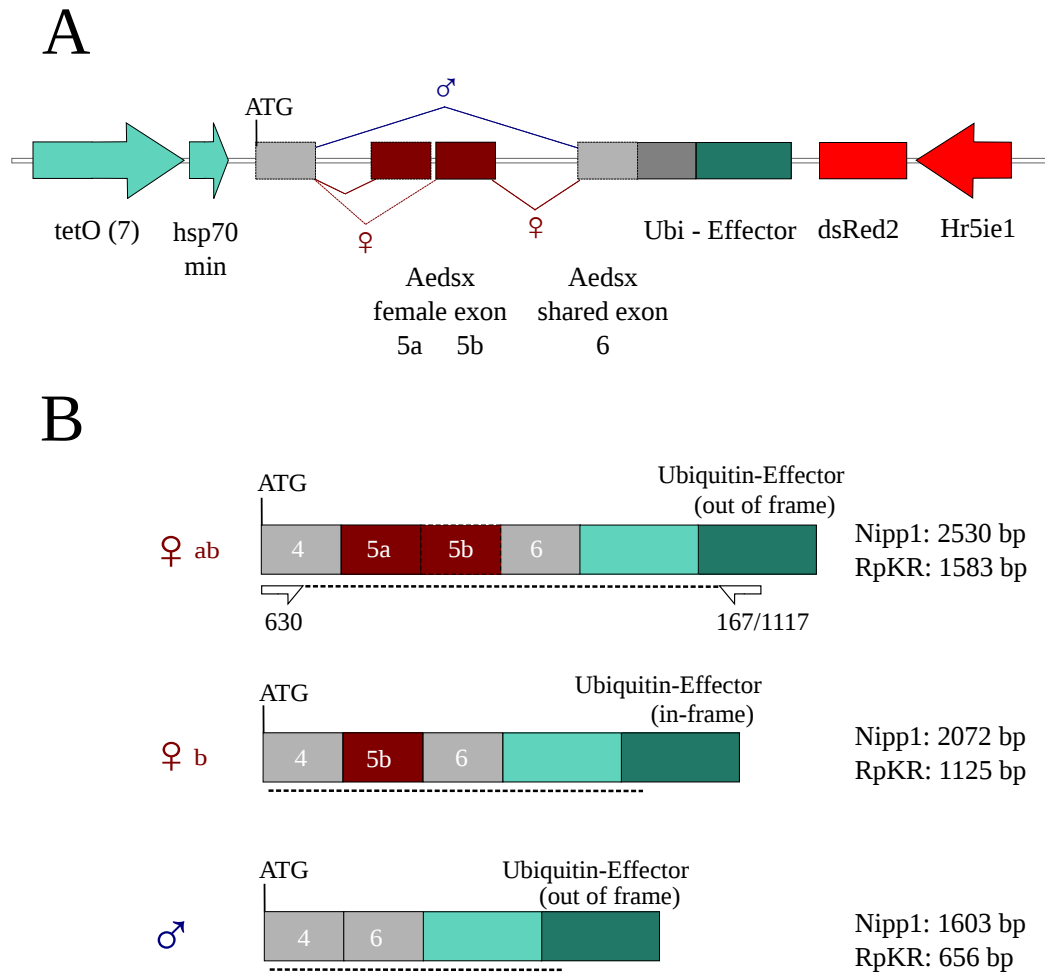


Figure 4.6: *tetO-dsx-Nipp1* and *-RprKR* Construct Design. Both constructs are identical apart from the effector gene. A) The *piggyBac* integrated construct contains a *DsRed2* fluorescent marker driven by the viral-derived *Hr5ie1* promoter, which expresses punctuated red fluorescence across the whole insect body. Expression of the *dsx-Nipp1* or *dsx-RprKR* cassette is driven by the binding of tTAV to the *tetO* operator adjacent to a *hsp70* minimal promoter. *Ae. aegypti dsx* is a mini-gene comprising two shared exons (4 and 6) flanking female specific exons 5a and 5b and separated by shortened introns. An engineered ATG site before shared exon 4 marks the start of translation. Further downstream in the construct is a fusion *Ubiquitin-effector* sequence, which will be cleaved by ubiquitinase at the N-terminus of ubiquitin post-translation to generate a free *Nipp1* or *RprKR* protein. Such ubiquitin fusion proteins can substantially increase expression of cloned gene products *in vivo* (Butt et al., 1989). B) The sex specific splicing pattern of the *Ae. aegypti dsx* mini-gene as described by Salvemini et al. (2011). Expected transcripts in transgenic males and females are shown along with 630 and 167/1117 primer binding sites (white arrows) and expected PCR products (dashed black line). *Nipp1* and *RprKR* are in-frame only in the female *dsx* exon 4-5b-6 transcript so only this spliceform should produce functional *Nipp1/RprKR*.

of primer-bound DNA. Some faint amplicons were also detected in tetO-*dsx*-RprKR and WT gDNA but these did not correspond to any band detected in the cDNA and may have been a result of non-specific binding. The largest band in tetO-*dsx*-RprKR gDNA may represent amplification of the transgenic construct, which is expected to be 4413 bp in unspliced form.

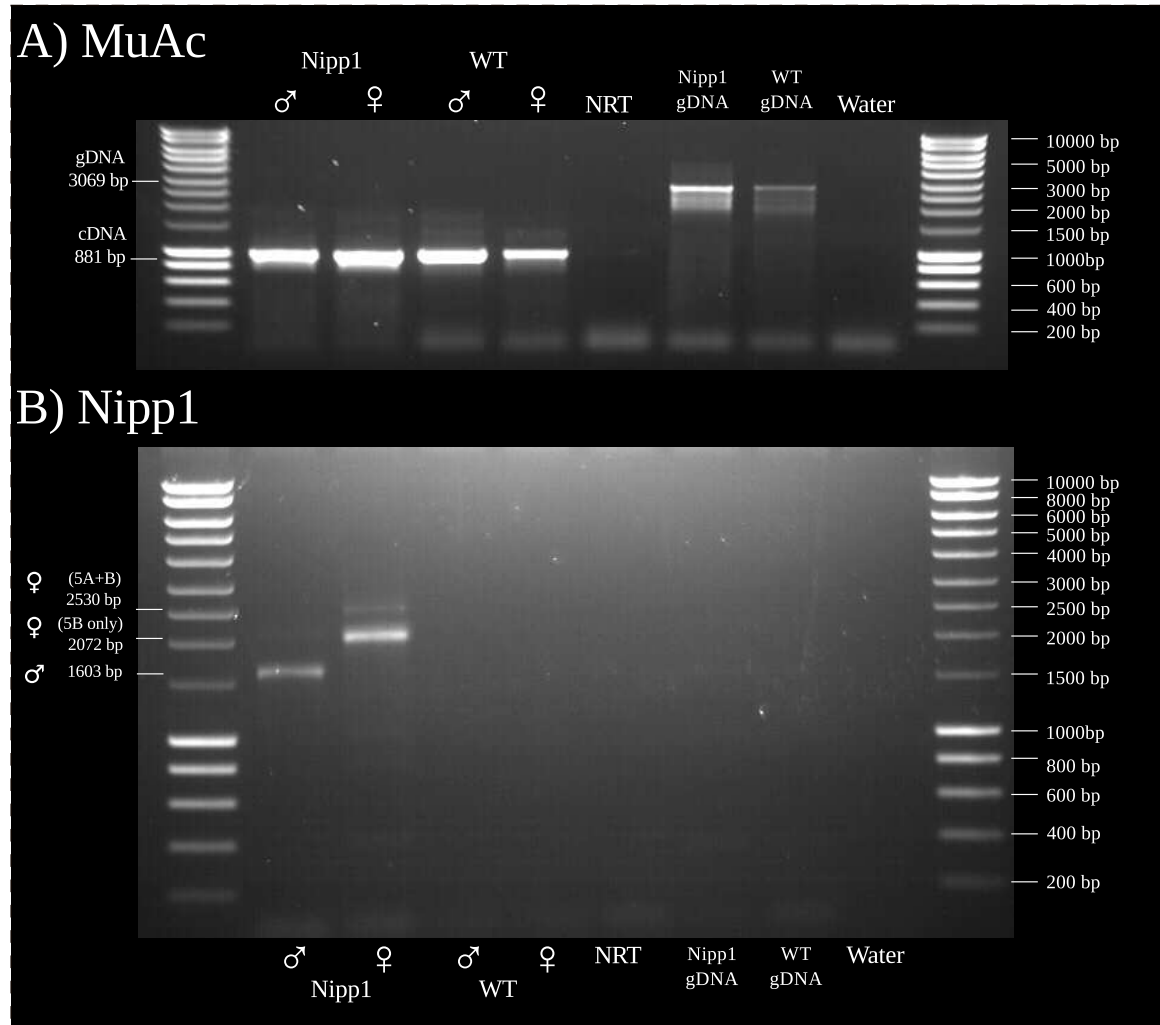


Figure 4.7: Gel electrophoresis of PCR products amplified by *Aedes* muscle actin (MuAc) primers as a positive control gene and 630/167 primers spanning the transgene *dsx* region from *dsx* exon-4 to Nipp1. Samples from left to right: Smart Ladder from Eurogentech 200 bp to 10,000 bp. cDNA from tetO-*dsx*-Nipp1 transgenic male and female pupae, WT male and female pupae. NRT: tetO-*dsx*-Nipp1 female mRNA with no reverse transcriptase included in the cDNA synthesis reaction as a control for gDNA contamination. gDNA from transgenic tetO-*dsx*-Nipp1 and WT as additional control for gDNA contamination, Water: negative control.

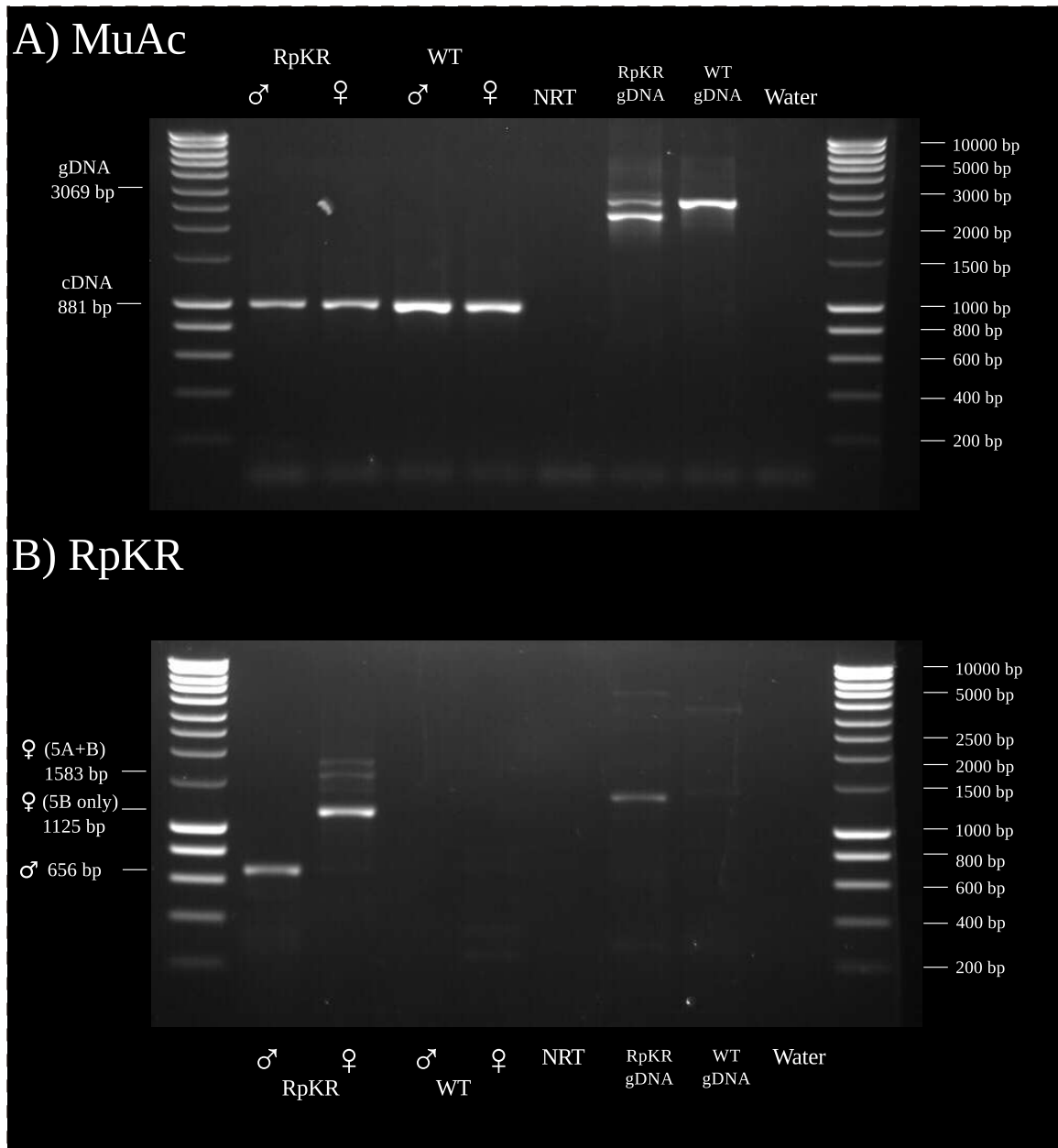


Figure 4.8: Gel electrophoresis of PCR products amplified by *Aedes* muscle actin (MuAc) primers as a positive control gene and 630/1117 primers spanning the transgene *dsx* region from *dsx* exon-4 to RprKR. Samples from left to right: Smart Ladder from Eurogentech 200 bp to 10,000 bp. cDNA from tetO-*dsx*-RprKR transgenic male and female pupae, WT male and female pupae. NRT: tetO-*dsx*-RprKR female mRNA with no reverse transcriptase included in the cDNA synthesis reaction as a control for gDNA contamination. gDNA from transgenic tetO-*dsx*-RprKR and WT as additional control for gDNA contamination, Water: negative control.

4.3.4 Female-lethal assay with tetO-*dsx*-Nipp1 and tetO-*dsx*-RprKR lines

Ten tetO-*dsx*-Nipp1 and two tetO-*dsx*-RprKR effector lines were crossed to pro-tTAV, an osiris promoter line, which previously caused lethality in late pupae or mid-eclosion in bisex-lethal lines (Conway, 2014). Survival to pupation and eclosion was assessed in WT and double-heterozygous transgenic individuals (double-transgenics). Single-transgenics expressing only the promoter or effector were not assessed as these were routinely reared off-tetracycline with no adverse effects (data not shown), but were counted at pupation to ensure that numbers of each genotype corresponded to Hardy-Weinberg equilibrium for a double-heterozygote cross (Figure 4.9). Expected ratios were 25% for each genotype with a 1:1 sex ratio resulting in a 12.5% prevalence for the most significant genotype, female double-heterozygotes. If the effector is expressed at lethal levels, this percentage should reduce off-tetracycline and no female double-heterozygote individuals should eclose if expression is 100% lethal. Due to the late-acting nature of the promoter, it was hypothesised that survival to pupation would be unaffected and lethality would only be apparent at eclosion.

In line with this prediction, survival to pupation (Nipp1: Figure 4.10, RprKR: Figure 4.12) was not affected by tetracycline treatment, sex or transgenic status in the majority of lines except tetO-*dsx*-Nipp1-O2, although data for this line was compromised by low overall survival in the on-tetracycline treatment resulting in exclusion of two replicates with <60% survival from the analysis. There was also an over-representation of males and WT in tetO-*dsx*-RprKR-T and tetO-*dsx*-RprKR-U crosses respectively (Line T-Male, coef = 0.336, $z = 3.653$, $p < 0.001$. Line U-WT, coef = 0.244, $z = 0.0879$, $p < 0.001$). All other lines showed no significant deviance from the null model (GLM with Poisson family distribution; significance taken as ANOVA χ^2 $p < 0.01$ during stepwise model fitting to account for multiple comparisons (Crawley, 2014)).

Eclosion was assessed as percentage of fully eclosed pupae and three lines showed significant lethality in female double-heterozygotes reared off-tetracycline: tetO-*dsx*-Nipp1-D, tetO-*dsx*-Nipp1-F and tetO-*dsx*-Nipp1-T (Figure 4.11). Three-way interactions were significant for all lines (Line D: coef = -2.343, $z = -3.722$, $p < 0.001$. Line F: coef = -0.000, $z = -3.722$, $p < 0.001$. Line T: coef = -2.343, $z = -3.722$, $p < 0.001$). The specific conditions of interest (female, double transgenic, off-tetracycline) were confirmed to be the only significant combination within this three-way interaction



Figure 4.9: Proportions of each genotype expected to eclose from offspring of cross between heterozygote promoter and effector lines driving lethal gene expression via a tTAV positive feedback loop in double-heterozygote individuals. Gene inheritance is expected to conform to Hardy-Weinberg equilibrium and population sex ratio is expected to be 1:1. The only genotype proportion expected to exhibit a reduction off-tetracycline is double-heterozygote female.

term using post-hoc testing with both pairwise contrasts between all factors and a cell means model, based on a new factor derived from the interaction between type, treatment and sex (Line D: Pairwise contrasts: $df = 1$, $\chi^2 = 15.494$, $p < 0.001$. Cell-means: Off:Nipp1:F, $df = 1$, $\chi^2 = 23.072$, $p < 0.001$. Line F: Pairwise contrasts: $df = 1$, $\chi^2 = 12.118$, $p < 0.001$. Cell-means: Off:Nipp1:F, $df = 1$, $\chi^2 = 32.0622$, $p < 0.001$. Line T: Pairwise contrasts: $df = 1$, $\chi^2 = 13.85$, $p < 0.001$. Cell-means: Off:Nipp1:F, $df = 1$, $\chi^2 = 28.4551$, $p < 0.001$. All other interactions in all lines: $df=1$, $\chi^2 < 0.2$, $p = 1$. All p-values reported are adjusted using the holm method).

The tetO-*dsx*-Nipp1-E2 line had mean 44.8% mortality in off-tetracycline female double-heterozygotes but this was not statistically significant due to high variability

between replicates. However, the lethal phenotype was consistent with that seen in line tetO-*dsx*-Nipp1-D and -F. All other lines showed no deviance from the null model (GLM with binomial family distribution, ANOVA χ^2 $p < 0.01$).

In all cases death occurred very late during pupation or mid-eclosion. No lines demonstrated 100% lethality and those individuals which did successfully eclose appeared to be able to walk and fly but their viability, longevity and fecundity was not assessed in this assay so it is unclear if fitness is affected post-eclosion. This data is in line with other work on the pro-tTAV promoter line (Conway, 2014) which appears to be highly expressed in the legs of the developing adult. A commonly observed sub-lethal effect was that legs remained stuck in the pupal case upon eclosion at a higher rate than observed for individuals reared on-tetracycline.

4.3.5 Female-lethal assay with low-, mid- and high-expression pupal promoter lines

The tetO-*dsx*-Nipp1-T line, which displayed a partially penetrant lethal phenotype in double-heterozygote females when crossed to the pro-tTAV line was next crossed to the putative pupal promoter lines generated to see if this lethal phenotype could be replicated and ideally improved.

In the putatively high pupal expression high-tTAV lines, transgene, treatment and sex had no significant effect on adult eclosion from pupae in any lines (Figure 4.16. GLM with binomial family distribution fitted step-wise and tested against null model. ANOVA, $p < 0.01$) but there were factors affecting survival to pupation in three lines: high-tTAV-G2, high-tTAV-C1 and high-tTAV-D (Figure 4.15. GLM with Poisson family distribution fitted step-wise and tested against null model, ANOVA, $p < 0.01$). In high-tTAV-G2 the interaction between type and treatment was significant, suggesting that tetracycline treatment differentially affected WT and transgenic pupation regardless of sex (coef = 0.452, $z = 2.714$, $p < 0.01$). Survival to pupation in transgenic lines was slightly lower than WT on-tetracycline and higher off-tetracycline (Figure 4.15). Crosses with high-tTAV-C1 and high-tTAV-D resulted in more WT pupae across all sexes and treatments than expected (high-tTAV-C1: coef = 0.296, $z = 3.520$, $p < 0.001$. high-tTAV-D: coef = 0.258, $z = 2.721$, $p < 0.01$. high-tTAV-M: coef = 0.314, $z = 3.665$, $p < 0.001$).

In mid-tTAV, the predicted mid-expression pupal promoter lines, no factors or interaction terms provided a significantly better fit to the data than the null model for

Survival to pupation on- and off-tetracycline in
WT vs *pro-tTAV* x *tetO-dsx-Nipp1*

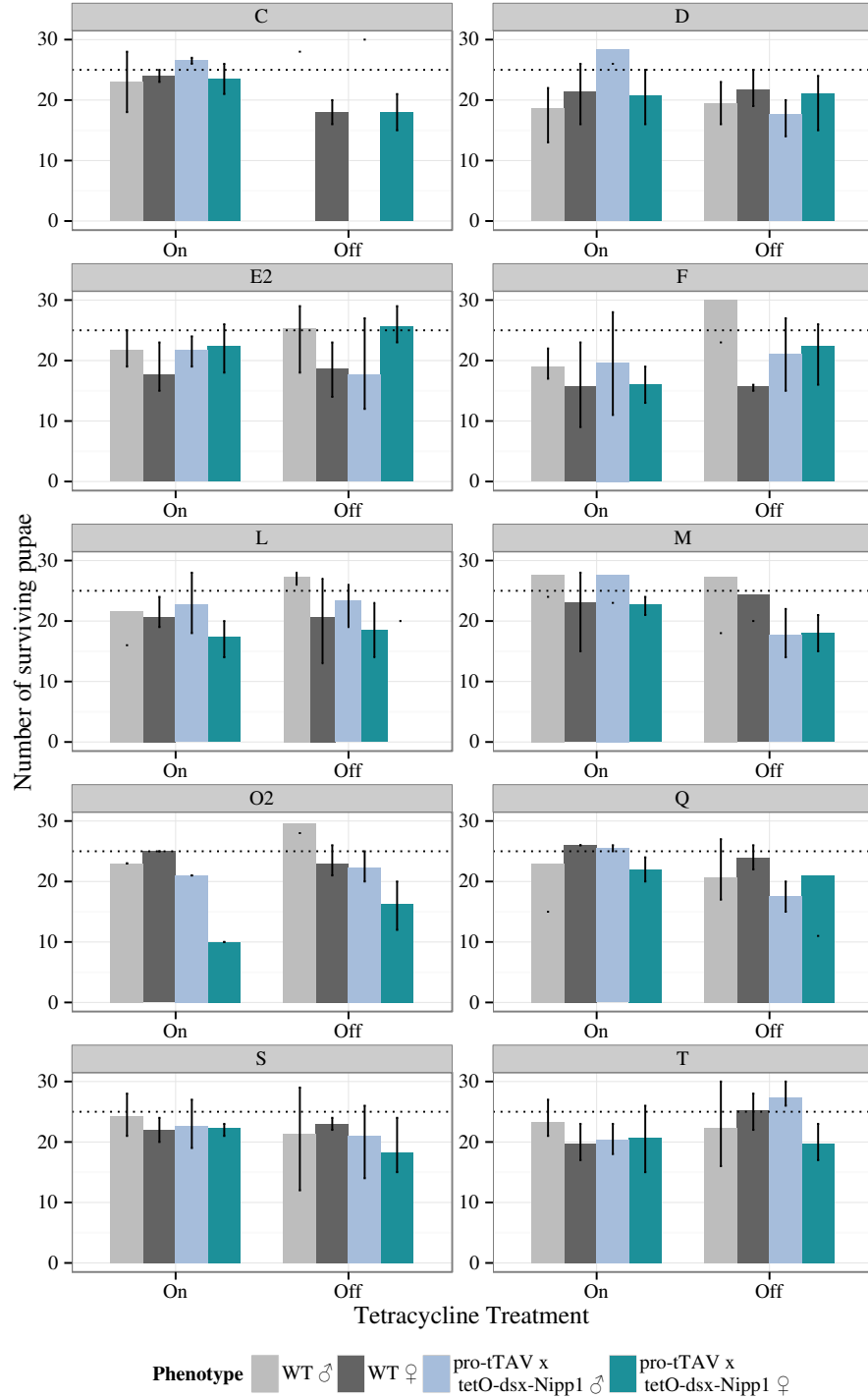


Figure 4.10: Count data for WT and double-heterozygote pupal survival on and off-tetracycline in *tetO-dsx-Nipp1* x *pro-tTAV* crosses. Bars show mean of three biological replicates with error bars indicating the range of results obtained. Dotted line indicates theoretical expected count assuming 100% survival, Hardy-Weinberg equilibrium and 1:1 sex ratio in the starting sample of 200 larvae.

Eclosion on- and off-tetracycline in
WT vs *pro-tTAV* x *tetO-dsx-Nipp1*

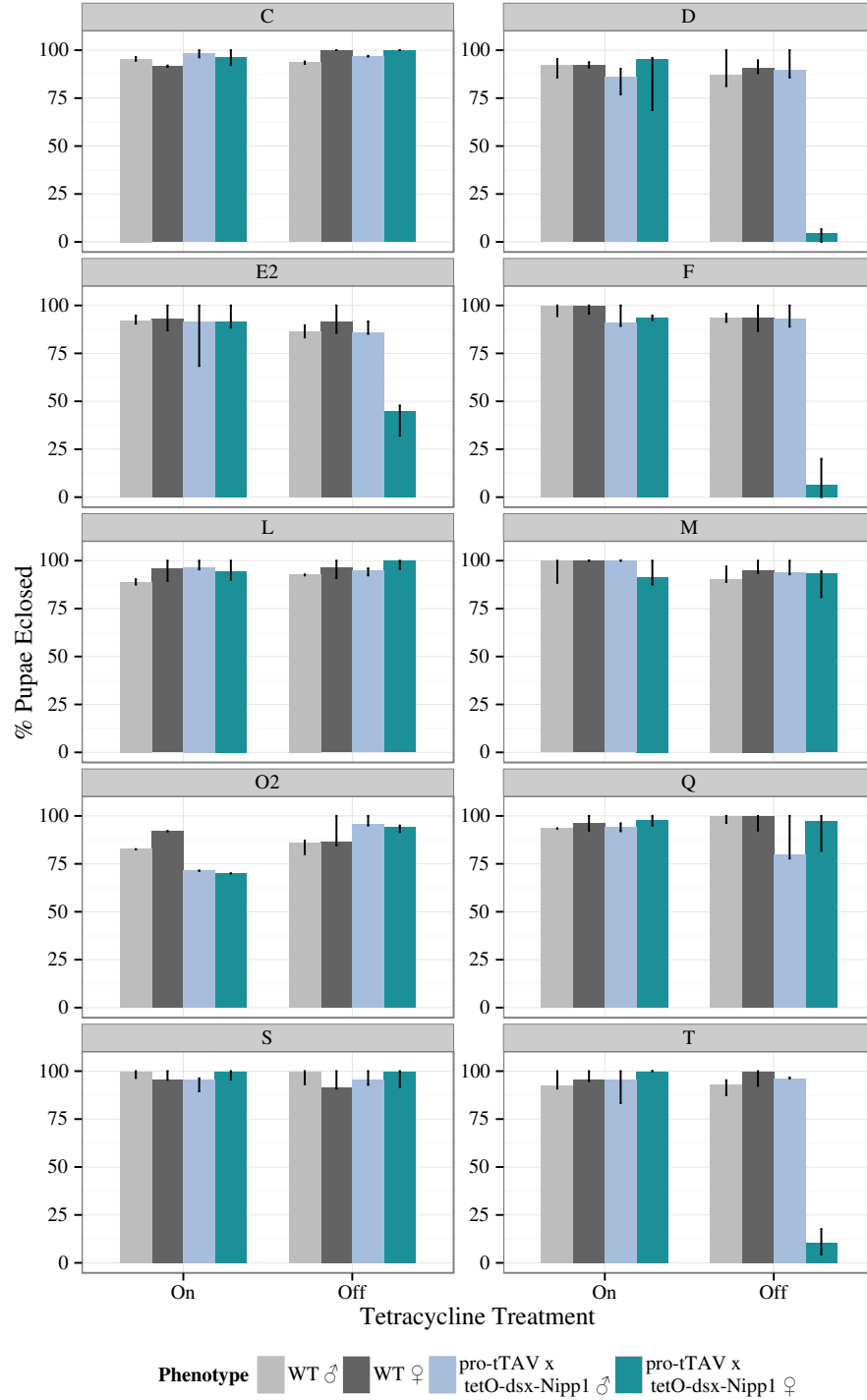


Figure 4.11: Proportion of WT and double-heterozygote adults eclosing from pupae of indicated phenotype on and off-tetracycline in *tetO-dsx-Nipp1* x *pro-tTAV* crosses. Bars show mean percentage eclosion across three biological replicates with error bars indicating range of values obtained.

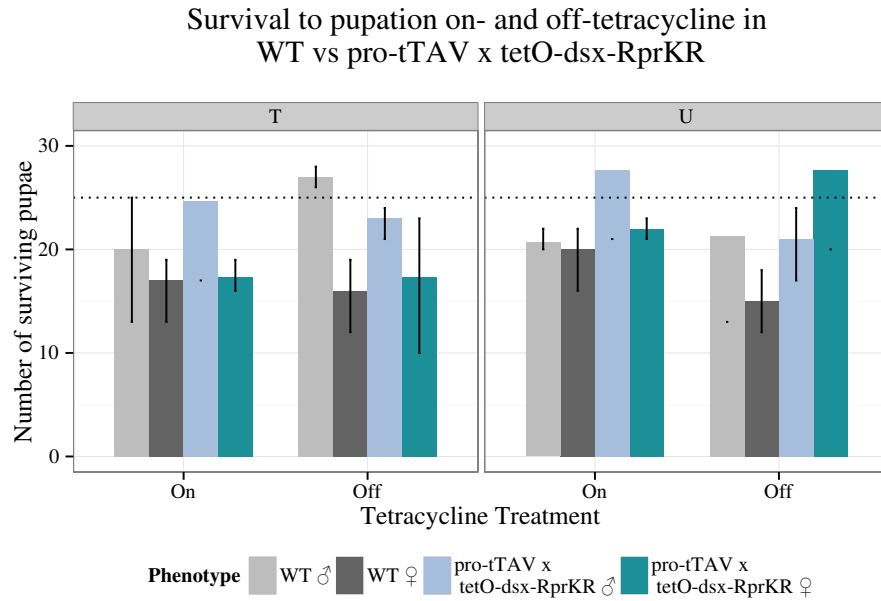


Figure 4.12: Count data for WT and double-heterozygote pupal survival on and off-tetracycline in *tetO-dsx-RprKR* x *pro-tTAV* crosses. Bars show mean of three biological replicates with error bars indicating the range of results obtained. Dotted line indicates theoretical expected count assuming 100% survival, Hardy-Weinberg equilibrium and 1:1 sex ratio in the starting sample of 200 larvae.

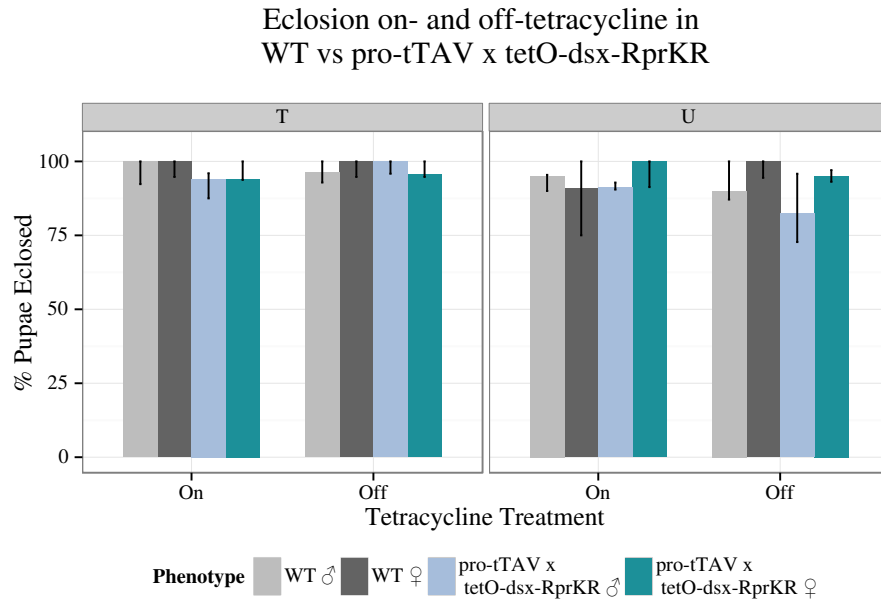


Figure 4.13: Proportion of WT and double-heterozygote adults eclosing from pupae of indicated phenotype on and off-tetracycline in *tetO-dsx-RprKR* x *pro-tTAV* crosses. Bars show mean percentage eclosion across three biological replicates with error bars indicating range of values obtained.



Figure 4.14: Illustration of lethal phenotype in double-heterozygote *tetO-dsx-Nipp1-F* x *pro-tTAV* females reared off-tetracycline. Death during this stage of eclosion is typical of the phenotype observed in all *tetO-dsx-Nipp1* lines demonstrating lethality.

pupal survival or eclosion (Figures 4.17 and 4.18). However, mid-tTAV-C3 and mid-tTAV-C2 crosses both had replicates containing double-heterozygote females reared off-tetracycline that died mid-eclosion, the same lethal phenotypes observed in effective crosses between *tetO-dsx-Nipp1* and *pro-tTAV* lines (Figure 4.14). mid-tTAV-C3 in particular had a mean survival from pupation to eclosion of only 54% but due to the range from 31-82% this was not statistically significant and is not functionally significant for the purposes of a genetic sexing system (GSS). However, it indicates that the mid-Pro promoter region drives pupal expression as anticipated.

In crosses using low-tTAV, the predicted low-expression pupal promoter lines, there was no effect of any factor on survival to pupation (Figure 4.19) except in low-tTAV-G, where low overall survival in the on-tetracycline treatment led to treatment being an explanatory factor in the fitted single factor model (coef = -0.378, $z = -3.529$, $p < 0.001$). Proportion of pupae eclosing showed no deviation from the null model in any lines (Figure 4.20).

4.3.6 Pupal-specific expression driven by candidate promoters

Two promoter lines tested did not drive sufficient expression of tTAV to cause pupal-stage death in combination with the *tetO-dsx-Nipp1* construct. This could be due to several factors:

Survival to pupation on- and off-tetracycline in
WT vs high-tTAV x tetO-dsx-Nipp1

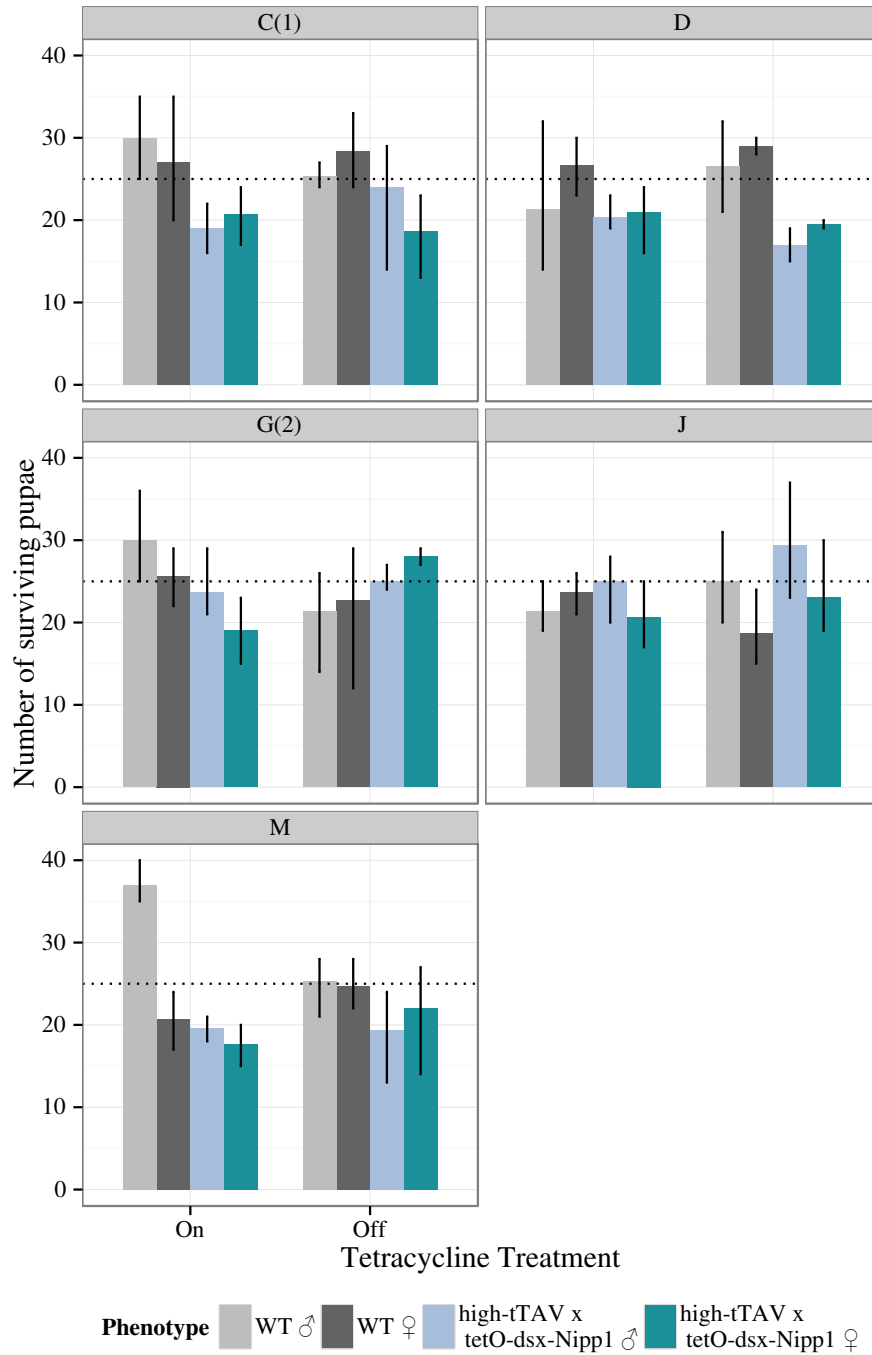


Figure 4.15: Count data for pupal survival on and off-tetracycline of offspring from crosses between heterozygous tetO-*dsx*-Nipp1 and high-tTAV lines. Bars show mean of three biological replicates with error bars indicating the range of results obtained. Dotted line indicates theoretical expected count assuming 100% survival, Hardy-Weinberg equilibrium and 1:1 sex ratio in the starting sample of 200 larvae.

Eclosion on- and off-tetracycline in WT vs high-tTAV x tetO-dsx-Nipp1

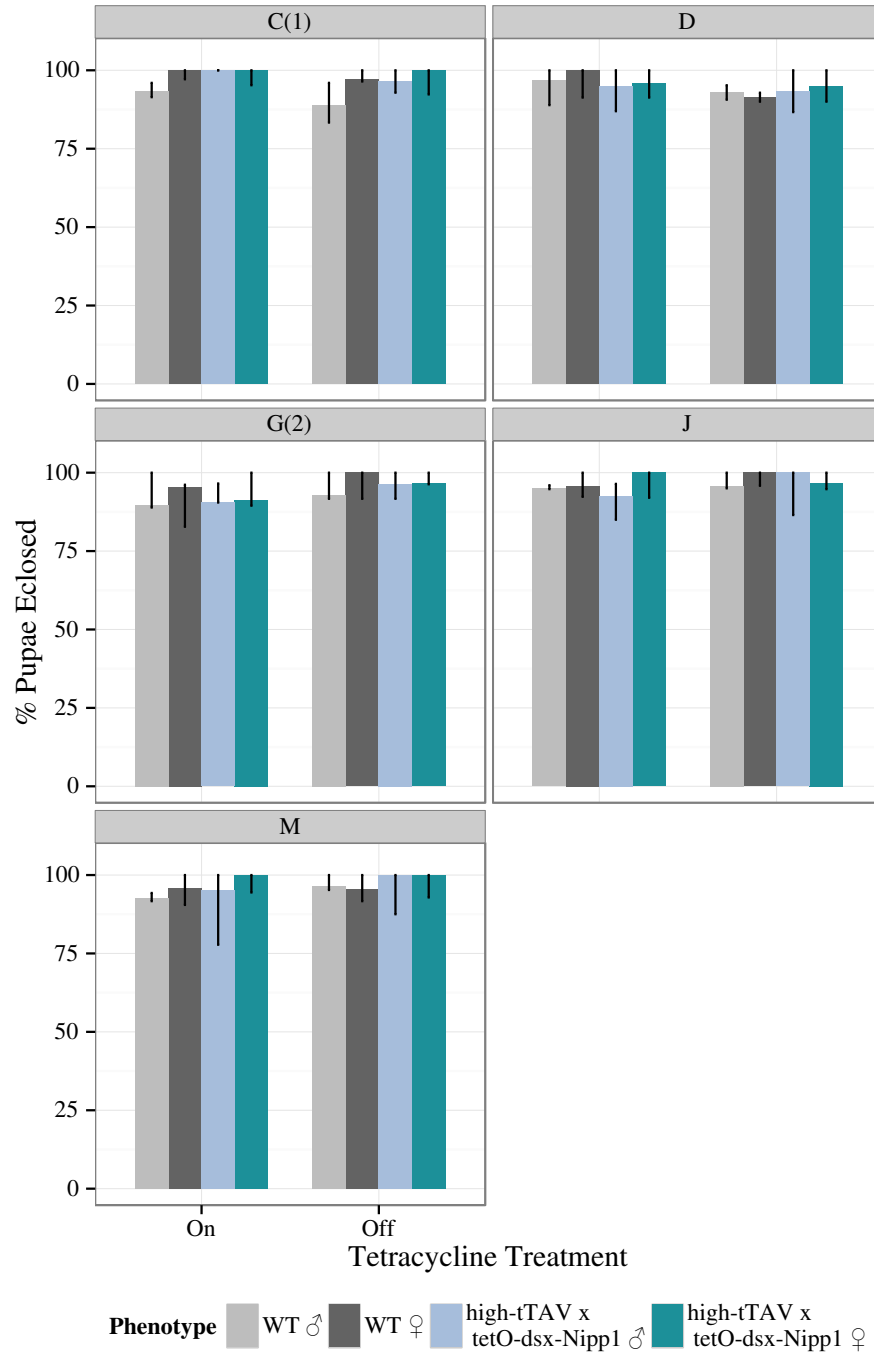


Figure 4.16: Proportion of adults eclosing from pupae on and off-tetracycline in tetO-*dsx*-Nipp1 x high-tTAV line crosses. Bars show mean percentage eclosion across three biological replicates with error bars indicating range of values obtained.

Survival to pupation on- and off-tetracycline in WT vs mid-tTAV x tetO-dsx-Nipp1

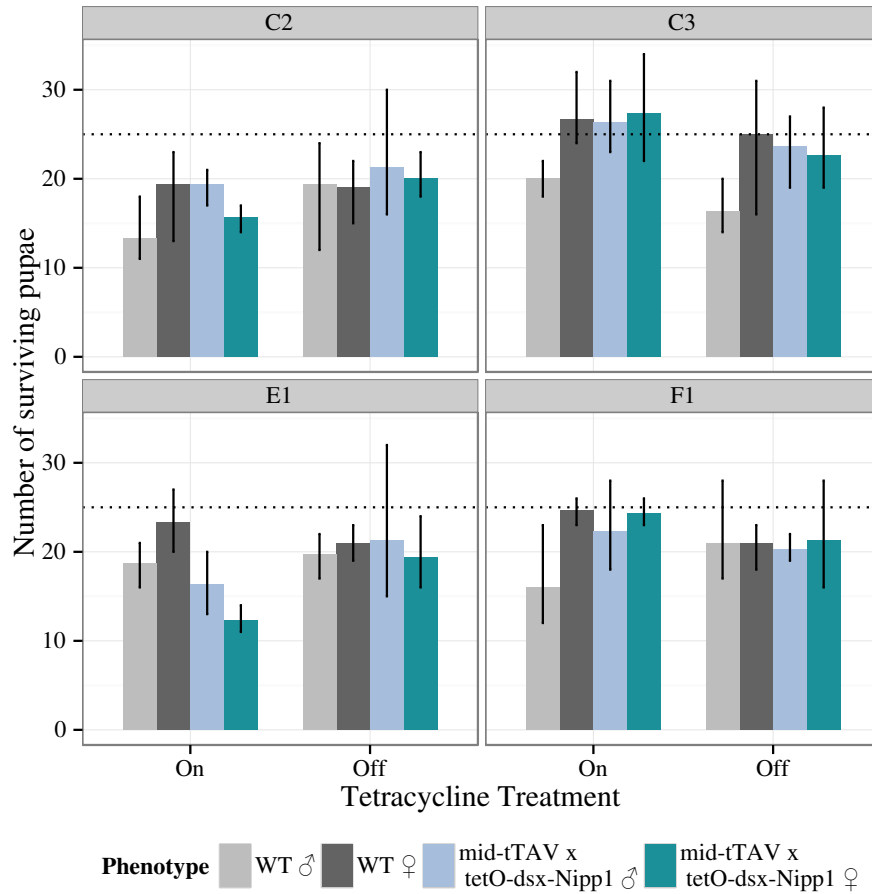


Figure 4.17: Count data for pupal survival on and off-tetracycline of offspring from crosses between heterozygous *tetO-dsx-Nipp1* and mid-tTAV high-expression pupal promoter lines. Bars show mean of three biological replicates with error bars indicating the range of results obtained. Dotted line indicates theoretical expected count assuming 100% survival, Hardy-Weinberg equilibrium and 1:1 sex ratio in the starting sample of 200 larvae.

1. The expression profile observed in data from Akbari et al. (2013a) is different in the WT strain used for these experiments.
2. Expression occurs for too short a time period or in the wrong tissues for effector-induced mortality.
3. The promoter sequence used is lacking some necessary control elements from the endogenous location e.g. a TFBS further upstream. Gene expression may also be dependent on enhancers or other more distant cis-regulatory elements.

Eclosion on- and off-tetracycline in WT vs mid-tTAV x tetO-dsx-Nipp1

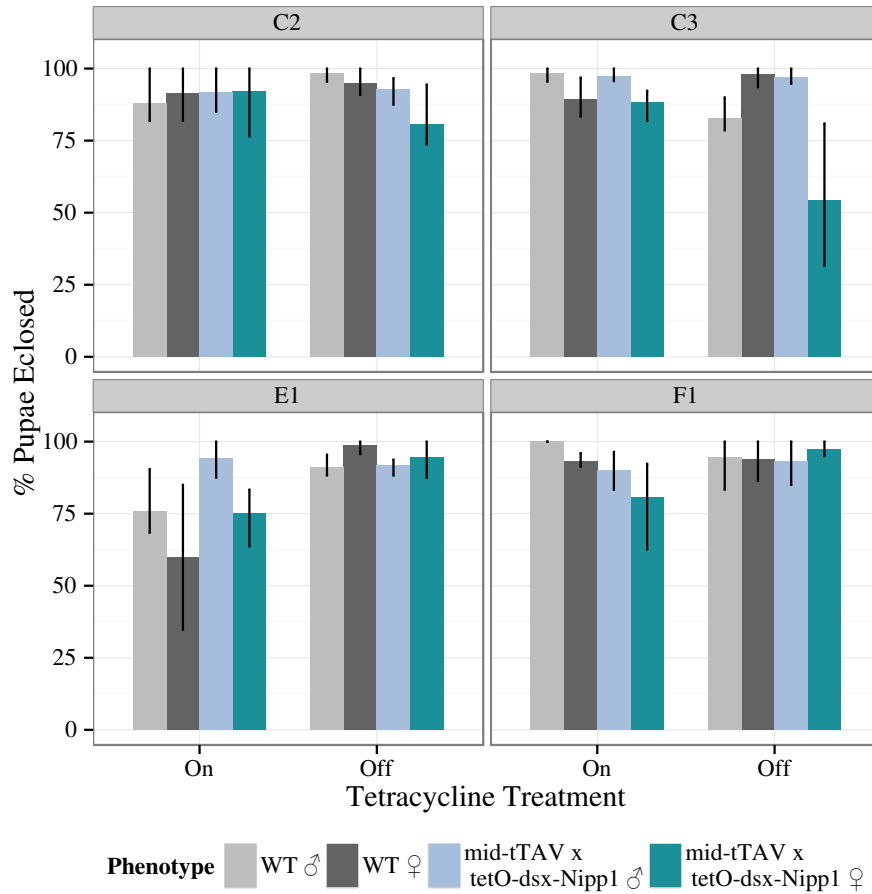


Figure 4.18: Proportion of adults eclosing from pupae on and off-tetracycline in *tetO-dsx-Nipp1* x mid-tTAV line crosses. Bars show mean percentage eclosion across three biological replicates with error bars indicating range of values obtained.

4. Position effects as a result of the construct's genomic insertion site constrain expression.

To address the first point, pupal-specific expression of the endogenous genes under the control of the chosen promoter was validated in the WT strain used in this study by PCR at various developmental stages (Figure 4.21). The muscle actin control gene (MuAc) was amplified in all stages except L1 larvae. The putative mid-expression gene mid-Pro, whose promoter gave a partially lethal phenotype in some mid-tTAV transformed lines, was strongly expressed in 6-18 h old pupae and at very low levels in 24-36 h pupae becoming undetectable by 48 h. This is in line with expression

Survival to pupation on- and off-tetracycline in WT vs low-tTAV x tetO-dsx-Nipp1

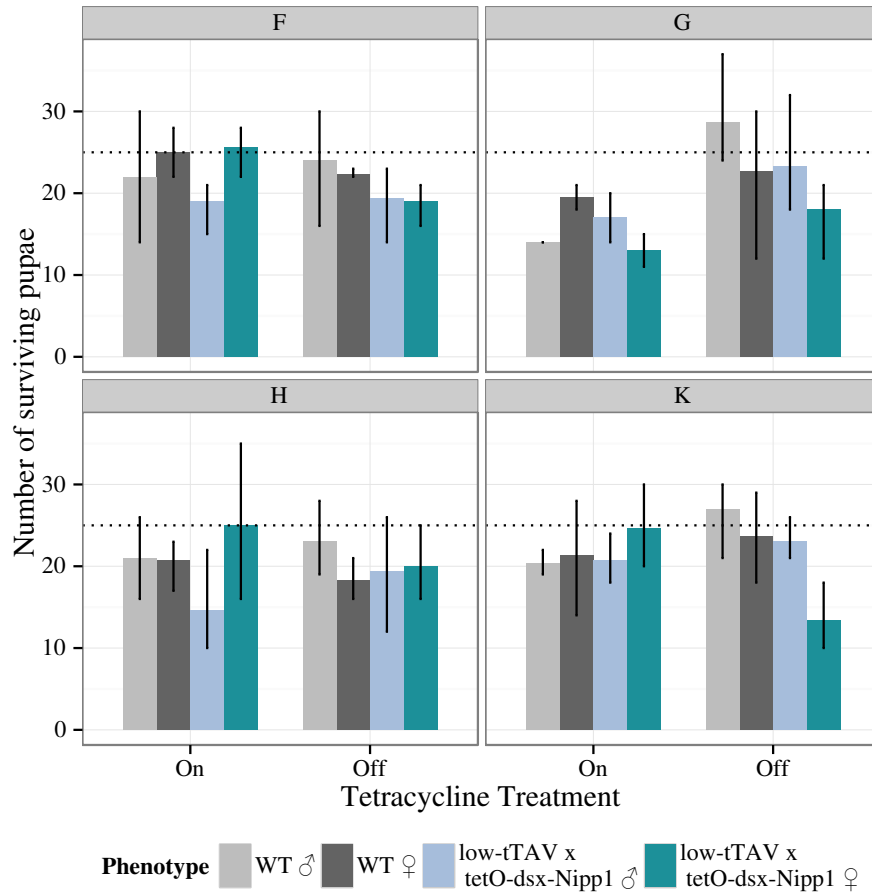


Figure 4.19: Count data for pupal survival on and off-tetracycline of offspring from crosses between heterozygous *tetO-dsx-Nipp1* and low-tTAV lines. Bars show mean of three biological replicates with error bars indicating the range of results obtained. Dotted line indicates theoretical expected count assuming 100% survival, Hardy-Weinberg equilibrium and 1:1 sex ratio in the starting sample of 200 larvae.

profiles for other osiris genes (Conway (2014) and Section 4.3.1). The high-Pro gene was only weakly expressed with a peak at 6-18 h and then again at 36 h, although fainter bands were more difficult to distinguish due to the short PCR product being very close to primer dimer bands. This short product was necessitated by a lack of primer binding sites available in the short 450 bp mRNA sequence. The low-Pro gene was only observed at 12 h and 36 h and bands were again very faint suggesting low expression given the 35 PCR cycles employed. The expression profile of the latter two genes is consistent with cuticle proteins but the strength of expression does not

Eclosion on- and off-tetracycline in WT vs low-tTAV x tetO-dsx-Nipp1

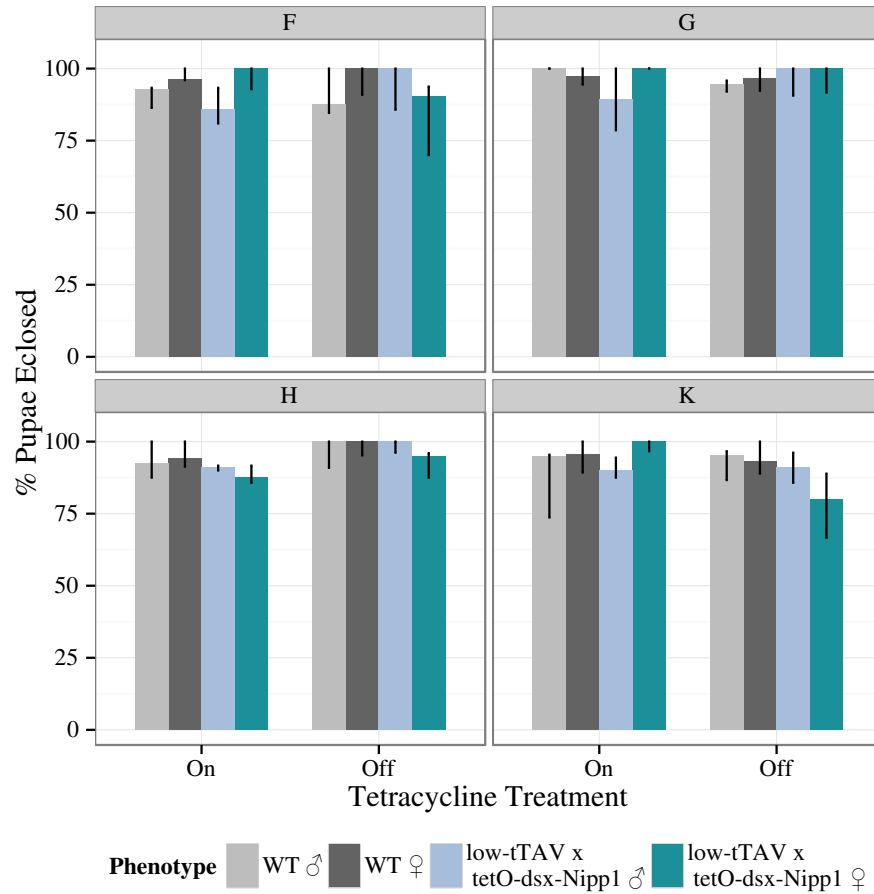


Figure 4.20: Proportion of adults eclosing from pupae on and off-tetracycline in *tetO-dsx-Nipp1* x low-tTAV line crosses. Bars show mean percentage eclosion across three biological replicates with error bars indicating range of values obtained.

reflect predictions from the Akbari et al. (2013a) dataset (Figure 4.21), highlighting the need to validate high-throughput data in experimental strains *a priori* in future.

To address the question of tissue-specific expression, 2-3 lines containing each construct were crossed to the OX3978-D marker line (*tetO-AmCyan*), which expresses an AmCyan marker under the control of a *tetO* operator and *hsp70* minimal promoter element. In mosquitoes reared off-tetracycline, bright blue fluorescence should therefore be observed in tissues where the putative pupal promoter is actively driving tTAV expression.

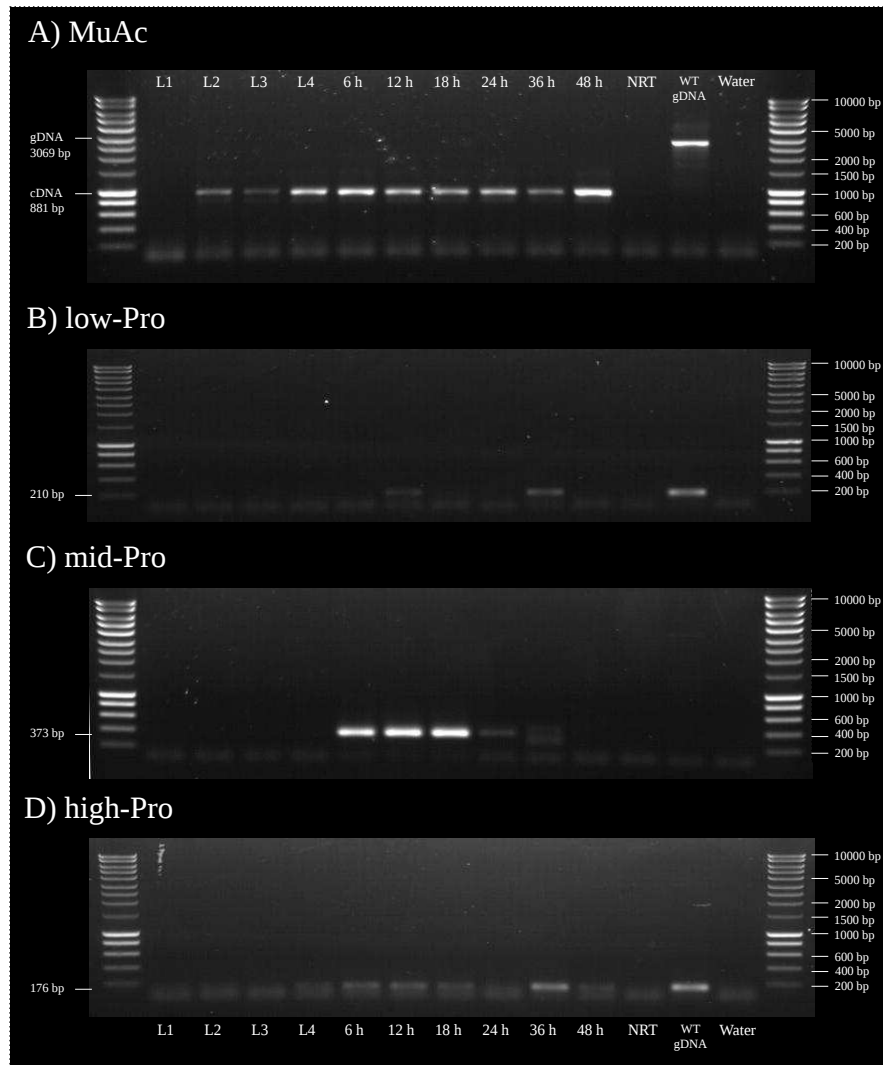


Figure 4.21: Gel electrophoresis image of PCR products from amplification of *MuAc* control gene and three putative pupal-specific genes low-, mid- and high-Pro. Samples from left to right: Smart Ladder from Eurogentech 200 bp to 10,000 bp. cDNA from L1, L2, L3 and L4 larvae. Female pupae at 6 h, 12 h, 18 h, 24 h, 36 h and 48 h. NRT: 24 h pupae mRNA with no reverse transcriptase included in the cDNA synthesis reaction as a control for gDNA contamination. gDNA as additional control for gDNA contamination, Water: negative control.

Neither high-tTAV-D nor high-tTAV-G crosses demonstrated AmCyan marker expression at any larval or pupal-stage. Low-tTAV-K reared off-tetracycline showed some fluorescent structures in the head at L4 larvae stage and faint flecks of fluorescence in pupae, consistent with sites of cuticle synthesis (Figure 4.22), but no such expression was observed in low-tTAV-H.

The mid-tTAV-C2 cross demonstrated AmCyan marker expression in a range of tissues and much fainter expression in the same pattern was observed in mid-tTAV-F but not mid-tTAV-E. The outline of the developing wing veins and structures such as legs and antennae was observed from 24 h to 36 h (Figure 4.23). Fluorescence in females after 36 h was only observable in the adult abdomen as at 48 h pupae were almost completely melanised, but in adult males expression was also observed in the antennae. This time series supports the mRNA expression pattern observed in Figure 4.21C.

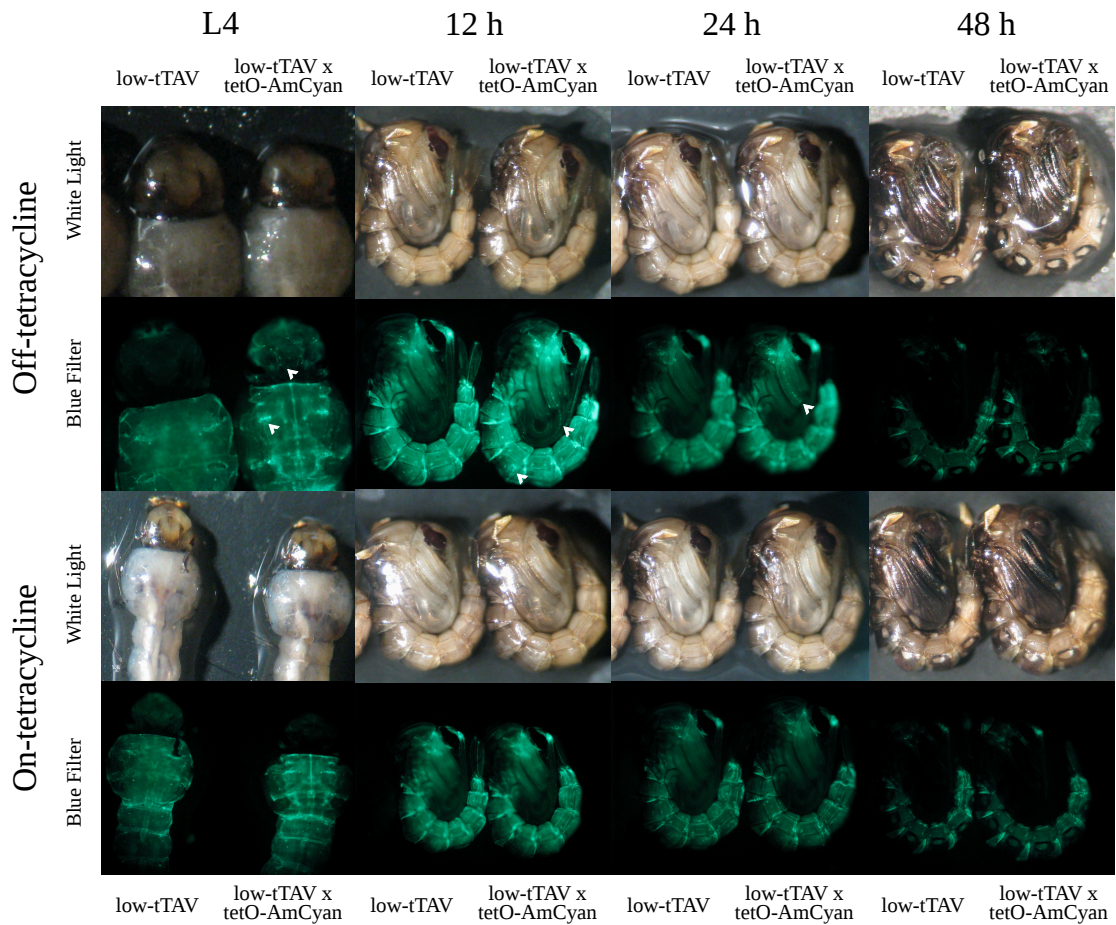


Figure 4.22: Photos under white light and blue fluorescent filter of transgenic larvae and pupae reared on and off-tetracycline. Left: low-tTAV-K transgenic mosquitoes expressing AmCyan under the control of a ubiquitous Hr5ie1 whole body promoter and tTAV driven by the promoter of the low-Pro gene. Right: Double transgenics in addition expressing AmCyan under a minimal tetO promoter, resulting in high expression of AmCyan off-tetracycline in the same pattern as tTAV expression. White arrows mark areas of AmCyan expression found only in the off-tetracycline double-heterozygote individuals.

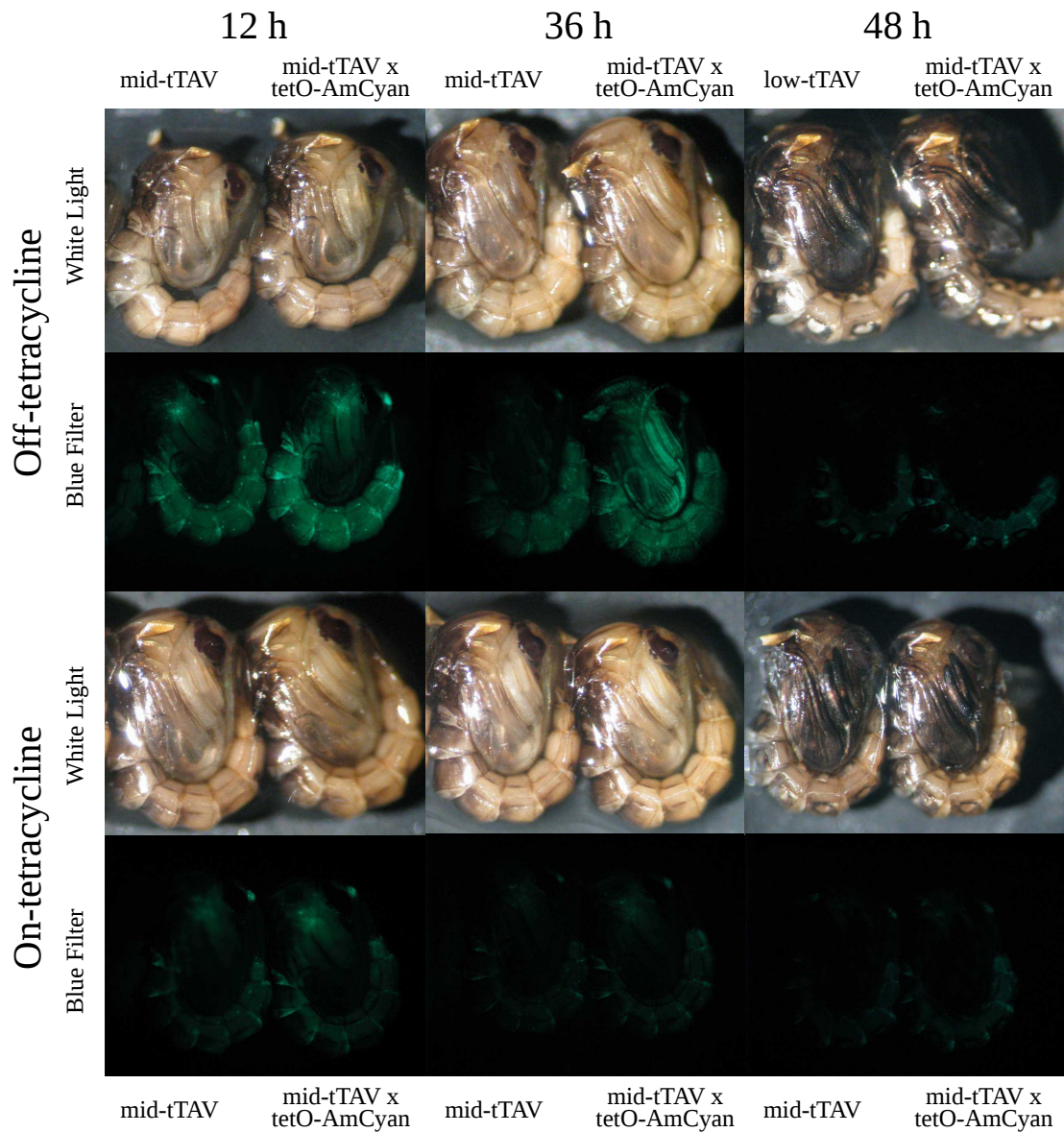


Figure 4.23: Photos under white light and blue fluorescent filter of transgenic larvae and pupae reared on and off-tetracycline. Left: mid-tTAV-C2 transgenic mosquitoes expressing AmCyan under the control of a ubiquitous Hr5iel whole body promoter and tTAV driven by the promoter of the mid-Pro gene. Right: Double transgenics in addition expressing AmCyan under a minimal tet-O promoter, resulting in high expression of AmCyan off-tetracycline in the same pattern as tTAV expression.

4.4 Discussion

4.4.1 Female-lethal *dsx*-effector systems

The results above demonstrate that tetO-*dsx*-effector constructs produce female specific expression of functional effector protein which, in the case of three Nipp1 expressing lines tested, can cause female-specific lethality off-tetracycline when coupled to a suitable promoter driving tTAV expression. No such phenotype was observed in lines expressing RprKR although splicing was almost identical to the tetO-*dsx*-Nipp1 construct and RprKR-induced lethality has been demonstrated previously in *Ae. aegypti*. As only two lines were tested it is likely that low expression was the cause of this failure and a stronger promoter, more favourable genomic position or other modification could result in a lethal phenotype. Potential variability in the effectiveness of constructs at different genomic positions was highlighted by the failure of seven further tetO-*dsx*-Nipp1 lines to induce lethality despite containing identical constructs and being crossed to the same promoter line.

Functional protein expression in tetO-*dsx*-effector systems is diluted by the non-functional female spliceform and also relies on availability of the *dsx*-splicing protein, although this is unlikely to be a limiting factor as no unspliced transcript was found here or by Collado (2013). Attenuation of lethality by non-functional spliceforms has been noted in systems using the *C. capitata tra* intron and was proposed as a potential reason for failure of *dsx-DmAdh* transgenic lines engineered by Collado (2013). The in-frame transcript was the most abundant based on PCR band strength, lending support to this construct design decision. It would be possible to re-engineer the spliceforms such that both female transcripts produce an open reading frame, but these results suggest that focusing on finding a more suitable promoter to drive tTAV would be most advantageous.

4.4.2 Pupal stage promoters in *Ae. aegypti*

An osiris gene *Osi21* orthologue (mid-Pro, AAEL004788) promoter was identified which drives pupal specific expression in similar tissues to the *Ae. aegypti* osiris gene promoter trialled by Conway (2014). The pattern of AmCyan fluorescent marker expression driven by tTAV under the control of this promoter is consistent with the expression of several endogenous osiris genes from other Dipteran species in non-neuronal ectoderm-derived tissues, as described by Shah et al. (2012), and in the

developing *D. melanogaster* wing, where Ren et al. (2005) detected >50-fold increase in several osiris genes in 24 h to 32 h old pupae. While lethality in double-heterozygote females reared off-tetracycline was not statistically significant based on the models used for analysis, the 30 - 81% of mosquitoes dying mid-eclosion suggests that this promoter is still a candidate for further development.

The high-tTAV and low-tTAV lines failed to result in lethality when driving expression of a tetO-*dsx-Nipp1* cassette. The strong expression pattern of these genes predicted from the Akbari et al. (2013a) transcriptome dataset was not reflected in PCR analysis of the WT strain used in this study, particularly in the case of high-Pro, which was expected to exhibit much higher expression than mid-Pro. Low expression peaking late in pupation, when extensive melanisation is already occurring, helps to explain the lack of observed AmCyan expression in marker line crosses as well as the absence of a lethal phenotype. The endogenous and transgenic expression observed under these promoters did appear to be pupal and late larval specific as expected. The high-Pro gene demonstrated expression in L4 larvae in PCR and the low-Pro promoter drove L4 AmCyan marker expression in crosses involving line low-tTAV-K, which also demonstrated non-significant but indicative lethality in double-heterozygote females reared off-tetracycline prior to pupation. Neither of these promoters will be taken forward for further testing or development.

These results demonstrate that validation of promoter candidates from high throughput transcriptome datasets is essential to corroborate expression patterns in the target strain, even where strong support is provided by datasets for orthologues in related species. This is likely especially important in datasets without biological replicates such as Akbari et al. (2013a). In the absence of further replicates, reanalysis using the increasingly sophisticated statistical methods developed for RNA-Seq may also have been helpful to provide better support for strong differential regulation between samples. For example, the Cufflinks normalisation method used by the authors to produce FPKM values and later to select candidate germline regulatory elements (Akbari et al., 2014) has since been updated to account for different sources of variation at the gene- and transcript level when calculating differential regulation (Trapnell et al., 2013).

Where pupal expression was predicted at a single timepoint, time series data gave far more useful information such as demonstrating the lag between effector gene expression and point of death in tetO-*dsx-Nipp1* lines crossed to mid-tTAV. This lag

suggests that promoter screens should identify genes expressed very early in pupal development or from late larval-stages to allow lethal levels of effector protein to build up and therefore increase the likelihood of pupal death occurring before eclosion, subject to other variables such as expression level and tissue-specificity.

Some differences in expression of endogenous genes and transgenes driven under the same promoter are expected as enhancer elements or important TFBSs may be missed from the promoter sequence extracted, in addition to other positional effects from the transgene insertion site. All of the promoters tested here contained a TFBS cohort characteristic of pupal-specific expression, with multiple Broad-complex binding sites and a small number of E74A sites. This information may be used to identify other pupal-specific promoters through bioinformatic methods and ensure that the relevant TFBSs are included in selected sequences.

4.4.3 Conclusion

The technical feasibility of a late-acting female-lethal genetic system for use in mosquito population suppression was demonstrated in this chapter, validating the tetO-*dsx*-effector genetic element and the promoter region of mid-Pro as additions to the genetic toolkit for the development of engineered *Ae. aegypti*.

Further work to develop a field-ready strain will involve the continued selection and screening of potential promoters to deliver spatio-temporal expression of lethal effectors that induce death at the pupal-stage rather than mid-eclosion. The chosen tTAV expressing and tTAV responsive genetic elements must also be combined in a single construct for further testing as an all-in-one system (Figure 4.2).

Chapter 5

Towards an ethanol-inducible genetic sexing system in *Ae. aegypti*

5.1 Introduction

Removal of females from release cohorts of *Ae. aegypti* carrying dominant lethal transgenes or infected with incompatibility-inducing *Wolbachia* increases the effectiveness of population suppression (Rendón et al., 2004) and reduces risk of biting and disease transmission by released females (see Section 1.5.2). Removal currently occurs at the pupal stage using mechanical sorting (see Section 1.6.1) but early removal is preferable to reduce wasted investment of time and resources in rearing females (Caceres, 2002). Some progress towards early sorting has been achieved through semi-automated segregation of larvae expressing sex-specific fluorescent markers (e.g. Catteruccia et al., 2005). However, inducing female-specific lethal gene expression using a xenobiotic agent or other stimulus (such as heat shock) introduced into the release generation larval rearing environment would remove the need for any handling.

Using an inducible gene expression system that will not interfere with the tetracycline-repressible dominant lethal transgenic technology employed by Oxitec Ltd. is currently not feasible due to a lack of suitable insect expression systems that are independent of tetracycline control. The most feasible current solution is constitutive expression of a male-specific resistance or female-specific susceptibility gene resulting in female death and male survival with minimal fitness costs on provision of an inducer. Engineering this system requires an effective inducible construct and inducer

combination plus a method of ensuring sex-specific expression; several successful implementations of both components can be found in different insect species.

Y-chromosome linked male expression of insecticide resistance genes or heat shock proteins to protect against sudden increases in temperature have been employed in several species including *An. gambiae*, both by classical genetic techniques such as chromosomal translocation and by transgenesis (Franz, 2005). This is not possible in *Ae. aegypti* as sex determination is autosomal and it lacks sex chromosomes. A transgenic sex-specific expression approach is therefore essential and several genes have been identified which are alternatively spliced in males and females (see Section 4.1). As per Chapter 4, I use the *dsx* cassette which is known to correctly splice female-specific transgenes (Collado, 2013). The next component required is an effective combination of an inducer and gene imparting resistance or susceptibility.

Insecticide resistance is the most common genetic sexing system (GSS) selection marker but in *D. melanogaster*, alcohol dehydrogenase (*DmAdh*) mutations have been extensively studied using simple alcohols as inducing factors. This system was chosen for further exploration in *Ae. aegypti* by Collado (2013) as it fits many criteria for a mass-rearing compatible inducible sexing technique: the inducing factor is not normally present in the rearing environment, it is minimally-toxic to exposed males, water-soluble, stable enough in aqueous solution to maintain lethal concentrations over the necessary time period, readily available commercially at low cost and safe for humans to handle.

Use of *Adh* as a GSS in mosquitoes builds on previous attempts in medfly and *D. melanogaster* by Robinson and Van Heemert (1981), Robinson et al. (1986) and Christophides et al. (2001). The initial attempt achieved female lethality in *D. melanogaster* with 4% ethanol treatment through creation of an *Adh*-null mutant line followed by translocation of *Adh* to the Y-chromosome, generating ethanol resistant males. Transfer of the same system to the medfly *C. capitata* failed as *Adh*-null mutants were homozygous inviable. The later transgenic attempt by Christophides et al. (2001) expressed a *D. melanogaster* *Adh* allozyme with high catalytic activity (*Adh*-Fast or *Adh*-F) in *C. capitata* under the control of a male-specific promoter, which resulted in two times higher *Adh* activity in males but was insufficient for discrimination between the sexes.

Collado (2013) demonstrated proof of principle for an ethanol inducible lethal system in *Ae. aegypti* by expression of *DmAdh* under the control of the *Ae. aegypti* *Hsp83*

promoter, which drives strong and ubiquitous expression (Xiao and Lis, 1989). However, contrary to expectations, *DmAdh*-expressing lines displayed far higher mortality on exposure to 0.5-1% ethanol than wild-type. Collado (2013) hypothesised that this could be due to the inability of *Ae. aegypti* to detoxify acetaldehyde, which is thought to be 10-30 times more toxic than ethanol itself (Akabane, 1970; Von Wartburg, 1971).

5.1.1 *Ae. aegypti* Adh and Aldh

There is currently little research on endogenous Adh or Aldh activity in *Ae. aegypti* to provide evidence for the above suggestion that low Aldh activity is the cause of ethanol-induced lethality in *DmAdh*-expressing *Ae. aegypti*. In addition, the idea of using *DmAdh* expression as a method of inducing sex-specific lethality in mosquitoes is predicated on the assumption that *Ae. aegypti* has limited endogenous capacity for metabolising ethanol, thus enabling transgenic expression to provide a significant differentiation in susceptibility. For instance, in a previous ineffective application of *DmAdh* expression for *C. capitata* sexing, the authors considered the need to create an *Adh*-null mutant background for transgenesis in order to generate sufficient differentiation between wild-type and transgenics (Christophides et al., 2001).

From an evolutionary perspective, mosquito larvae typically inhabit aquatic environments and *Ae. aegypti* have a preference for water filled man-made containers (Christophers et al., 1960), it is therefore unlikely that they are exposed to anything other than trace alcohol concentrations, for example from decomposing vegetation. The suggestion by Collado (2013) that *DmAdh*-expressing *Ae. aegypti* are incapable of detoxifying acetaldehyde is therefore plausible and interesting as it relates to a long-standing question on the mechanism of downstream acetaldehyde metabolism.

Adh metabolises alcohols to aldehydes or ketones (Figure 5.1) and is best known for imparting high ethanol tolerance to *D. melanogaster* and other fruit flies (Carvalho et al., 2009; Heinstra et al., 1983), enabling them to inhabit their ecological niches feeding and ovipositing on soft or fermenting fruits. Wild type *D. melanogaster* show no loss of fitness at ethanol concentrations of up to 4-5% (Gibson et al., 1981) and are able to use some alcohols as energy sources (Van Herrewege et al., 1980).

It was long thought that *Adh* was the primary enzyme involved in insects (e.g. Geer et al., 1985; Heinstra et al., 1983, 1989), although a significant role for acetaldehyde dehydrogenase (*Aldh*) had been demonstrated in mammals (e.g. Hinson and Neal (1972), reviewed in Riveros-Rosas et al. (1997)). However, earlier evidence

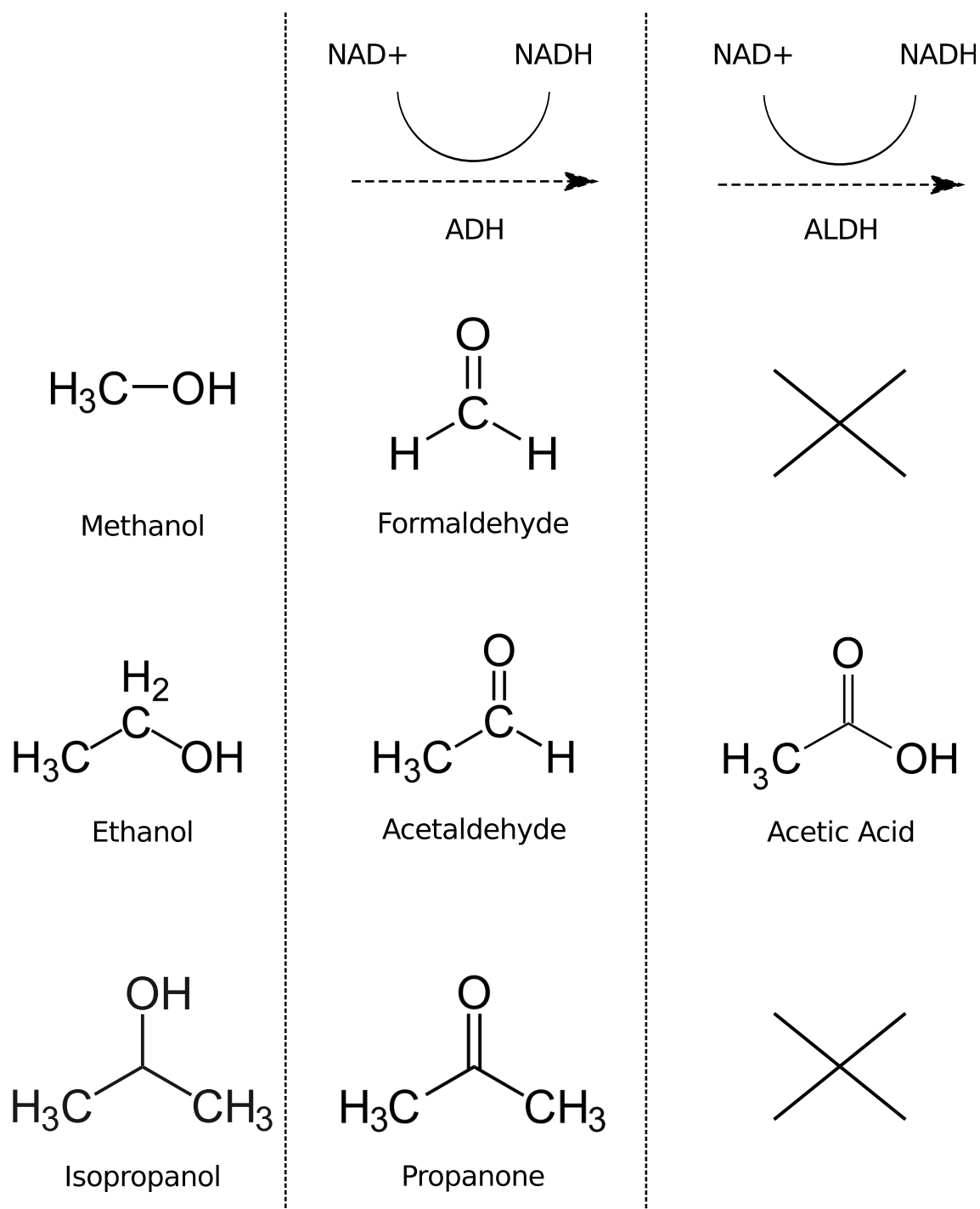


Figure 5.1: Adh catalyses the oxidation of primary alcohols such as methanol and ethanol to their corresponding aldehydes (formaldehyde and acetaldehyde). Aldehydes with more than two carbons can then be further oxidised to carboxylic acids by aldehyde dehydrogenases such as Aldh and also by Adh. Secondary alcohols such as isopropanol are catabolised to their corresponding ketones, which cannot be further oxidised. Tertiary alcohols cannot be oxidised by Adh as they lack a hydrogen on the carbon atom of the hydroxyl group, therefore they were not considered for testing.

emphasising the lesser role of Aldh in *D. melanogaster* only examined the effect of enzyme inhibition on ethanol flux in lipids (Heinstra and Geer, 1991; Heinstra et al., 1989) and when *D. melanogaster* larval fitness assays were performed by Leal and Barbancho (1992) and Fry and Saweikis (2006), Aldh inhibition drastically reduced survivorship in 5% ethanol. *Aldh*-null mutants were as ethanol intolerant as *Adh*-null mutants, suggesting that both enzymes are necessary for ethanol detoxification. These results need not be contradictory, as pointed out by Fry and Saweikis (2006) it is possible that ethanol flux in particularly sensitive tissues could have a significant impact on viability or fitness while not being picked up in biochemical analyses of the whole larva. A greater role for Aldh makes sense in light of the kinetic inefficiency of Adh-mediated acetaldehyde metabolism, which at steady state constantly regenerates ethanol molecules via a dismutase reaction (Fry et al., 2004).

Ae. aegypti genes with Adh and Aldh related domains were examined to determine likely baseline metabolic capabilities. Adh domains reside in the short-chain dehydrogenase/reductase (SDR) family and come in short- medium- and long-chain forms. Only the former is thought to be involved in ethanol metabolism (Hernández-Tobías et al., 2011). DmAdh itself contains a type-II short chain insect alcohol dehydrogenase domain (InterPro IPR002424, Alcohol dehydrogenase, insect-type), which will denote the most likely candidates for ethanol metabolic activity. A BLAST comparison of DmADH (GenBank: CAA77330.1) and the VectorBase *Ae. aegypti* protein library revealed 36 proteins with an E-value of <0.001 and identities ranging from 24.1% to 34.5% similarity to DmAdh. Six of these were found to contain the type-II short chain insect alcohol dehydrogenase domain (Table 5.1) while all others were members of the broader SDR family. This annotation using standard protein databases contrasts with Hernández-Tobías et al. (2011), who classified only one *Ae. aegypti* as a type II short-chain Adh, but how their categorisation was made is not documented.

The low similarity of *Ae. aegypti* polypeptides to DmAdh suggests a reduced likelihood of functional homology but the Tephritidae Adh, which is thought to be non-orthologous to the Drosophilidae protein having independently evolved from a common ancestral gene (Ashburner, 1998), also displays only 40% similarity and is known to be able to effectively metabolise simple alcohols. Therefore, this result does not provide strong evidence for or against significant endogenous Adh activity in *Ae. aegypti*.

VectorBase ID	Genomic Location	E-value	% ID to DmAdh
AAEL017270	supercont1.581:640234-642408	2E-025	29.7
AAEL017337	supercont1.581:620205-625070	1E-028	34.1
AAEL017396	supercont1.779:589637-591081	2E-022	35.6
AAEL017547	supercont1.634:249211-250318	2E-022	30.2
AAEL011450	supercont1.581: 632,399-633,740	2E-022	30.2
AAEL017308	supercont1.581:608849-609408	8E-010	30.9

Table 5.1: All genes in *Ae. aegypti* genome assembly AaegL3 identified in VectorBase as containing the InterPro protein domain IPR002424 (Alcohol dehydrogenase, insect-type). E-value and % ID are BLASTP results for *DmAdh* (GenBank: CAA77330.1).

The evolutionary history, genomic location and expression profile of the six putative Adh genes were then reviewed. Comparison via the VectorBase gene tree function (Figure 5.3) showed two duplication events within the *Ae. aegypti* lineage giving rise to AAEL017337 and AEL017396 plus AAEL017547 and AAEL011450. One of each pairing is located in a cluster of four Adh domain containing genes within a 40 kb stretch of supercontig 1.581 (AAEL017308, AAEL017337, AAEL017270 and AAEL011450), with their counterparts being located in different supercontigs. AAEL017308 does not appear to have duplicated since the divergence of *Aedes* and *Culex* and is clustered both phylogenetically and genomically with two genes containing only a glucose/ribitol dehydrogenase domain (InterPro: IPR002198), indicating that while they are part of the SDR family, they have no alcohol dehydrogenase function. All six genes are expressed at low levels through development but none exhibit more than minimal larval expression and the most highly expressed (AAEL017337) peaks at fewer than 60 transcripts in very early embryos and pupae (Figure 5.2, Akbari et al. (2013a)). This is very different to the continuous high expression profile of *DmAdh* (<http://www.modencode.org>, Roy et al. (2010)). Adh genes are known to play a role in detoxification of xenobiotics in some organisms (Choi et al., 2002) but of the type II genes reported here only AAEL017547 had a corresponding reporter in microarrays exploring response to insecticide exposure in resistant versus susceptible strains. It demonstrated 1.2-fold upregulation in permethrin-resistant strains (Poupardin et al., 2010) but equivalent down-regulation in the LiTOX Bti resistant strain (Tetreau et al., 2012) and no change in Imidacloprid-resistant larvae and adults (Riaz et al., 2013), suggesting it is unlikely to play a major role.

The next stage of analysis was to examine aldehyde dehydrogenases in *Ae. aegypti* to look for evidence regarding the hypothesis that unexpected mortality in *DmAdh*

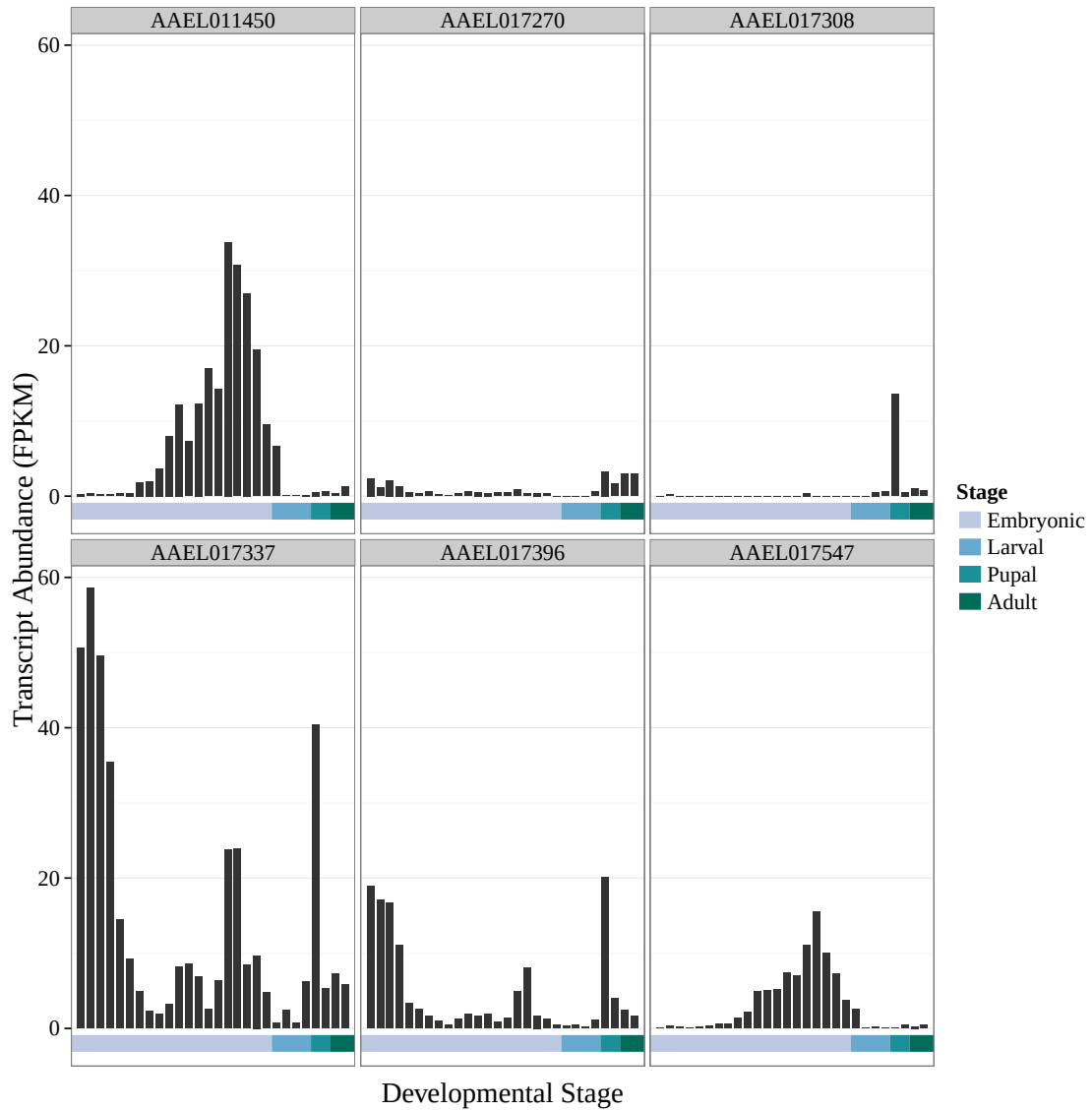


Figure 5.2: Expression profile over the course of development of six *Ae. aegypti* genes identified in VectorBase as containing the insect-type Adh protein domain (InterPro IPR002424). RNA-Seq transcript abundance data Akbari et al. (2013a) is normalised and expressed as fragments per kb of exon per million fragments mapped (FPKM). Embryonic, larval, pupal and adult lifestyles are shown with left bars representing males and right bars females in pupal and adult samples. Adult data is taken from carcass tissues only and all samples representing gonads or blood fed females were excluded.

expressing individuals was due to lack of acetaldehyde detoxification (Collado, 2013).

The aldehyde dehydrogenase superfamily is a broadly distributed group of NAD(P)⁺-dependent enzymes with a diverse set of substrates and functions, so it was unsurprising that 15 *Ae. aegypti* proteins contain an Aldh domain (InterPro IPR015590, Aldehyde_DH_dom). A BLAST analysis of *Ae. aegypti* with the primary *D. melanogaster*

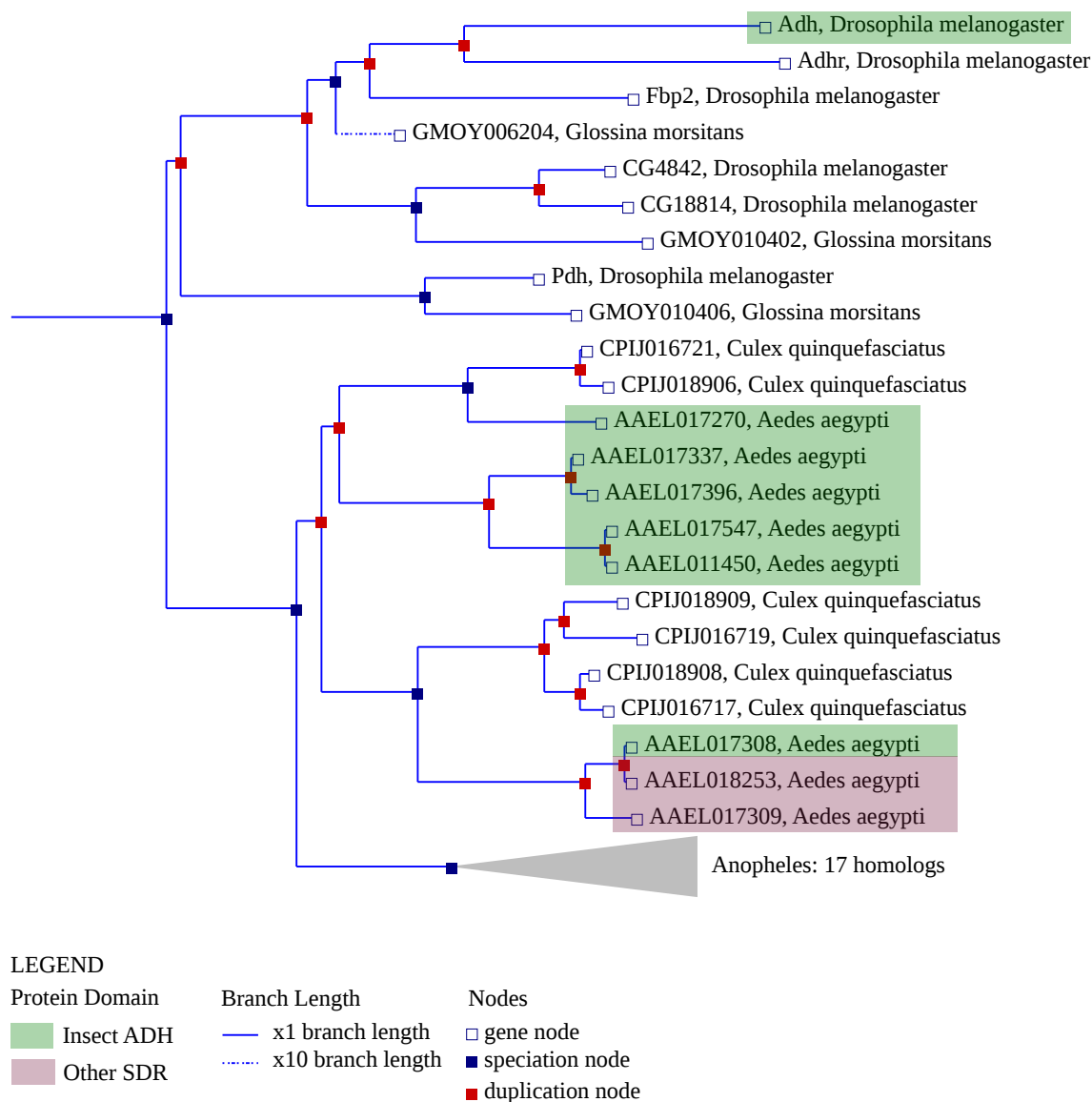
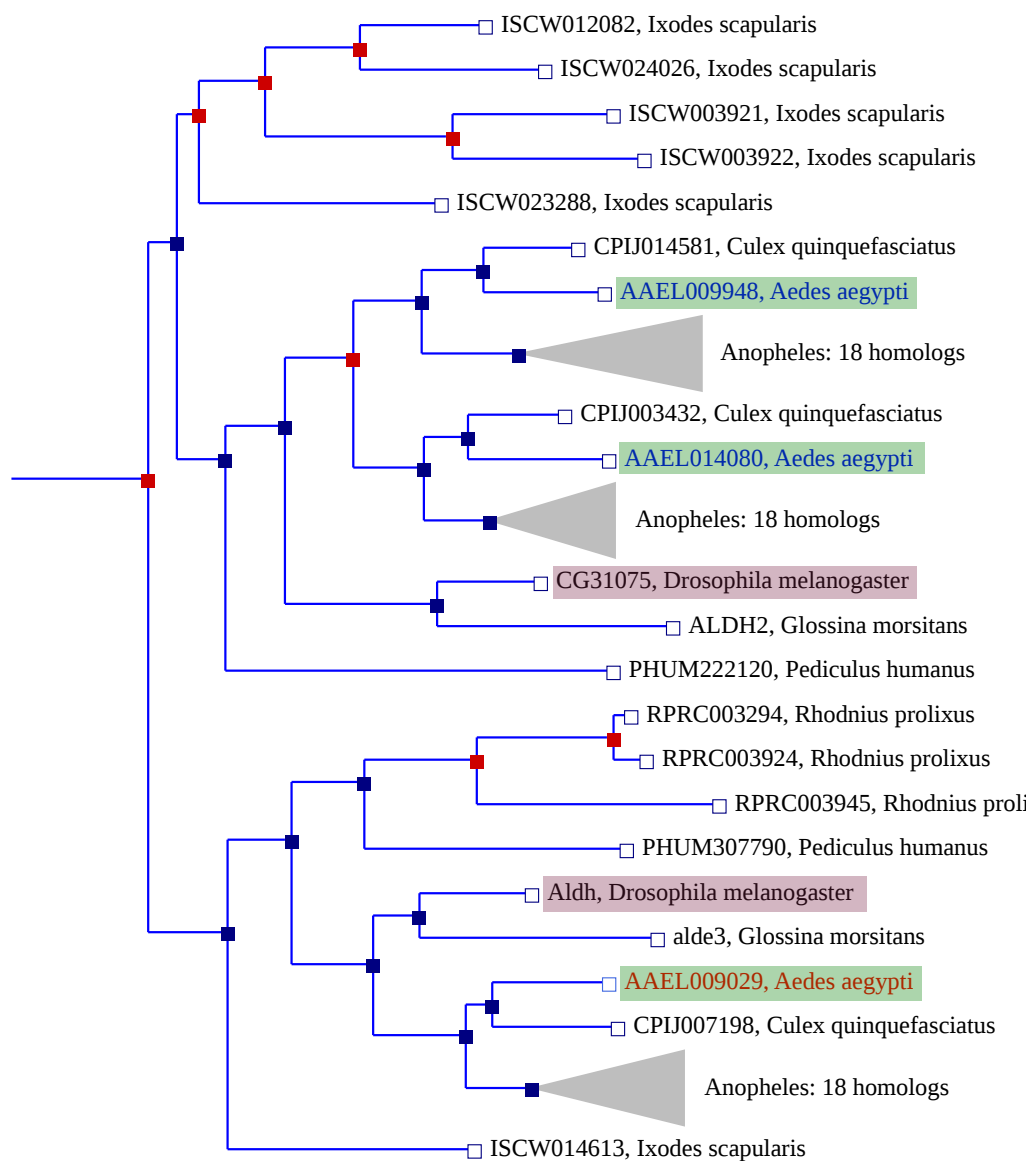


Figure 5.3: *Ae. aegypti* genes containing putative insect-type Adh domains (green) and SDR domains (pink), showing their evolutionary relationship to *DmAdh*. Modified from a gene tree produced by the VectorBase gene orthology and paralogy prediction pipeline using maximum likelihood phylogenetic gene trees generated by TreeBeST (Vilella et al. (2009); <https://www.vectorbase.org/Multi/GeneTree?gt=VBGT00190000009906>).

Aldh (GenBank: NP_001260290.1, encoded by Aldh/CG3752) returned 14 proteins with E-values <0.01. One particular gene, AAEL009029, had 80% identity at the protein level and is firmly placed as an orthologue of *DmAldh* (Figure 5.4). The protein sequence is highly conserved across a range of taxa, with 67% identity to ALDH2 in humans, several other mammals and the zebrafish *Danio rerio*. Two proteins with 58% identity to *DmAldh*, AAEL014080 and AAEL009948, appear to have arisen from



LEGEND

Genes of interest

- Ae. aegypti ALDH
- D. melanogaster ALDH

Nodes

- gene node
- speciation node
- duplication node

Figure 5.4: *Ae. aegypti* AAEL009029 (green) is the direct orthologue of *D. melanogaster* Aldh (pink). Other genes also contain a putative Aldh domain. Modified from a gene tree produced by the VectorBase gene orthology and paralogy prediction pipeline using maximum likelihood phylogenetic gene trees generated by TreeBeST (Vilella et al., 2009).

VectorBase ID	Description	Genomic Location	E-value	% ID
AAEL009029	aldehyde dehydrogenase	supercont1.363: 720199-733814	0	80%
AAEL014080	aldehyde dehydrogenase	supercont1.1002: 133920-177403	0	58.5%
AAEL009948	aldehyde dehydrogenase	supercont1.440: 61468-70016	0	58.4%
AAEL010764	aldehyde dehydrogenase	supercont1.507: 569856-582271	4E-143	44.4%
AAEL004685	succinate semialdehyde dehydrogenase	supercont1.127: 2247574-2253203	8E-091	35.8%
AAEL011756	aldehyde dehydrogenase	supercont1.608: 518341-540207	2E-055	28.3%
AAEL001134	methylmalonate-semialdehyde dehydrogenase	supercont1.23: 440020-448114	2E-052	28.5%
AAEL012162	aldehyde dehydrogenase	supercont1.662: 590574-603157	2E-037	27.6%
AAEL009664	aldehyde dehydrogenase	supercont1.417: 904388-906479	6E-037	27.4%
AAEL015630	pyrroline-5-carboxylate dehydrogenase	supercont1.3892: 3040-4434	9E-029	26.8%
AAEL005422	pyrroline-5-carboxylate dehydrogenase	supercont1.155: 1662252-1728445	6E-027	25.2%
AAEL013831	pyrroline-5-carboxylate dehydrogenase	supercont1.934: 297052-303289	5E-026	25.1%
AAEL012165	aldehyde dehydrogenase, putative	supercont1.662: 500144-535730	2E-017	35.3%
AAEL012161	hypothetical protein	supercont1.662: 535736-565043	4E-010	28.2%

Table 5.2: All genes in *Ae. aegypti* genome assembly AaegL3 identified in VectorBase as containing the InterPro protein domain IPR015590 (Aldehyde_DH_dom). BLAST E-value and % amino acid similarity to the *D. melanogaster* Aldh gene (GenBank: NP_001260290.1, encoded by Aldh/CG3752).

a duplication within the Culicidae and are orthologous to another *D. melanogaster* aldehyde dehydrogenase (CG31075). This gene appear to be a duplicate of *DmAldh* (Gallach et al., 2010), is also highly expressed and has a complementary temporal expression pattern to the primary *Aldh*, which could be an example of subfunctionalisation (<http://www.modencode.org>, Roy et al. (2010)). It is not clear if this expression pattern is mirrored in *Ae. aegypti* due to the lack of granularity in pupal time course data but all three *Ae. aegypti* genes described above are highly expressed in larvae (Figure 5.5, Akbari et al. (2013a)), when any alcohol treatment would take place for sexing during mass-rearing.

All three genes are represented in xenobiotic exposed resistant vs susceptible microarrays. AAEL009029 is 1.2–1.3-fold upregulated in both permethrin and Imidicloprid resistant strains but shows no expression changes in the Bti resistant LiTOX strain. AAEL014080 shows 1.4-fold upregulation in permethrin resistance but a similar down-regulation in LiTOX and no change in Imidicloprid assays. AAEL009948 is 1-1-fold up-regulated in Imidacloprid exposed resistant larve but down-regulated in

adults, permethrin exposed resistant strains and the LiTOX strain. The same three genes were recently identified as *DmAdh* orthologues by Lumjuan et al. (2014) and both AAEL014080 and AAEL009948 displayed upregulation in permethrin resistant strains at larval stages as assessed by qRT-PCR.

These results show that Aldh-domain containing genes are expressed in larvae and have a potential role in xenobiotic detoxification but no evidence was found for their affinity for acetaldehyde as a substrate. This genetic and evolutionary analysis has thus been inconclusive in providing evidence for the endogenous ability of *Ae. aegypti* to metabolise ethanol and acetaldehyde.

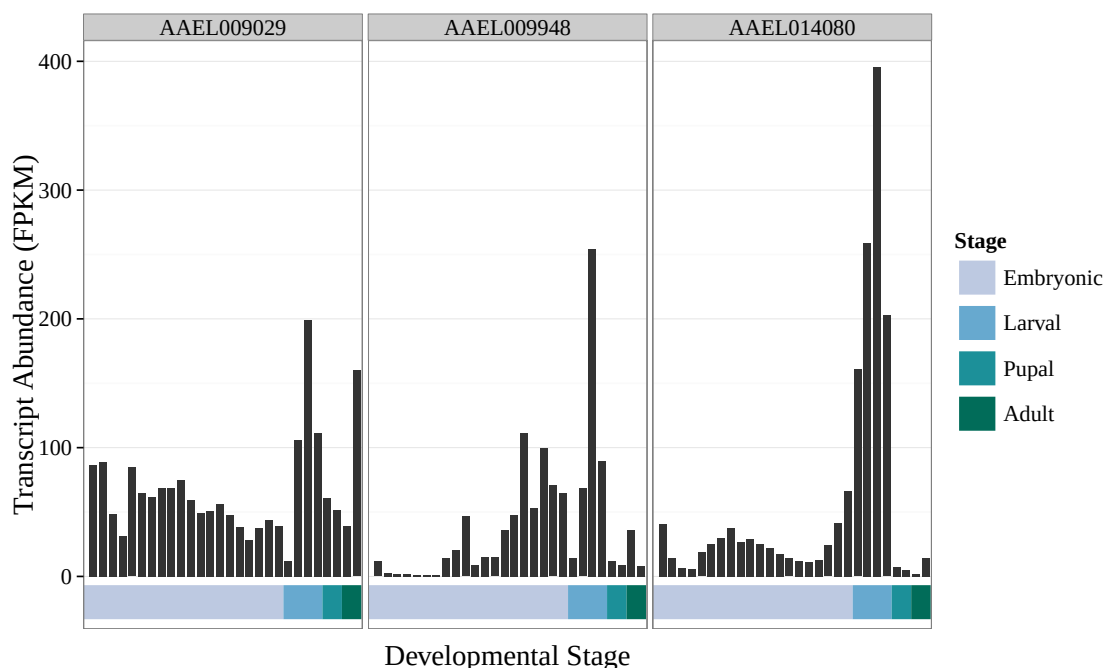


Figure 5.5: All three *Ae. aegypti* orthologues of *DmAdh* are highly expressed in larvae, when any alcohol treatment would take place for sexing during mass-rearing. Normalised RNA-Seq transcript abundance data obtained from Akbari et al. (2013a).

5.1.2 Towards an alcohol-induced GSS

Collado (2013) then combined *DmAdh* with the *dsx* mini-gene, designing the splice sites to generate in-frame translation of *DmAdh* exons in females only to induce female-specific lethality on addition of ethanol. However, despite detection of the expected male and females spliceforms, the desired lethal effect was not seen. Examination of transcripts suggested that although the male splicing pattern excluded the engineered *DmAdh* start codon, an endogenous start codon in the *DmAdh* sequence

could be enabling translation and thus DmAdh protein synthesis in males, resulting in the lack of sex discrimination observed.

This chapter builds on the work of Collado (2013). Firstly, I survey the literature to identify suitable alcohols for testing and then perform a dose response assay with a selection of four alcohols to ascertain the NOAEL - no observable adverse effect level. I use this as a starting point for experiments in any *dsx-DmAdh* transgenic lines generated. Finally, I use a redesigned *dsx-DmAdh* construct with endogenous ATG sites removed via site-specific mutagenesis to develop and characterise eight transgenic *Ae. aegypti* lines for ethanol-inducible female lethality. I determine DmAdh enzyme activity and *dsx-DmAdh* transgene expression in all lines to establish possible reasons for the lack of lethal effects observed in these experiments.

5.2 Materials and Methods

5.2.1 Analysis of *Ae. aegypti* Adh and Aldh

DmAdh protein sequence (GenBank: CAA77330.1) was located in the NCBI protein database using search criteria alcohol dehydrogenase [term] *Drosophila melanogaster* [organism]. A BLASTp search was performed against the VectorBase *Ae. aegypti* protein collection (<http://www.vectorbase.org>) with a maximum E-value cut-off of 0.001. A protein domain analysis was performed on the top hit using built-in VectorBase functions. All genes containing the InterPro domain of interest IPR002424 (Alcohol dehydrogenase, insect-type) were located and a gene evolutionary tree was generated by VectorBase. Genomic location and expression were explored in VectorBase, the Caltech *Ae. aegypti* genome browser (<http://aedes.caltech.edu>) and the *Ae. aegypti* developmental transcriptome data generated by Akbari et al. (2013a). The same procedure was undertaken for Aldh, this time using the Aldh sequence from FlyBase (<http://flybase.org>) and searching for InterPro domain IPR015590 (Aldehyde_DH_dom).

5.2.2 *dsx-DmAdh* Transgenic Construct (OX5010)

The *dsx-DmAdh* transgenic construct (Figure 5.6A) uses the PiggyBac transposon integrase system and is engineered after the similar OX4597 construct described by Collado (2013). It was built by Tarig Dafaalla of Oxitec Ltd and contains an *Ae. aegypti*

doublesex (*dsx*) mini gene controlling the alternative splicing of a fused *Ubiquitin-DmAdh* element driven by the *Ae. aegypti* *Hsp83* ubiquitous promoter as well as a DsRed2 marker driven by the 3xP3 promoter. Transgenic individuals should display red fluorescence in the eye at all life stages and additionally in the larval anal papillae and ventral segmental ganglia.

The *dsx* mini gene comprises two shared exons (4 and 6) flanking two female-specific exons (5a and 5b) as described in Salvemini et al. (2011), these are separated by shortened introns to reduce construct size and the gene is expected to produce one male-specific spliceform and two female-specific spliceforms (Figure 5.6C). *dsx-DmAdh* has three modifications compared to OX4597. Firstly, endogenous start codons in the male transcript of OX4597 were removed via site specific mutagenesis. Secondly, the engineered start codon was moved one base before shared exon 4 from its original position within the female-specific exon 5b. This produces a single open reading frame across the entire female transcript only in the *dsx* exon 4-5b-6 spliceform, which will thus be the only spliceform producing an in-frame transcript for translation to Adh. Finally, the promoter driving expression of the *dsx-DmAdh* cassette was changed from Hsp70 to Hsp83, as this proved to produce reliable and successful ubiquitous expression in the bisex *DmAdh* construct developed by Collado (2013).

5.2.3 Microinjections and mosquito rearing

An injection mixture containing 300 $\mu\text{g} \cdot \mu\text{l}^{-1}$ *dsx-DmAdh* plasmid DNA and 700 $\mu\text{g} \cdot \mu\text{l}^{-1}$ mRNA helper encoding piggyBac transposase was suspended in injection buffer and microinjected into WT eggs from which survivors were reared to pupation as per Section 4.2.4.

1-2 G_0 males were mated to 10-20 WT females or 3-4 G_0 females mated with two WT males. Eggs resulting from crosses were reared, screened for transgenic G_1 larvae and resulting lines were established as described in 4.2.4.

5.2.4 General mosquito rearing

See Section 4.2.5.

5.2.5 Analysis of transgene expression and splicing pattern

To confirm splicing pattern, larval and pupal samples from the representative *dsx-DmAdh*-B line were collected at different life stages for PCR analysis (Table 5.3).

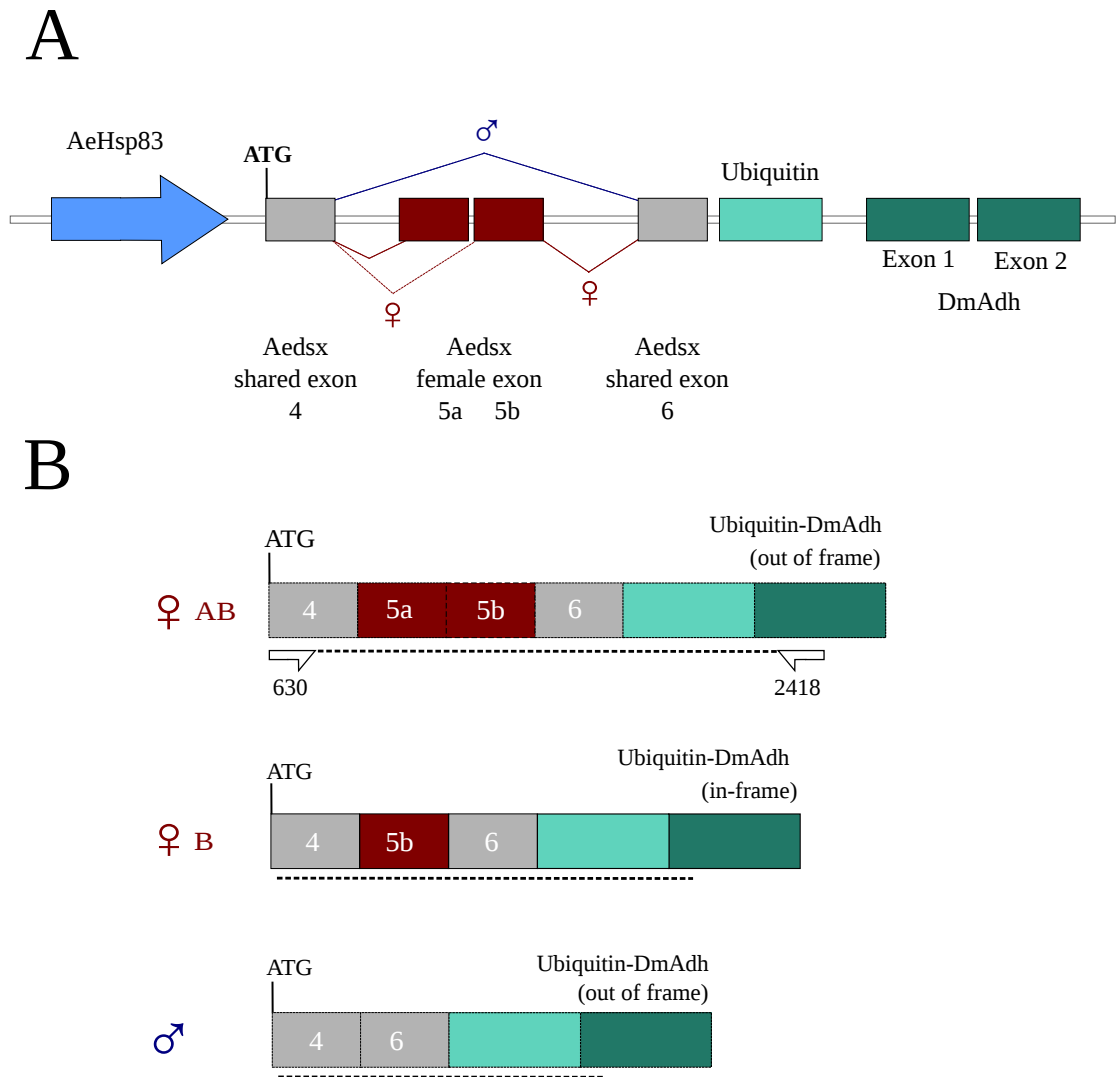


Figure 5.6: *dsx-DmAdh* Construct Design: A) The piggyBac integrated construct contains a DsRed2 fluorescent marker driven by the 3xP3 promoter (not shown), which expresses red fluorescence in the larval eyes, anal papillae and intersegmental ganglia and is then restricted to the eyes of pupae and adults. Expression of the *dsx-DmAdh* cassette is driven by the ubiquitous *AeHsp83* promoter. *Ae. aegypti dsx* is a mini-gene comprising two shared exons (4 and 6) flanking female-specific exons 5a and 5b and separated by shortened introns. An engineered ATG site before shared exon 4 marks the start of translation. Further downstream in the construct is a fusion *Ubiquitin-DmAdh* sequence, which will be cleaved by ubiquitinase at the N-terminus of ubiquitin post-translation to generate a free Adh protein. Such ubiquitin fusion proteins can substantially increase expression of cloned gene products *in vivo* (Butt et al., 1989). B) The sex-specific splicing pattern of the *Ae. aegypti dsx* mini-gene as described by Salvemini et al. (2011). Expected transcripts in transgenic males and females are shown along with 630 and 2418 primer binding sites (white arrows) and expected PCR products (dashed black line). *Ubiquitin-DmAdh* is in-frame only in the female B transcript so only this spliceform should produce functional Adh.

RNA extraction, cDNA synthesis, PCR, cloning and sequencing was undertaken as per Section 4.2.6 using the primers in Table 5.4.

Stage	Quantity	<i>dsx-DmAdh</i> -B/WT
L1 (12 h)	1mg	Mixed
L2 (48 h)	1mg	Mixed
L3 (72 h)	8 larvae	<i>dsx-DmAdh</i> -B
L4 (140 h)	5 larvae	<i>dsx-DmAdh</i> -B
♂ Pupae	3 pupae	<i>dsx-DmAdh</i> -B
♀ Pupae	2 pupae	<i>dsx-DmAdh</i> -B

Table 5.3: Sampled stages of *dsx-DmAdh*-B for RNA extraction and PCR. Earlier stages were mixed transgenic and wild-type individuals due to the sample size required and difficulty of screening and separating L1-2 larvae.

Primer Name	Primer Sequence
<i>Aed</i> <i>dsx</i> -F (630)	5'-GAGATGATGCCCCTGATGTACG-3'
DmAdh-F (2416)	5'-CACCAAGCTGCTGAAGACCATCTTC-3'
DmAdh-R (2418)	5'-ATGCCGGGGTTCACAGTGTAAG-3'
AeMuAcRTF (1098)	5'-GGCATTCATTCTAGTTCCGAAACCG-3'
AeMuAcR (895)	5'-CCCAGGAAGGATGGCTGGAAGAG-3'
pJET F	5'-CGACTCACTATAGGGAGAGCGGC-3'
pJET R	5'-AAGAACATCGATTTTCCATGGCAG-3'

Table 5.4: Primers to amplify *DmAdh* and *Ae. aegypti* muscle actin (*AeMuAc*) as well as *dsx-DmAdh* cassette spanning primers to identify spliceforms.

5.2.6 Toxicity of alcohols to wild-type mosquitoes

WT eggs were hatched overnight under low pressure in deionised water before L1 larvae were aliquoted into pots containing the relevant alcohol treatments (Table 5.5), which were then covered loosely with Parafilm to reduce evaporation while avoiding the creation of anaerobic conditions which could lead to bacterial growth in the rearing water.

Four replicates of 200 larvae were used and within each experiment pots were randomly assigned to tray locations to reduce variability in proximity to humidifiers and heaters in the insectary.

Larvae were fed using Tetramin[®] following a diet devised by Irka Bargielowski (Oxitec Ltd, Table 4.8). Where there were delays in development and plentiful food remained in the pot, larvae were not fed and the feeding regime was continued from

the same point on the following day. This was to avoid overfeeding, which is associated with growth of microorganisms in the rearing water and larval mortality. Pupae were counted and sexed on alternate days from the start of pupation until all larvae had pupated.

Data was analysed in R with dose-response models selected and fitted using the drc package (Ritz and Streibig, 2005). Model testing was performed using log-likelihood, AIC and lack of fit measures.

Alcohol	Concentration % v/v						
	1	2	3	4	5	6	7
Ethanol	2.5	2.25	2	1.75	1.5	1.25	1
Methanol	0.8	0.4	0.2	0.1	0.05	NA	NA
Isopropanol	1.5	1.25	1	0.75	0.5	0.25	NA
Ethylene glycol	0.5	0.4	0.3	0.2	0.1	NA	NA

Table 5.5: Alcohol treatments for wild-type toxicity testing.

5.2.7 Toxicity of ethanol to *dsx-DmAdh* lines

For preliminary testing, *dsx-DmAdh* G₄ heterozygous eggs were hatched overnight and L1 larvae were aliquoted into five replicate pots containing 200 larvae and 300 ml deionised water or 1% v/v ethanol and reared as per the protocol for WT toxicity testing (Section 5.2.6). Pupae were picked on alternate days, screened for fluorescence and sexed.

Data for each line was analysed using a log-linear model by implementing a poisson family GLM in R. A saturated model containing the three factors treatment, transgenic status and sex plus all interactions was tested against the null model using a χ^2 ANOVA with interactions and factors removed in a step-wise manner.

5.2.8 Adh Activity Assay

Twenty male or female pupae from WT and *dsx-DmAdh* lines A, B, C3, K and M were homogenised with a pestle in 750 μ l 50 mM Tris-HCl (pH 8.5). The homogenate was centrifuged at 10,000 xg for 30 min and the supernatant filtered through glassfibre (Whatman GF/A 21 mm glassfibre filters, Sigma Aldrich, St Louis, MO, USA) into a fresh 1.5 ml tube. 50 μ l samples were pipetted in duplicate into a 96-well plate along with a 2-fold dilution series of NADH (2 mM to 31.25 μ M). 150 μ l reagent

solution (330 μ M NAD⁺, 330 μ M Nitro Blue Tetrazolium (NBT) and 8 μ M phenazine methosulphate (PMS), 100 mM EtOH in 50 mM Tris-HCl with 0.13% gelatine) was added and the plate was incubated in the dark at room temperature. Colorimetric readings at 590 nm were taken every 30 s for 60 min using an FLUOstar Omega microplate reader (BMG Labtech GmbH, Ortenberg, Germany) and a final reading was taken at 120 min. During the pilot experiment five pools of only five pupae from *dsx-DmAdh*-B were used and the final reading was at 60 min.

In order to normalise results to sample protein content, 5 μ l of each sample was added to 150 μ l Bradford reagent in duplicate. Plates were incubated at room temperature for 20 mins, read at 450 and 595 nm using the FLUOstar Omega microplate reader and the ratio of these two readings was used for normalisation of NADH concentration produced (Zor and Selinger, 1996).

All chemicals were sourced from Sigma Aldrich (St Louis, MO, USA) and the protocol was adapted from Fibla and González-Duarte (1993).

5.2.9 qRT-PCR for *DmAdh* and *dsx-5a* transcript expression

RNA extraction and cDNA synthesis was performed as per section 5.2.5 using three pools of four female transgenic pupae from *dsx-DmAdh*-A, B, C(3), I, K and M, four WT female pupae and mixed pools of four *D. suzukii* adults.

Primers were designed to amplify both *DmAdh* and *DsAdh*. The *DsAdh* sequence was obtained from the *D. suzukii* genome (<http://spottedwingflybase.oregonstate.edu>) by a BLASTn search using the *DmAdh* mRNA sequence (CG3481, Genbank NT_033779.4). A region displaying 79% nucleotide similarity (scaffold1:1865028..1868458) and a partial transcript (DS10_00000162) displaying 94 % similarity to the *DmAdh* CDS were returned, annotated as alcohol dehydrogenase related protein (*Adhr*). *Adh* and *Adhr* fall in the same genomic region in *D. melanogaster* so a modified CDS for *Adh* only was extracted by mapping both the annotated *DsAdh* exons and the *DmAdh* CDS to the *D. suzukii* genomic region. Once translated, this protein sequence was 99.6% similar to *DmAdh*. Intron spanning qRT-PCR primers were then designed using Primer-BLAST to allow amplification of *Adh* transcript from both species. Primers to the *dsx-5a* exon were also designed using Primer-BLAST and all primers (Table 5.6) were confirmed to have efficiencies between 1.88-2.05 and a single peak in the melting curve of the PCR products.

Primer	Sequence
qDmAdh_6F	5'-GCTGGTATCCTGGACGATCA-3'
qDmAdh_6R	5'-GTTGACCAGGCCAGTGTAGT-3'
qAe_dsx5A_F2	5'-CGCCAGTCCGGATGATAGA-3'
qAe_dsx5A_R2	5'-AATCGATCTGATCTTTCGTGGTTTA-3'

Table 5.6: Primer sequences for qRT-PCR of *DmAdh/DsAdh* and *dsx* exon-5a.

To generate a plasmid standard curve of known copy number, the molecular weight of the *dsx-DmAdh* construct was calculated and maxi-prep concentration was determined using a Geneflow NanoPhotometer (Geneflow Ltd., Lichfield, UK). A 1:2 serial dilution was created from approximately one billion to 10,000 copies of the plasmid. This copy number can only be an estimate due to the likely contamination of the plasmid with RNA, but the primary aim was to determine relative expression of *Adh* in *Ae. aegypti* compared to *D. suzukii* so measurement of absolute expression was not required.

(a)		(b)			
Reagent	Volume μ l	Step	Temp $^{\circ}$	Time	Cycles
cDNA	2	1	94	2 min	1
2x SYBR JumpStart	5	2	94	15 s	
10 mM Primer F	0.25	3	59	60 S	40
10 mM Primer R	0.25				
dH ₂ O	2.5				
Total	10				

Table 5.7: a) qRT-PCR reagent recipe. b) Thermocycling conditions for qRT-PCR.

qRT-PCR was performed using SYBR Green JumpStart Taq ReadyMix (St Louis, MO, USA) as per Table 5.7 on a Stratagene Mx3005P Real-Time PCR System (Agilent Technologies, Inc., Santa Clara, CA) with a dissociation curve taken reading every 1 $^{\circ}$ C from 59 $^{\circ}$ C to 94 $^{\circ}$ C. After data was collected using MxPro software, a linear model was fitted to the log copy number of the plasmid standard curve in R and sample expression was approximated by prediction from Ct values. Log data was tested for normality and equal variance before an ANOVA and Tukey post-hoc testing were performed. The exponent of the log predicted values is displayed in graphs for clarity of scale.

5.3 Results

5.3.1 Secondary data analysis for selection of alcohols

Adh was a key early genetic selection marker for experiments because it allows both positive and negative selection via the use of different alcohols. *Adh*-null mutants show high sensitivity to primary alcohols but reduced sensitivity to secondary alcohols, which are oxidised to ketone products that are more toxic than the original alcohol and unable to be further metabolised (Figure 5.1). If this proves true in mosquitoes, it would improve the versatility of the tool as a separation method, allowing either sex to be selected by altering the alcohol supplied. A range of suitable alcohols was thus sought and tested. The key features of a successful sex discriminating alcohol for use with the *dsx-DmAdh* expression system are low toxicity of the alcohol treatment such that males are unharmed and high toxicity of the product of *Adh* metabolism to ensure 100% lethality in females. Secondary data was collected on toxicity of a range of alcohols and where possible the corresponding oxidation product in order to choose candidates for testing alongside ethanol as potential lethality inducers. A review of the literature and online toxicology databases was undertaken and four alcohols were taken forward for analysis via long term exposure of wild-type L1 larvae under standard rearing conditions.

Alcohol	Adh Product	Type	Alcohol LC50	Product LC50	Ratio	Ref
Methanol	Formaldehyde	Aldehyde	8.5	0.25	34.0	1
Ethanol	Acetaldehyde	Aldehyde	3.8	0.27	14.1	1
1-Propanol	Propanal	Aldehyde	1.3	0.25	5.2	1
2-Propanol	2-Propanone	Ketone	3.2	4.5	0.7	2,3
1-Butanol	Butanal	Aldehyde	0.45	0.2	2.2	1
2-Butanol	2-Butanone	Ketone	0.9	1.3	0.7	1
1-Heptanol	1-Heptanal	Aldehyde	160000 ppm	1000000 ppm	0.2	2,3
2-Methyl-2-propanol	2-Methylpropanal	Aldehyde	2.4	0.25	9.6	1
Ethylene glycol	Glycolaldehyde	Aldehyde	21	-	-	1

Table 5.8: Toxicity of a range of alcohols to *Ae. aegypti* larvae. Aldehydes are more toxic than their corresponding primary alcohols across all chain lengths, with the highest relative LC50 different being the 34-fold increase in toxicity of formaldehyde compared to methanol, versus a 14-fold difference between ethanol and acetaldehyde. Data retrieved from the Environmental Protection Agency EcoTox database (<http://cfpub.epa.gov/ecotox/>). LC50 is the concentration which is lethal to 50% of the treated population, recorded at % volume/volume (% v/v) or parts per million (ppm). References: 1) Kramer et al. (1983) 2) Slooff et al. (1983) 3) Slooff (1982).

In *D. melanogaster* both carbon chain length and saturation of the hydroxyl-bound

carbon affect toxicity in *Adh*-positive flies, with secondary alcohols being more toxic than primary and toxicity generally increasing with chain length, with the exception of ethanol (Figure 5.7, David and Bocquet (1976)). It was not known if this result would hold true in *Ae. aegypti* due to uncertainty about their capacity for Adh activity and detoxification of alcohol metabolites. Secondary data indicated that toxicity of primary alcohols, this time including ethanol, increases with carbon chain length (Table 5.8, Figure 5.7). Aldehydes were more toxic than their corresponding primary alcohols across all chain lengths, with the highest relative LC50 difference being the 34-fold increase in toxicity of formaldehyde compared to methanol, versus a 14-fold difference between ethanol and acetaldehyde (Table 5.8). These results support a lack of endogenous ethanol detoxification ability and suggest that methanol may be a better sex discriminating factor than ethanol. However, this effect would be diminished by the poor affinity of DmAdh for methanol as a substrate (Benach et al., 2001). In terms of primary and secondary alcohols, there are only two comparisons available, for propanol and butanol, both of which show an approximately two-fold decrease in the LC50 value for the secondary alcohol versus the primary. Interestingly, the ketone oxidation products show reduced toxicity compared to their equivalent aldehyde and to their original alcohol, suggesting that transgenic DmAdh could even have a protective effect on treatment of *Ae. aegypti* with secondary alcohols.

The least toxic alcohol described in the literature for *Ae. aegypti* was ethylene glycol, with fourth instar larvae reported as surviving for four hours in up to 21% v/v concentration (Table 5.8). However, no information was available on the toxicity of its metabolite glycoaldehyde in *Ae. aegypti* or of ethylene glycol itself in an Adh expressing insect such as *D. melanogaster*. It is known that *D. melanogaster* produce oxalic acid as a product of ethylene glycol metabolism (Chen et al., 2011), which is promising as oxalic acid poisoning in mammals is treated using an Adh inhibitor to prevent the glycoaldehyde forming (Barceloux et al., 1999; Leth and Gregersen, 2005). Ethylene glycol may therefore be a candidate to induce lethality in DmAdh expressing insects.

Secondary data presented in Figure 5.7 was obtained under different assay conditions in each species, which is one complication of comparing toxicity results across organisms with very different food sources and opportunities for exposure. All *Ae. aegypti* secondary data was collected from four hour treatments of L4 larvae, with concentrations increased until all individuals died in that time period (Kramer et al.,

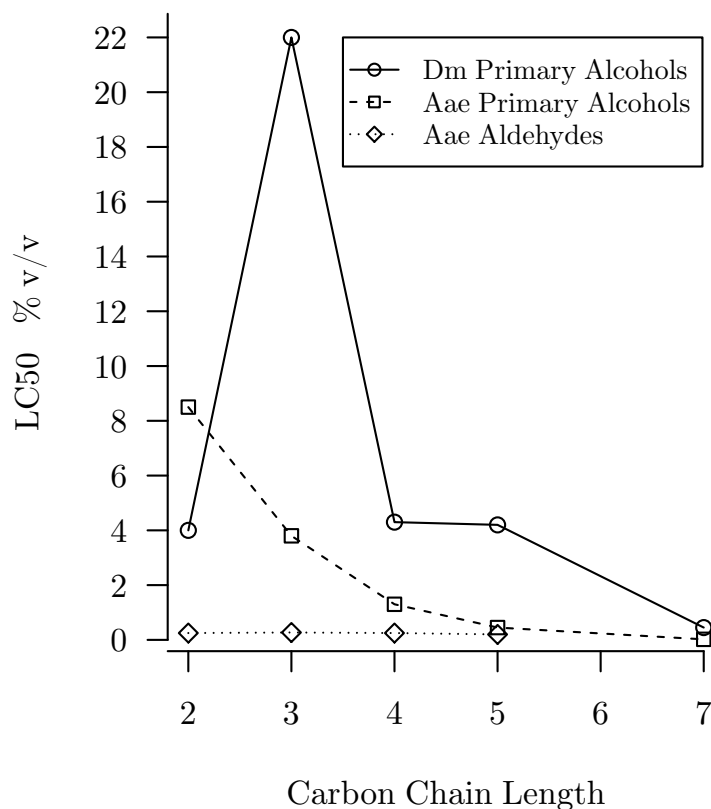


Figure 5.7: Graph showing LC50 values for increasing primary alcohol carbon length in *D. melanogaster* and *Ae. aegypti* alongside corresponding aldehyde activity in *Ae. aegypti*. *Ae. aegypti* data retrieved from the EPA EcoTox database (<http://cfpub.epa.gov/ecotox/>), *D. melanogaster* data from David and Bocquet (1976).

1983; Slooff, 1982; Slooff et al., 1983). While this is useful in terms of gauging trends in toxicity it was necessary to collect data for long term exposure from L1 under standard rearing conditions. This reflects the most likely use case for an early inducible GSS for mass-rearing. although short term treatments followed by rinsing are also a possibility, particularly with developments in automated, continuous-flow mass-rearing devices.

5.3.2 Toxicity of ethanol, methanol, isopropanol and ethylene glycol to wild-type *Ae. aegypti*

Based on the data analysis above, ethanol, methanol, isopropanol and ethylene glycol were chosen for further testing with wild-type *Ae. aegypti* (WT). This selection was also based on other properties such as being easily miscible with water, readily available as common laboratory reagents and comparatively safe for human handling at low concentrations in contrast to some more complex alcohols. These assays were designed to establish concentrations with minimal fitness effects for testing with transgenic lines.

Alcohol	LC50	95% Confidence Interval	NOAEL
Ethanol	1.54	1.52 - 1.55	1
Methanol	NA	NA	0.05
Ethylene Glycol	0.35	0.34 - 0.35	0.1
Isopropanol	0.80	0.79 - 0.82	0.25

Table 5.9: Table of LC50 values and 95% confidence intervals predicted from fitted models of dose response. NOAEL was the lowest experimental dose which did not produce a biologically significant decrease in survival to pupation, which was taken as >5% lower survival than controls.

A number of pilot experiments (data not shown) were performed to determine the spread of concentrations necessary to obtain a full dose response curve, followed by a single assay for each alcohol with four biological replicates at each concentration. Data for methanol is incomplete and variable as pots were prone to bacterial contamination in each of four independently attempted assays, even though parallel treatments with other alcohols were unaffected.

Dose-response models were fitted using the *drc* package in R and evaluated by AIC, log-likelihood and lack of fit. Chosen models were further tested by χ^2 goodness of fit. Subfigure 5.8a: Ethanol dose-response fitted with a three-parameter Weibull 1 model (df = 19, $\chi^2 = 68.5$, p = <0.001). Subfigure 5.8b: Isopropanol dose-response fitted with a three-parameter Weibull 1 model (df = 21, $\chi^2 = 114.2$, p = <0.001). Subfigure 5.8c: Ethylene glycol dose-response fitted with a three-parameter log-normal model (df = 19, $\chi^2 = 67.6$, p = <0.001). Subfigure 5.8d: Methanol dose-response fitted with a two-parameter log-logistic model (df = 17, $\chi^2 = 121.5$, p = <0.001).

The NOAEL and LC50 was obtained for each alcohol excluding methanol, where the LC50 could not be determined from the data available (Table 5.9). Contrary to

the secondary data collected on toxicity to L4 larvae (Table 5.8), ethylene glycol was the most toxic alcohol on long term exposure to L1s with a LC50 of 0.35%, although the NOAEL was 0.1%, which is higher than methanol at 0.05%. Ethanol was the least toxic of the four alcohols with a LC50 of 1.54% while isopropanol fell mid-way at 0.8%. Ethanol, isopropanol and ethylene glycol all displayed very steep reductions in survival over a small increase in concentration (Figure 5.8). The steeper the curve in quantal response models, the more concentrated the underlying tolerance distribution of the population, suggesting that there is probably little variation in resistance to these alcohols between larvae (Finney, 1971), which is advantageous for use on large populations in mass-rearing where 100% sex discrimination would be ideal.

The NOAEL data points are the most significant results as they provide a starting point for future assays comparing mortality between wild-type and *dsx-DmAdh* transgenic females without compromising male survival. However, mortality is an extreme outcome to measure when sub-lethal effects such as reduced male longevity and mating competitiveness would also be detrimental in releases for genetic control. Assays to assess these traits need to be carried out in any functioning alcohol inducible GSS.

5.3.3 *dsx-DmAdh* Transgenic Lines

To generate transgenic *dsx-DmAdh* lines a total of 1841 eggs were microinjected with the plasmid construct *dsx-DmAdh* (Figure 5.6), which contains *DmAdh* downstream of a *dsx* mini-gene which is alternatively spliced in males and females and should produce an in-frame *DmAdh* transcript in females only. Expression of this cassette is driven by the *Ae. aegypti* Hsp83 promoter and a DsRed2 fluorescent marker is driven by the eye-specific 3xP3 promoter to enable identification of transgenic individuals.

45 G₀ larvae emerged, equating to a 2.4% injection survival rate. 91% of injection survivors pupated of which 63% (19 males and 7 females) demonstrated transient expression of the DsRed2 marker. This demonstrates successful injection although transgenic lines can still be derived from non-transiently expressing individuals, therefore all survivors were used in crosses to WT. In total, 31 adults were crossed in 12 paired male pools and two female pools of 3-4 G₀ survivors.

Eight pools yielded transgenic G₁ individuals and separation by phenotype led to 13 lines in total from which two single male crosses were established per line. Where phenotypes from the same parent pools differed only in terms of marker expression strength rather than pattern, only one line was picked to go forward as this was

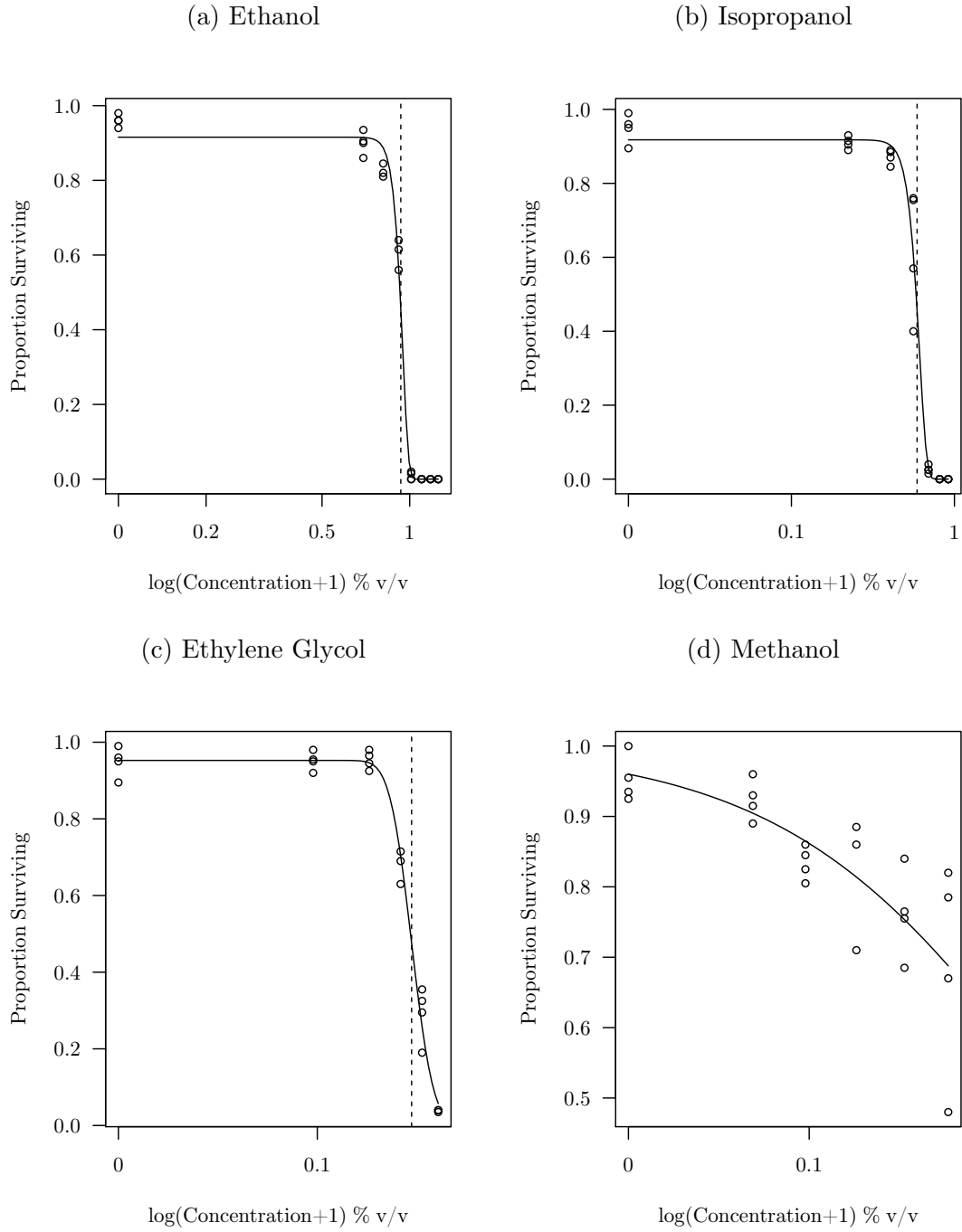


Figure 5.8: Data points and fitted models representing proportional survival to pupation of *Ae. aegypti* in four biological replicates for each dose of ethanol, methanol, isopropanol and ethylene glycol. Dashed vertical line represents the LC50 value (Table 5.9).

considered likely to be due to multiple insertions. At G_2 , one line (*dsx-DmAdh-L*) was found to show transgenic female-specific lethality and discarded while five lines showed signs of multiple insertions of the construct and required single male

outcrossing for further generations. No lines demonstrated sex-linked inheritance but only two lines originated as single insertions and also showed no adverse effects on transgenic individuals, all others had to be outcrossed.

By G₄ of outcrossing to WT, five lines (A, B, D, I and M) demonstrated single insertion inheritance patterns and by G₅ the final two lines (C and K) had been isolated as single insertions. Two lines derived from G₄ single male crosses of line C were retained for testing as different marker intensity suggested they may represent independent insertions. All lines expressed the DsRed2 marker in the expected pattern for the 3xP3 promoter, with expression in larval eyes and anal papillae in all lines alongside ventral segmental ganglia in a subset of lines (Figure 5.9).

5.3.4 Expression of *DmAdh* and sex-specific splicing of the *dsx-Adh* construct

dsx-DmAdh-B was selected from the eight successfully transformed lines to confirm expression of the *DmAdh* gene in larvae, which is essential for an early sexing system. The *Aedes* muscle actin (*AeMuAc*) gene chosen as a positive control was expressed at all life stages, as was the *DmAdh* transcript (Figure 5.10). The *DmAdh* amplicon was expected to be 336 bp in all samples but the band did not migrate below the 400 bp marker on the ladder used. The genomic DNA band expected to be 80 bp larger was observed in the correct relative position so the gene appears to be splicing correctly and this was later confirmed by sequencing. Therefore, the minor difference in relation to the ladder appears to be an artefact. A faint 406 bp amplicon derived from genomic DNA was observed in the male pupae sample, which was also used for the no reverse transcriptase control. However, no genomic contamination was observed in any other samples. Expression of *DmAdh* in L1-L2 demonstrates that alcohols could be provided for metabolism soon after hatching and corroborates expression data from previous lines generated by Collado (2013).

The region spanning from *dsx* exon-4 to *DmAdh* exon-2 was amplified and sequenced to confirm correct sex-specific splicing was occurring. One spliceform of 1006 bp was observed as expected in male pupae and two spliceforms of the expected sizes, 1450 bp and 1930 bp were observed in female pupae, with a third additional band present that did not correspond to any known *dsx* splicing pattern (Figure 5.11). The most abundant female spliceform was the smaller *dsx* exon-4-5b-6 transcript, which should produce functional Adh, while the two larger transcripts produced much less

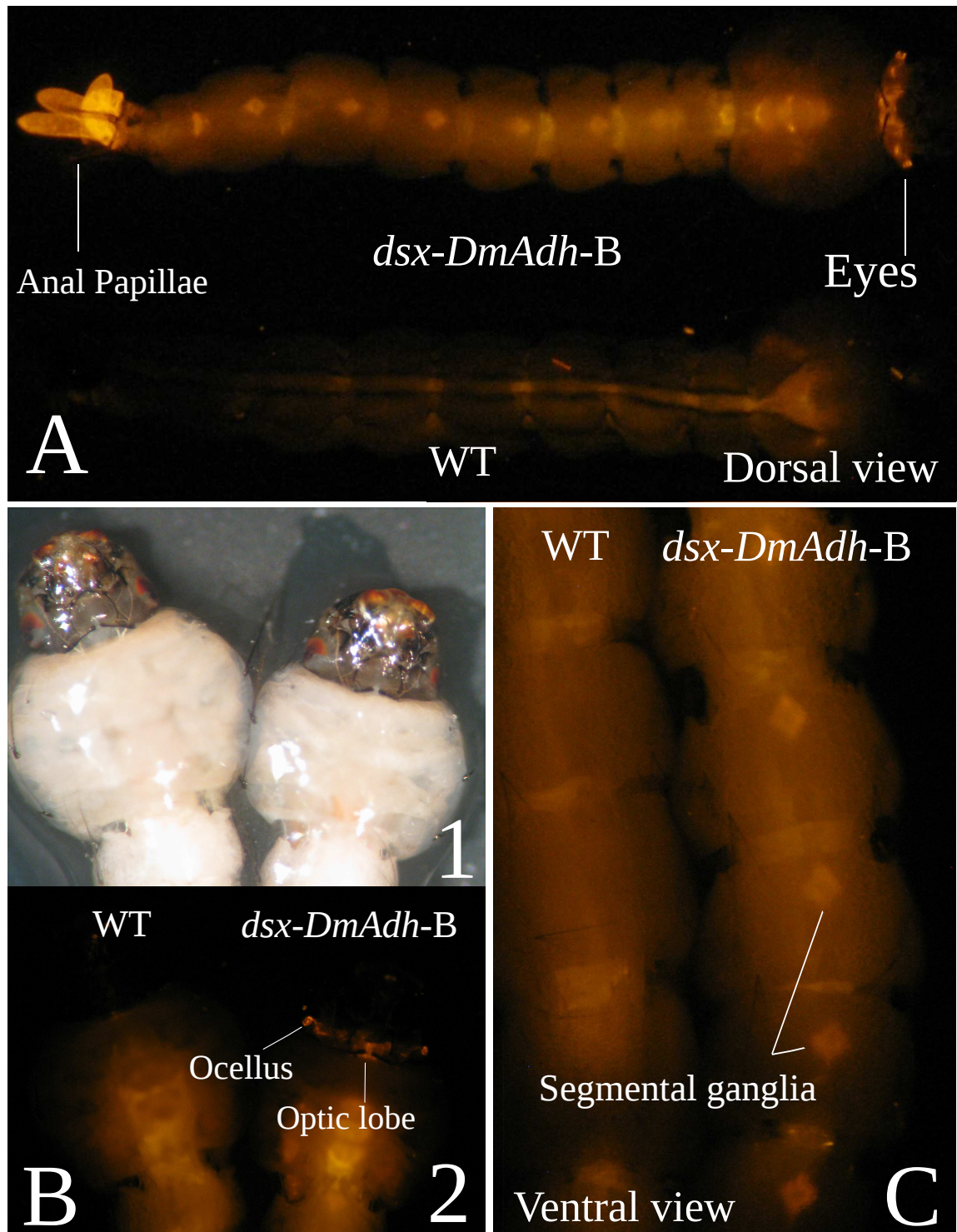


Figure 5.9: Images of DsRed2 fluorescence in *dsx-DmAdh-B* and WT L4 larvae. A, B2 and C were taken under a red filter and B1 under white light. As expected under the control of the 3xP3 promoter, fluorescence is evident in the anal papillae (A), eyes (A and B2) and ventral segmental ganglia (C).

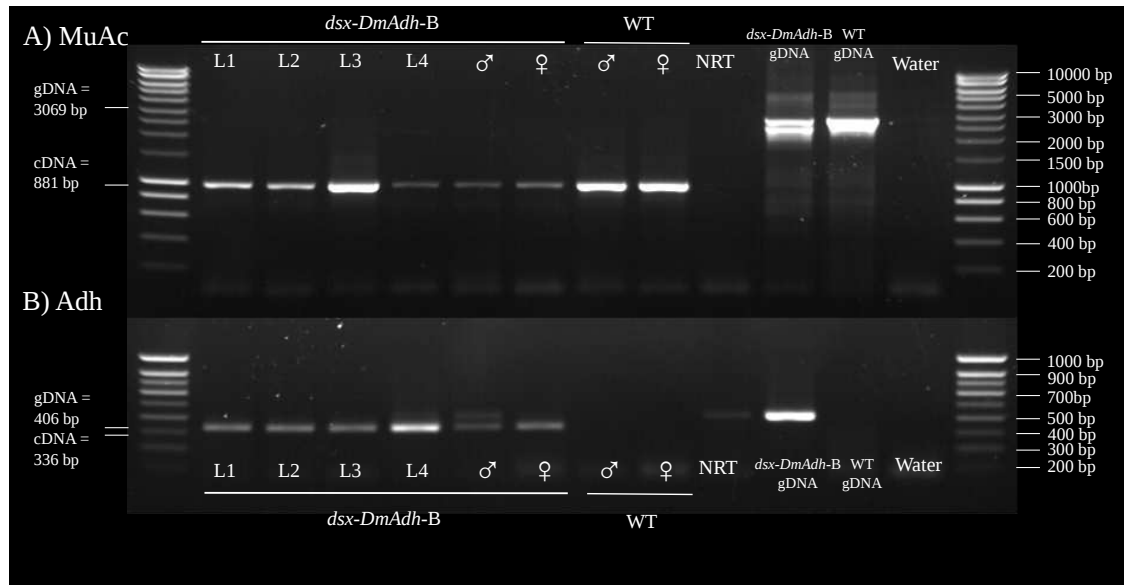


Figure 5.10: A) Electrophoresis gel image of PCR products amplified with *AeMuAc* primers. B) Electrophoresis gel of PCR products amplified by *DmAdh* primers, detecting sequence common to all spliceforms. The *DmAdh* product was expected to be 336 bp in all samples but the band did not migrate below 400 bp, however the gDNA amplicon appears in the correct relative position. A faint gDNA contamination band was observed in the male pupae sample, which was also used for the no reverse transcriptase (NRT) control. Samples from left to right: A) Smart Ladder from Eurogentech, 200 to 10,000 bp B) Smart Ladder (small fragment) from 100 bp (not visible on gel) to 1000 bp. A and B) cDNA from pools of *dsx-DmAdh-B* transgenic larvae at L1 (18 h), L2 (48 h), L3 (72 h) and L4 (140 h), *dsx-DmAdh-B* male and female pupae, WT male and female pupae. NRT: *dsx-DmAdh-B* male pupae RNA with no reverse transcriptase included in cDNA synthesis reaction as negative control. gDNA from *dsx-DmAdh-B* and WT as additional control for gDNA contamination. Water: negative control.

intense bands and the third spliceform in particular was not observed after every PCR reaction. As the standard number of PCR cycles employed was 35, amplification of even very low abundance transcripts would be expected so the irregularity with which this band was obtained suggests that it may represent an interim transcript or other low frequency spliceform. Due to the infrequent occurrence of the band it was not possible to clone the PCR product and obtain a sequence.

On sequencing the three expected spliceforms and aligning to the *dsx-DmAdh* construct sequence (Figure 5.6), all were confirmed to contain the correct exons with a maximum of two nucleotide differences across the whole sequence giving 99.9% identity.

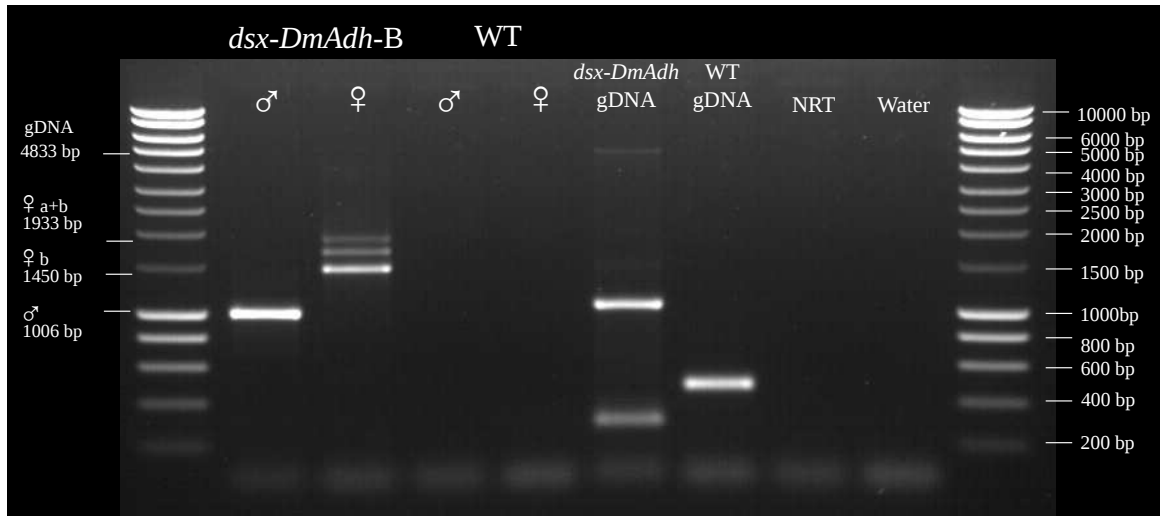


Figure 5.11: Electrophoresis gel of PCR products amplified by 630/2418 primers spanning the transgene *dsx* region. One spliceform of 1006 bp was observed as expected in male pupae and two spliceforms of the expected sizes, 1450 bp and 1930 bp were observed in female pupae. A third additional band that did not correspond to any known *dsx* splicing pattern occurred but was inconsistent in repeated PCRs so no sequence could be obtained. Samples from left to right: Smart Ladder from Eurogentech, 200 to 10,000 bp. cDNA from *dsx-DmAdh-B* male and female pupae, WT male and female pupae. NRT: *dsx-DmAdh-B* male pupae RNA with no reverse transcriptase included in cDNA synthesis reaction as negative control. gDNA from *dsx-DmAdh-B* and WT as additional control for gDNA contamination. Water: negative control.

5.3.5 Long term exposure of *dsx-DmAdh* transgenic larvae to 1% ethanol

1% ethanol was chosen for long term toxicity assays in *dsx-DmAdh* transgenic larvae based on its previous use by Collado (2013) and NOAEL results from the toxicity assays above (Table 5.9). All eight heterozygous *dsx-DmAdh* transgenic lines were treated with 1% ethanol from L1 stage and transgenic larvae were scored for survival to pupation compared to WT larvae in the same pot (Figure 5.12). 1% v/v ethanol concentration was chosen based on the NOAEL and also as Collado (2013) had demonstrated that it induced early lethality in a bisex *DmAdh* expressing system. Poisson family GLMs were fitted stepwise to pupation count data for each line with three factors, sex, transgenic status and treatment (significance testing of models used a χ^2 ANOVA, $p < 0.01$ to account for multiple testing). No lines showed a significant interaction between all three factors, which would have indicated transgenic female lethality on ethanol. Survival in lines *dsx-DmAdh-C3*, *dsx-DmAdh-K* and *dsx-DmAdh-M* showed no deviation from the null model. Transgenics overall showed

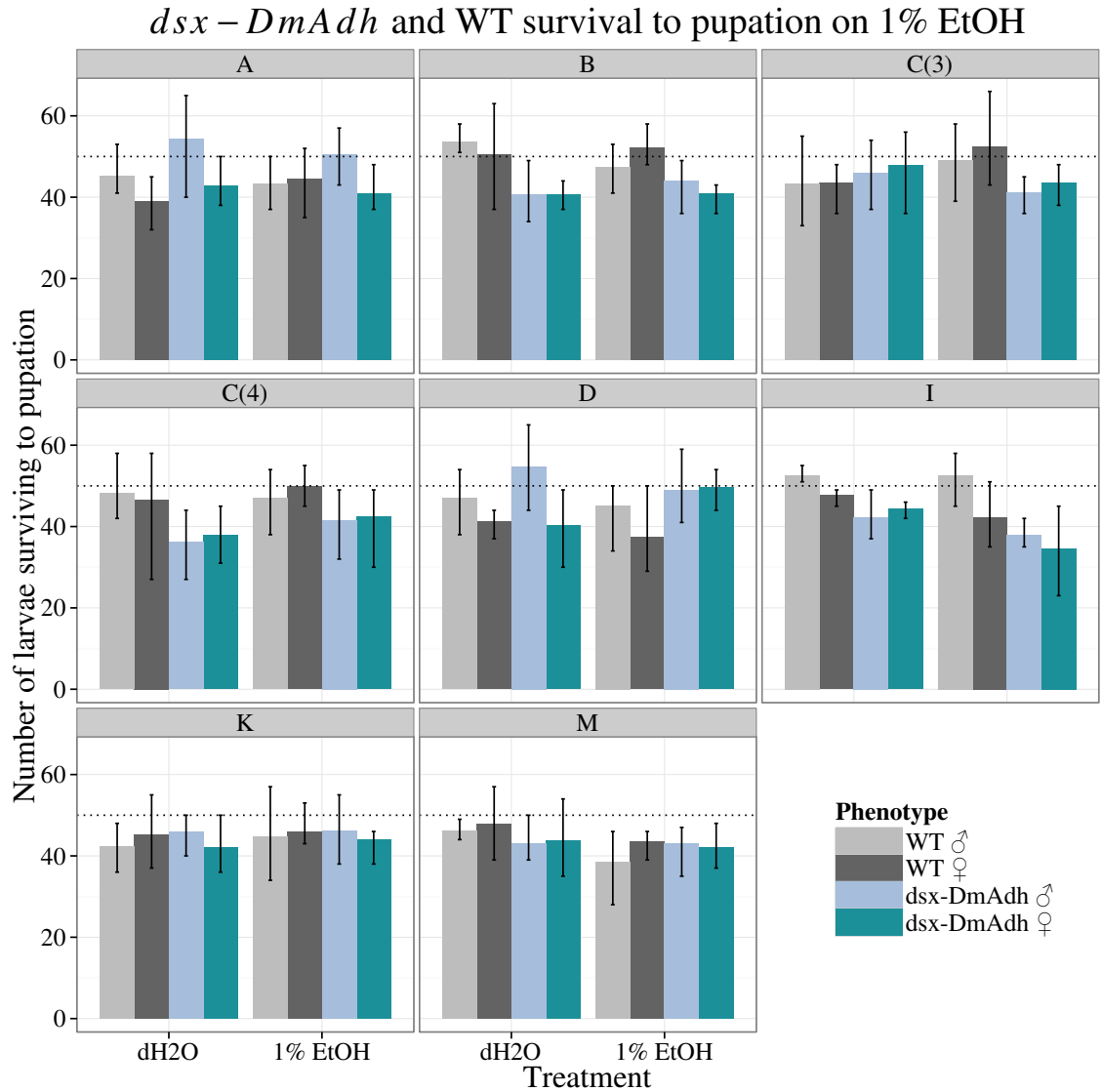


Figure 5.12: Count data showing number of larvae surviving to pupation per phenotype in each *dsx-DmAdh* line tested. Bars represent mean of five replicates of 200 heterozygous larvae with error bars representing range of data. Dotted line indicates maximum expected number of survivors given 100% survival and exact theoretical distribution of genotypes (50% expressing the transgene and a 1:1 sex ratio).

variable survival compared to WT, being slightly increased in lines *dsx-DmAdh*-A and *dsx-DmAdh*-D across all sexes and treatments (*dsx-DmAdh*-A: coefficient = 0.093, $z = 1.978$, $p < 0.05$. *dsx-DmAdh*-B: coefficient = 0.125, $z = 2.669$, $p < 0.01$) but reduced in *dsx-DmAdh*-B, *dsx-DmAdh*-C4 and *dsx-DmAdh*-I (*dsx-DmAdh*-B: coefficient = -0.204, $z = -4.366$, $p < 0.001$. *dsx-DmAdh*-C4: coefficient = -0.193, $z = -4.012$, $p < 0.001$. *dsx-DmAdh*-I: coefficient = -0.203, $z = -3.305$, $p < 0.001$). Lines *dsx-DmAdh*-

A and *dsx-DmAdh*-D also showed increased male survival in both transgenics and WT averaged over treatments (*dsx-DmAdh*-A: coefficient = 0.145, $z = 3.060$, $p < 0.01$. *dsx-DmAdh*-D: coefficient = 0.152, $z = 3.230$, $p < 0.01$). Overall, the results show that inducible lethality is not occurring but in general, transgenesis with the *dsx-DmAdh* construct was not detrimental to survival.

5.3.6 *DmAdh* transcript levels in *dsx-DmAdh* lines versus *D. suzukii*

As no transgenic lines displayed a lethal phenotype despite the correct spliceform being detected in *dsx-DmAdh*-B female pupae, two hypotheses to account for this observation were examined. Firstly that the lack of activity was due to low transcript abundance of *DmAdh* overall and specifically of the *dsx*-exon-4-5b-6-*DmAdh* transcript that should generate functional Adh. Secondly, that there was limited DmAdh enzymatic activity. *Drosophila suzukii* was chosen as a positive control and comparator for all assays as *Drosophila* are known to produce physiologically significant levels of DmAdh (Section 5.1.1).

qRT-PCR was undertaken on pools of females from *dsx-DmAdh* transgenic lines to determine if low levels of transcription could provide an explanation for the lack of observed activity. Transgenic *DmAdh* transcript levels were compared to endogenous *D. suzukii* Adh (*DsAdh*) using identical primers and expression was predicted from a standard curve of *dsx-DmAdh* plasmid DNA of known copy number (Figure 5.13). Due to the use of DNA as a standard for cDNA samples, this assay is suitable for determining relative expression in *Ae. aegypti* compared to *D. suzukii* but raw copy number should only be regarded as an estimate. Expression of the *dsx*-5a exon was then determined in the same manner to estimate the approximate percentage of *DmAdh* that is expressed as an out-of-frame spliceform.

Mean transgenic *DmAdh* expression was between 15.5 and 42.9% of endogenous *D. suzukii* Adh expression but at least 14.5 - 21.6% of that total is expected to be out of frame for Adh translation. Differences in expression were statistically significant between *D. suzukii* and the *Ae. aegypti* lines A, B, C and K (ANOVA on log predicted values: $F = 9.365$, $p = 0.000792$. Tukey post-hoc: *Ds* - *dsx-DmAdh* lines, $p < 0.005$). Although *dsx-DmAdh*-M *DmAdh* expression was less than half (42.9%) *DsAdh* expression, this was not statistically significantly different (Tukey post-hoc, $p = 0.194$). There was also no significant difference in expression between any of the

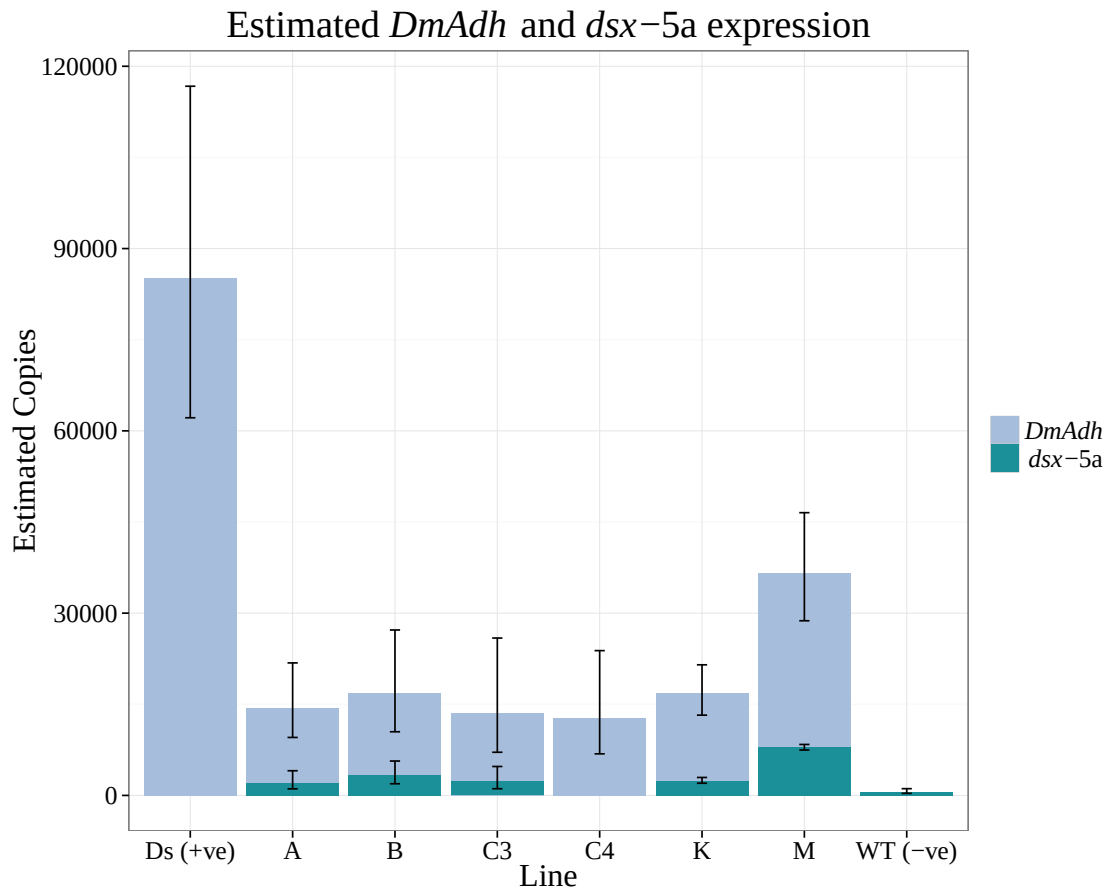


Figure 5.13: Estimated copy number of *DmAdh* and *dsx-5a* transcript in pools of female heterozygote *dsx-DmAdh* lines compared to an *dsx-DmAdh* plasmid standard curve of known copy number. Positive control: mixed sex pool of *D. suzukii* adults (Ds). Negative control: Non-transgenic WT females.

transgenic lines. Physiologically relevant levels of protein are therefore expected in at least one line and some activity might reasonably be expected in the other lines based on this result.

5.3.7 *DmAdh* enzyme activity in *dsx-DmAdh* lines versus *D. suzukii*

To assess *DmAdh* enzymatic activity, a colourimetric assay was adapted from Fibla and González-Duarte (1993). Crude extracts of 20 male or female *Ae. aegypti* pupae were incubated for two hours with an ethanol substrate in the presence of Nitro Blue Tetrazolium, a colourimetric indicator of NADH, the reduced form of NAD⁺ generated during *Adh* mediated catabolism of ethanol (Figure 5.14). Initial experiments conducted with five pupae did not yield positive results over 60 mins (data not

shown) and even a five-fold increase in biomass plus doubling incubation time did not produce any detectable production of NADH, in contrast to a visibly striking colour change in wells containing *D. suzukii* extract.

The assay was sensitive to as little as 31.25 μ M using NADH standards but this was likely impaired in mosquito samples because there was background absorbance from melanin retained in the crude extract, particularly at higher biomass. To account for this background, NADH production was predicted from a standard curve using change in absorption at 590 nm over the course of the experiment. To account for differences in biomass, total NADH produced was normalised to protein content measured using a Bradford assay, taking the ratio of 590 nm/450 nm absorption which scales linearly with protein concentration (Zor and Selinger, 1996). In mosquito samples absorption at 590 nm decreased over 120 min so negative values were returned. This is an experimental artefact and likely related to changes in absorbance of melanin over the course of measurement. It is not related to Adh activity because the same pattern is seen in all WT and male samples, but it does suggest that sensitivity of the assay is decreased in mosquito samples.

To ensure that activity at higher levels could be detected if present, a female mosquito sample was spiked with increasing concentrations of NADH. A change in absorbance was easily detectable at 0.4 mM, which is just 40 % of the 0.95 mM detected in *D. suzukii* sample. As total protein concentration in female mosquito extracts was four times that in *D. suzukii*, this suggests that a difference should have been detected if *Ae. aegypti* was expressing as little as 10 % of the functional Adh produced by *D. suzukii*, assuming that DsAdh and DmAdh have comparable affinity for ethanol. This is highly likely as the protein sequences are 99.6% similar with only one amino acid mismatch.

Taken together, these results suggest that at least one of the *dsx-DmAdh* expressing lines is producing functional transcript at levels that should show detectable activity, even accounting for impaired sensitivity due to melanin content. This is not observed, suggesting that the protein is either not translated or is not functional.

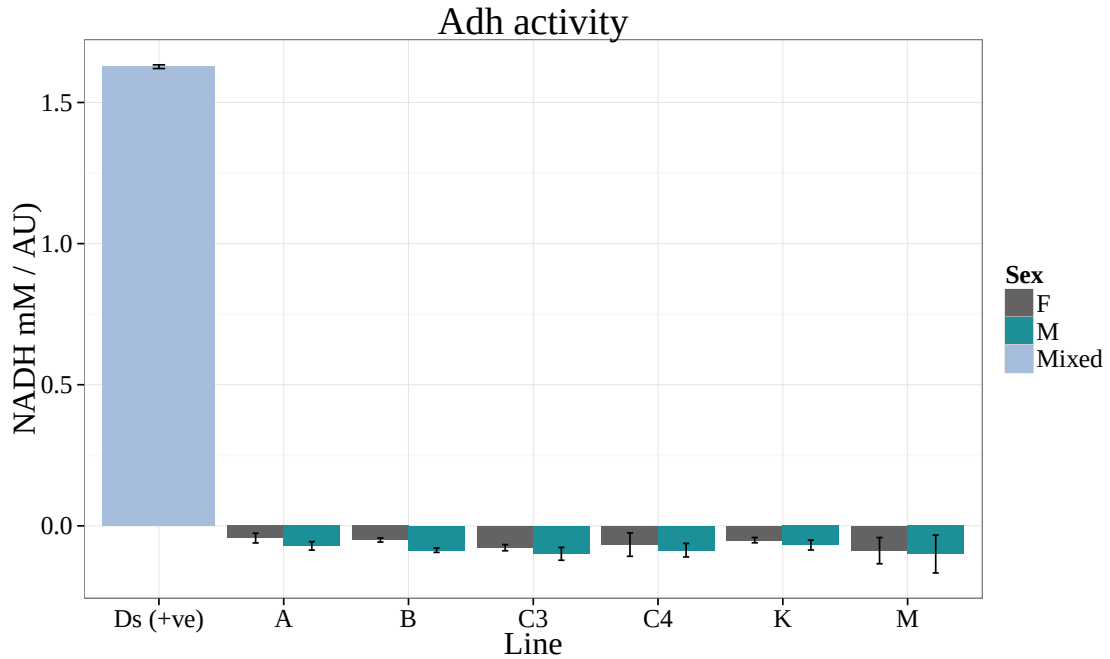


Figure 5.14: Enzyme activity assay performed with male and female pools of heterozygote *dsx-DmAdh* lines. Positive control: mixed sex pool of *D. sukukii* adults (Ds). Males provided the negative control as they were not found to express an in-frame *DmAdh* spliceform. Bars show mean faanal NADH concentration from three biological replicates as predicted from change in absorbance at 590 nm over 120 min using a linear model based on a standard curve of pure NADH. This was normalised to protein concentration using the Bradford assay A590/A450 ratio of absorbance to give NADH mM / Absorbance Units (AU). Error bars show standard deviation of the mean. Absorption at 590 nm decreased in mosquito samples over 120 min, resulting in negative values. This appears to be an experimental artefact due to high melanin content in the samples, which absorbs in the same range. Spiking of samples with pure NADH indicated that a change would still be detectable at 0.4 mM, meaning that that a difference should have been detected if *Ae. aegypti* was expressing as little as 10 % of the functional Adh produced by *D. sukukii*.

5.4 Discussion

5.4.1 *Ae. aegypti* alcohol metabolism

Alcohol metabolism in *Ae. aegypti* has understandably not been studied in any detail, which makes it difficult to predict the consequences of moving one component of the complex *D. melanogaster* metabolic network and embedding it in another species which has evolved under very different conditions. While *Ae. aegypti* has several genes containing Adh domains as documented in this chapter, they have very different expression profiles to *DmAdh*, significantly not being expressed highly in larvae. They are phylogenetically related to *DmAdh* but dissimilar at the protein

level, suggesting a lack of functional homology. The most compelling evidence that these genes are unlikely to have evolved the ability to efficiently metabolise simple alcohols is the ecology and evolution of *Ae. aegypti*, which diverged from the Dipteran branch leading to Drosophilidae and other fruit flies more than 270 million years ago (timetree.org, Hedges et al. 2006). This divergence predates the evolution of angiosperms and their fruits that are thought to have provided the selection pressure for the evolution of Adh in fruit-ovipositing flies such as the Drosophilidae and Tephritidae (hypothesised by Ashburner 1998 and supported by Brogna et al. (2001); Hernández-Tobías et al. (2011)). This evidence alongside the documented examples of Adh domain containing proteins in *D. melanogaster* which are only incidentally involved in ethanol metabolism, for instance photoreceptor dehydrogenase (Pdh) involved in retinol metabolism *in vivo*, suggests that significant involvement in alcohol metabolism is unlikely and any interference with *dsx-DmAdh* as a GSS can be discounted, although functional testing would be required to confirm this.

The high expression and conserved protein sequence of at least three Aldh domain containing genes in *Ae. aegypti* suggests that it may have endogenous capacity to oxidise acetaldehyde to less toxic acetic acid. However, in addition to expression levels being lower than the corresponding genes in *D. melanogaster*, acetaldehyde levels in *Ae. aegypti* cells are unlikely to reach the levels seen in *Drosophila* because the main process of production is catabolism of ethanol or fermentation which do not normally occur in the species. In the absence of selection pressure for high affinity to acetaldehyde as a substrate, these enzymes may be optimised to other aldehydes and therefore have sufficiently limited ability to process acetaldehyde that capacity was overwhelmed by the quantity produced in the original bisex lethal DmAdh expressing lines generated by Collado (2013). There are more likely biological roles for DmAldh orthologous proteins in *Ae. aegypti* and in the many other animal species with little natural exposure to ethanol, for instance Chakraborty and Fry (2011) demonstrate that DmAldh also plays a vital role in detoxifying reactive aldehydes that are generated by lipid peroxidation and Lumjuan et al. (2014) identify a potential role of *Ae. aegypti* Aldh in permethrin resistance.

The results presented here thus provide no additional conclusive evidence for the hypothesis that lethality caused by DmAdh catalysed ethanol metabolism in *Ae. aegypti* is due to the build up of toxic acetaldehyde which the mosquito has insufficient

endogenous capacity to detoxify. The identification of potential genes involved in alcohol metabolism and experimental methods developed in this chapter will contribute to future work on the molecular mechanism of lethality. For example, the affinity of *Ae. aegypti* Aldh enzymes for acetaldehyde as a substrate could be tested using minor modifications to the Adh enzyme activity assay described in section 5.2.8 as Aldh also reduces NAD⁺ to NADH (Figure 5.1) and thus the same colourimetric reporter can be used. The NOAEL values of various alcohols in addition to ethanol also provide a resource for future experiments to both explore the metabolic capabilities of *Ae. aegypti* and hone a *dsx-DmAdh* GSS larval treatment regime to achieve maximal female lethality with minimal male fitness effects.

5.4.2 Developing *dsx-DmAdh* expression as a GSS

None of the eight putatively independent transgenic lines generated in this chapter demonstrated a female lethal effect on long term exposure to ethanol at the NOAEL dose for wild-type *Ae. aegypti*. The removal of endogenous ATG sites and other minor modifications made to the *dsx-DmAdh* construct following failure of inducible sexing in lines generated by Collado (2013) were thus insufficient to restore the functionality seen in the earlier bisex *DmAdh* expressing system, suggesting that erroneous translation from multiple spliceforms was not the primary reason for the previous lack of sex discrimination, although it may have played a role.

In the newly generated lines, transcript coding for Adh was present in females and not males, albeit at less than 15 - 40% of levels observed in *D. sukukii*, which was used as a positive control and comparator. Spliceforms containing the *dsx*-5a exon and hence out of frame *DmAdh* contributed to a minority of total expression, which is in line with evidence from end-point PCR and lends support to the *dsx-DmAdh* construct design using *dsx*-exon-4-5b-6 as the in-frame spliceform. Despite correct splicing and mRNA expression, no evidence was found for Adh enzyme activity using a colourimetric assay, which indicates that the protein is non-functional or produced at levels which are unlikely to be physiologically relevant. Sensitivity of the assay was unfortunately reduced due to interference by melanin pigment meaning that the 15 - 20% relative transcription observed in the majority of lines would put corresponding protein levels on the threshold of detectability even assuming a very high correlation between transcript and protein level. However, the *dsx-DmAdh*-M line showed 40% relative transcription compared to *D. sukukii* and enzyme activity

was still undetectable. In conjunction with the lack of any lethal effect following 1% ethanol treatment, this result clearly demonstrates that any existing Adh activity in the transgenic heterozygote lines is below the threshold where it is biologically relevant for a GSS. The two priorities for future development of the *dsx-DmAdh* early inducible sexing system are thus ensuring that the DmAdh produced is functional and increasing transcript levels in transgenic lines.

The simplest assay modification to determine DmAdh activity with greater sensitivity would be to use crude extracts of transgenic larvae rather than pupae to reduce interference from melanin. Further purification of DmAdh could also be useful, such as the separation protocol followed by on-gel activity assay developed by Ursprung and Leone (1965) and later variants. DmAdh in the original bisex expressing construct developed by Collado (2013) was functional, so if this version proves not to be then the modifications made in developing the *dsx-DmAdh* construct tested by Collado (2013) and carried forward to the current *dsx-DmAdh* construct are obvious targets for causing mis-folding or other protein level defects resulting in loss of function. The major difference expected to affect translation and post-translational processing was the inclusion of a fused Ubiquitin-DmAdh element. Ubiquitin is cleaved at the N-terminus by specific proteases to free the protein of interest and is thought to enhance expression of transgenic proteins (Butt et al., 1989). However, the original bisex construct was able to produce lethal levels of DmAdh without fusion to Ubiquitin, therefore it could be removed, although this would leave a nonsense N-terminal tag due to the placement of the ATG start site ahead of the splice junctions. Alternatively, an amino acid linker could be inserted between the fused sequences to enable greater access for proteases to the N-terminus cleavage site, as also suggested by Collado (2013) following Franco et al. (2011).

If functionality of DmAdh is established by the more sensitive assays discussed above, transcript level could be increased in several ways, the simplest being to in-breed the highest expressing transgenic line produced in this chapter (*dsx-DmAdh-M*). If a viable homozygous line is generated carrying two transgenic alleles then DmAdh expression could be increased, potentially as much as two times. The threshold activity for lethality may be sudden as observed in the alcohol toxicity studies with wild-type *Ae. aegypti* where the range of concentrations between the NOAEL and 100% lethality was relatively small. In particular if the lethal mechanism is build up

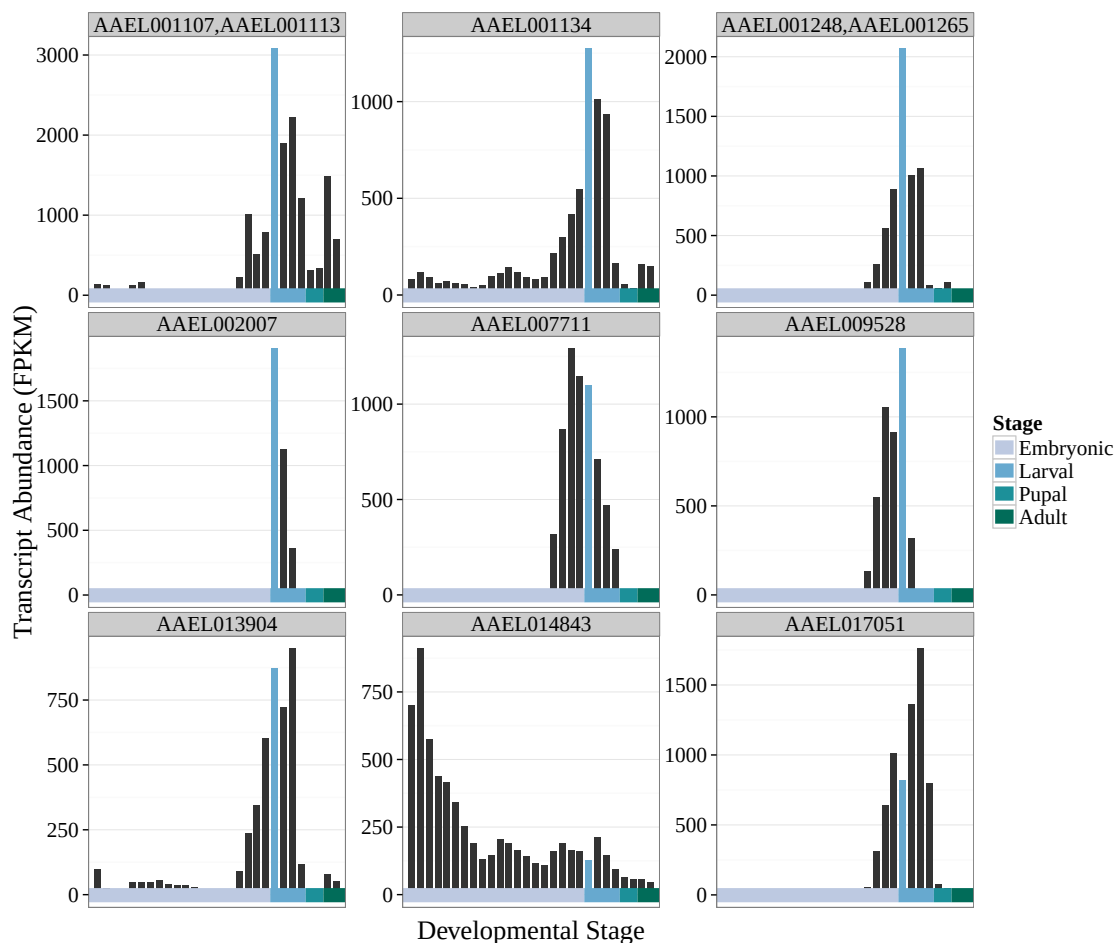


Figure 5.15: Normalised RNA-Seq transcript abundance data for a selection of putative late embryo/early larval stage promoters. The developmental stage highlighted in blue corresponds to L1 larvae, when alcohol would be administered to induce female lethality during mass-rearing. Expression data for *Hsp83* (AAEL014843), which is under the control of the current promoter driving expression of *dsx-DmAdh*, is provided for comparison.

of toxic acetaldehyde, there may be a sharp transition at which endogenous detoxification capacity is overwhelmed and thus a doubling of *DmAdh* expression or less may be sufficient to produce sex discrimination.

Another potential solution is to continue injecting the *dsx-DmAdh* construct to generate more lines for testing, as expression may be constrained by positional effects arising from the genomic environment of the transgene due to cis-regulatory elements, chromatin structure and a host of other variables. Insertion into another position might hence produce a more favourable level of transcription.

The final methods involve modifying the construct and could therefore be pursued even if modifications need to be made to the Ubiquitin-DmAdh fusion element. The

first is to re-engineer the spliceforms so all female transcripts produce in-frame *DmAdh* for translation. Based on qRT-PCR results this would mean that 20% more transcript is translated to functional Adh. This comparatively slight gain could be bolstered by using a stronger promoter than *Hsp83*. The other two commonly used promoters in Oxitec Ltd. transgenic strains are from *AeHsp70*, which shows similar expression to *AeHsp83* and *Hexamerin 1.2*, which was thought to specifically induce expression in female larvae from L4 onwards (Totten et al., 2013) but also shows early larval activity (Oxitec Ltd, unpublished data).

The *Hexamerin 1.2* promoter should therefore replace *AeHsp83* in the next construct design but alternatives would be desirable. Combining a genetic engineering approach with recent high-throughput *Ae. aegypti* transcriptome datasets provides an opportunity to select candidate promoters tailored to specific expression profiles matching the intended application of the transgenic system. Considering the practical use of inducible early sexing within a mass-rearing context, it is likely that eggs would be hatched overnight and alcohol supplied at L1 stage before larvae are aliquoted into trays. Ideally the long term treatment tested in this chapter could be replaced by a shorter exposure to the chosen alcohol, potentially at a higher concentration. Therefore, late embryo expression would be advantageous as protein would build up prior to treatment and continuation of expression through at least early larval stages would also be important. Later expression would be unnecessary but most likely unproblematic. Several candidate genes for promoter extraction which demonstrate the desired expression profile were selected from the Akbari et al. (2013a) transcriptome dataset (Figure 5.15). All could potentially drive expression an order of magnitude higher than that recorded for the *AeHsp83* promoter (AAEL014843). Verification of expression patterns by PCR is necessary and further research into their likely tissue specification would be required, with high expression in fat body being ideal as this is the canonical site of ethanol metabolism in insects.

5.4.3 Conclusions

Early genetic sexing is a priority for the mass-rearing of *Ae. aegypti* for male-only release programmes due to cost, space, time and resources savings gained by early removal of females. As orthogonal expression systems for use in conjunction with tetracycline-repressible approaches are under development but some way from completion, inducible sexing using a simple metabolic approach such as DmAdh induced

susceptibility to environmental alcohols remains an attractive option. This chapter does not demonstrate a functional inducible sexing system but builds on the work of Collado (2013) in generating eight new transgenic lines, confirming sex-specific expression of *dsx-DmAdh* spliceforms and providing additional data for future genetic and biochemical investigations of ethanol metabolism in *Ae. aegypti*. I also highlight the need for further verification of DmAdh functionality while making suggestions to address low transcript expression which in combination should improve future iterations in the development of *dsx-DmAdh* expression as a GSS.

Chapter 6

General Discussion

The arbovirus vectors *Ae. albopictus* and *Ae. aegypti* represent a significant threat to human health, particularly through transmission of DENV and CHIKV in their expanding range. As vector control is the only effective preventative measure currently deployed, improving methods to suppress or replace *Aedes* mosquitoes is vital for disease control. This thesis sought to improve understanding and application of two heritable control methods for *Aedes*: *Wolbachia*-induced viral refractoriness for population replacement in *Ae. albopictus* and release of insects carrying a female-specific dominant lethal mutation to suppress *Ae. aegypti* populations.

Chapter 2 examined a hypothesis for the molecular mechanism underpinning *wMel* induced DENV refractoriness in *Ae. albopictus*. Results from *Ae. aegypti* led Pan et al. (2012) to hypothesise that raised ROS levels caused by upregulation of a Duox enzyme were inducing the Toll innate immune pathway and targeting pathogens via AMPs. Investigations in *Ae. albopictus* revealed no evidence for increased ROS production or significant upregulation in any of the innate immune pathways in either *Ae. albopictus* Aa23-T cell lines infected with *wMel* or *wMelPop* or *wMel*-infected Uju adult strains. ROS generating pathways, antioxidant genes and regulators similarly showed no sign of *Wolbachia*-induced modulation, suggesting that there is an alternative dominant causal factor in *Ae. albopictus*.

Chapter 3 then sought evidence for the hypothesis that lipid and cholesterol metabolism could be altered by *Wolbachia* infection and thus hold potential for impacting DENV replication. Some evidence for a reduction in cholesterol was found from qRT-PCR of genes implicated in cholesterol metabolism and mass spectrometry of lipid extracts from *Ae. albopictus* Aa23-T cell lines. *Wolbachia*-host resource competition over cholesterol was indicated, as demonstrated by reduced *Wolbachia* titres

on cholesterol depletion in Aa23-T.*wMel* cells. There was little evidence for major changes in lipid pathways or autophagy at the transcriptional level but DIMS and LCMS analysis of the Aa23-T lipidome compared to cells infected with *wMel* and *wMelPop* revealed substantial shifts in the cellular lipid profile. Most significantly, sphingolipids were depleted across all classes and some reduction in diacylglycerol fatty acids and phosphatidylcholines was noted. These lipid classes are selectively enriched in DENV-infected mosquito cells (Perera et al., 2012), thus suggesting that *Wolbachia* may produce a cellular lipid environment that is hostile to viral replication. This result provides a promising avenue for future experiments to uncover the functional significance of the observed changes for host interactions with both *Wolbachia* and DENV. It is clear that greater use of modern high-throughput analysis techniques such as lipidomics and other 'omics approaches will enhance our understanding of basic virology and host-symbiont interactions.

Existing data from *Ae. aegypti* and *D. melanogaster* indicates a role for innate immunity and cholesterol respectively, so this novel data on lipids suggests a multifactorial mechanism could be underlying *Wolbachia*-induced viral refractoriness, which may include a common dominant factor that is yet to be verified. This would be beneficial for longer term population control strategies as it would reduce the risk of resistance arising in either virus or host, capitalising on the co-evolution advantage of biological control compared to static chemical controls, which frequently lead to population level resistance (Section 1.3.1). However, resistance remains a risk and without a detailed understanding of the mechanism of viral refractoriness it is difficult to model the contexts in which it could arise and therefore plan appropriate monitoring of all three organisms or formulate a strategy for prevention or rescue, such as the release of an alternative *Wolbachia* strain.

One key concern is that fluctuations in density could reduce symbiont-mediated protection. Viral refractoriness has already been shown to be density dependent (Lu et al., 2012) and results in Chapter 3 show that lipid profile changes are more profound at higher *Wolbachia* densities. Cholesterol depletion via cyclodextrin was shown in Chapter 3 to potentially reduce *Wolbachia* densities, indicating that basic ecological parameters such as larval diet could have an impact. Work is ongoing to better understand key regulators of *Wolbachia* density (reviewed in Section 1.5.5) and release populations are being closely monitored in recognition of the importance of infection density, which so far has not been attenuated in openly released *wMel* infected *Ae.*

aegypti (Frentiu et al., 2014). Another scenario is incomplete blocking leading to natural selection for resistant viruses, particularly as modelling suggests increases in virulence are likely in the co-evolution of pathogens and protective symbionts (Jones et al., 2011). Better understanding of these interactions through mechanistic knowledge and potentially experimental evolution to screen for resistant mutant hosts or viruses is therefore useful for planning the future of *Wolbachia*-based population replacement within an integrated vector management system combined with public health measures targeting the virus, including surveillance and future vaccines.

Release of mosquitoes infected with highly invasive *Wolbachia* without mechanistic understanding of the system has only been possible from a regulatory perspective as *Wolbachia* transinfection is not considered a genetic modification due to neither organism containing transgenes. In addition, a precedent is provided by certain naturally occurring *Wolbachia* strains in mosquitoes e.g. *wAlbA/B*. In open releases in Australia, *Wolbachia*-infected *Ae. aegypti* was regulated as a ‘Veterinary Chemical product’ (De Barro et al., 2011). In contrast, GM risk assessments require data on the modification and its mechanism in the species (Mumford, 2012).

Chapter 4 examined an engineered female-specific dominant lethal genetic system which like *Wolbachia* is clearly effective but uncertainties about the exact molecular mechanism of its lethal effector remain, albeit to a far lesser extent. This made it desirable to test new lethal effectors with better characterised modes of action as well as specific promoters to give finer grained control of their spatio-temporal expression, in order to produce reliable late-acting lethality that would be reproducible in future lines without relying on random positional effects in the genome. A suitable promoter from the Osiris gene family was found and shown to induce female-specific, late pupal lethality in combination with the Nipp1 lethal effector and sex-specific alternative splicing of *dsx*. Lethality did not reach 100% but proof of principle was obtained.

Chapter 5 attempted to solve a major practical barrier to male-only releases as utilised in dominant lethal and *Wolbachia* CI-mediated population suppression strategies: the need for genetic sexing to remove mechanical sorting requirements and free up space and resources for rearing larger cohorts of males. An ethanol inducible, female-specific genetic sexing system was trialled in *Ae. aegypti* using female-specific expression of *DmAdh*. No functional lines were generated and assays analysing Adh activity and gene expression suggested that a stronger promoter may be necessary to

deliver a detectable result. Data on alcohol toxicity in *Ae. aegypti* was collected to parameterise future experiments using ethanol inducible systems.

Scientific understanding is required at all stages in the vector control process to improve both effectiveness, economy and safety. Risk mitigation by manual sexing was specifically cited in the Malaysian Government report on regulation of dominant lethal *Ae. aegypti* (Malaysia, 2010) and a robust genetic method could deliver more assurance that release of biting females will be minimised. The major time and resource saving as a result of a successfully implemented genetic sexing mechanism could result in greater uptake of the technology when fed into cost-benefit models for policy makers (e.g. the model framework produced by Alphey et al., 2011).

The control approaches in this thesis are only part of a growing toolbox for mosquito genetic control and are not themselves mutually exclusive, for instance reducing wild type populations through genetic population suppression using dominant lethal lines prior to a *Wolbachia* population replacement programme could increase the likelihood of successful replacement. Hancock et al. (2011) produced models to support this strategy and also found that a heavily male-biased release required the overall release of fewer females to achieve the same level of *Wolbachia* invasion, which is positive as it reduces biting and disease transmission risk. Genetic sexing as discussed here could be used on a subsection of the *Wolbachia*-infected release generation to achieve this. However, while a *Wolbachia* ‘effector’ is clearly orthogonal to the female-lethal approach in Chapter 4, a tetracycline-repressible system would be problematic because *Wolbachia* are susceptible to this antibiotic, therefore an alternative expression system or an inducible sexing method as explored in Chapter 5 would be required.

Other tools currently under development will increase the future versatility of the transgenic sexing approaches explored here. For example, sperm lethal systems would allow early genetic sexing by female-specific dominant lethal expression to be coupled with bisex lethality that is controlled under the same expression system. However, sperm lethal systems remove the possibility of late-acting lethality in the F_1 generation, illustrating the trade-offs that have to be considered in design of genetic control approaches.

In conclusion, genetic methods hold great potential for the control of *Aedes* mosquitoes. Challenges remain in terms of public acceptance, regulation, scaling of mass production and optimising the implementation and monitoring of releases, but both ap-

proaches investigated in this thesis are currently deployed in the field with promising results. The findings here provide a basis for improving their implementation through greater knowledge of the underlying mechanism of action and proof of principle for more targeted design and engineering of lethal phenotypes, although in all cases further work is required to deliver a corroborated and complete picture or to generate fully functional systems.

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