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OXITEC LTD & DEPT OF ZOOLOGY, UNIVERSITY OF OXFORD

Improving Transgenic Approaches to Mosquito Population Control

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ABSTRACT

The disease vectors *Aedes aegypti* and *Aedes albopictus* are serious and growing threats to global health. As vectors of the arboviruses dengue fever and chikungunya, these mosquitoes are responsible for hundreds of millions of cases and thousands of deaths each year. Absent specific treatments or vaccines, effective control of mosquito populations remains the only option for tackling a growing public health challenge. More effective control tools are urgently needed. Recently, a novel approach to pest population control has been developed based on the release of insects carrying a repressible, dominant lethal allele. This approach has achieved dramatic reductions in *Ae. aegypti* populations in regulated open field experiments. Despite this success, there remains scope to improve upon the current technology. It is proposed that an 'ideal' strain would combine the following features: (i) repressible lethality in late juvenile phases; (ii) a mechanism for removing females at an early developmental stage in the release generation; and (iii) orthogonal expression control mechanisms allowing both these systems to be combined in a single strain.

This thesis describes research undertaken in pursuit of a 'next generation' strain. Two novel promoters from putative *Osiris* genes have been identified which confer a 10^2 - 10^3 – fold up-regulation in transgene expression specific to late pupal stages. One of these '*Osiris*' promoters has been used to develop transgenic *Aedes aegypti* strains. 5 lines showed pupal-specific lethality of 98-100% penetrance, which was repressed in the presence of tetracycline. An *Ae. albopictus* orthologue of the sex-determining gene *doublesex* (*dsx*) has been isolated and characterised and a female-specific expression system developed. Transgenic lines show female-specific expression of a transgene; however, there remains some 'leaky' expression in male insects. Finally, a potential expression control tool based on an auxin-inducible expression system has been investigated. 11 different transgenic lines were developed based on three different construct designs. None showed auxin-inducible expression of a transgene.

AUTHOR'S DECLARATION AND STATEMENT ON CONFIDENTIALITY

I declare that all work here presented is my own, subject to those specific exceptions which are indicated in the text

Elements of the work presented here are of a commercially sensitive nature. This document should therefore be treated as strictly confidential and neither the document itself nor any part thereof distributed to unauthorised parties.

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1. INTRODUCTION

Mosquito-borne diseases infect hundreds of millions of people, and are responsible for close to a million deaths each year. Recent decades have witnessed an explosion in the global case load resulting from mosquito-borne arboviruses, in particular dengue fever and chikungunya, as increasing urbanisation, the expansion of global trade and travel, and a changing climate, have all contributed to the rapid population growth and range expansion of their principal vectors, *Ae. aegypti* and *Ae. albopictus*. With no vaccine, and absent the treatment and control options which have begun to prove effective in the battle against malaria (WHO, 2013), reducing populations of these mosquito vectors remains the only viable option for meeting a growing global health challenge. With conventional approaches continuing to prove inadequate to meet this challenge, new, more effective solutions are urgently needed.

1.1 THE MOSQUITO-BORNE VIRUSES DENGUE FEVER AND CHIKUNGUNYA ARE MAJOR THREATS TO GLOBAL HEALTH

Dengue fever is the world's most widespread arbovirus, responsible for up to 390 million infections every year (Bhatt et al., 2013). It is vectored primarily by two mosquito species, *Aedes aegypti* and *Aedes albopictus*. These species are also the primary vectors of chikungunya. While outbreaks were rare prior to 2000, in recent years chikungunya has emerged as a major public health threat.

1.1.1 SYMPTOMS AND PATHOLOGY

Both dengue viruses (DENV) and chikungunya virus (CHIKV) are transmitted between human hosts through the bite of an infected *Aedes* mosquito. On biting an infected person, the mosquito ingests the virus with the blood meal. The virus first infects the mosquito mid-gut, before spreading to other organs, including the salivary glands. This dissemination can take 8-12 days for DENV, but can be much more rapid for CHIKV (Vazeille et al., 2007).

Dengue fever causes flu-like symptoms which can range from mild fever to severe aching, nausea, rash, headache and vomiting. It is rarely fatal, but dengue haemorrhagic fever (DHF), a more severe complication, can occur in a small percentage of cases. Patients suffering from DHF can experience bleeding from the nose, mouth and gums. These symptoms can progress into circulatory collapse and shock (Dengue Shock Syndrome), which is fatal in about 1-5% of cases (WHO, 2009). The pathology of dengue is complicated by at least four related but distinct viral serotypes; DENV 1-4. The possibility of a fifth serotype was recently announced (Normile, 2013). Infection of a person with a given serotype confers life-long immunity to that type, but long term protection is not conferred for the other three serotypes. The advent of severe disease has been shown to be more common in secondary infections (Halstead et al., 1970).

Chikungunya also causes flu-like symptoms including nausea, fever and severe joint pain, which can often be confused with those of dengue fever. The acute joint pain (arthralgia) triggered by the infection can in some cases evolve into chronic pain and arthritis (de Andrade et al., 2010). A mortality rate of 1/1000 has been reported from past epidemics (Burt et al., 2012).

1.1.2 TREATMENT AND CONTROL

There is currently no specific treatment for either dengue fever or chikungunya. Treatment is symptomatic for mild fever, whereas fluid replacement therapy can be a vital intervention in the more severe forms of the disease (WHO, 2009).

Development of a dengue vaccine is complicated by the existence of four antigenically distinct dengue serotypes, and consequently the need for a vaccine to be tetravalent. The recent report of a possible fifth serotype (Normile, 2013) further complicates the landscape of dengue virology and consequently that of vaccine development. Nonetheless, several such vaccine candidates are in various stages of development (Cassetti and Halstead, 2014). The furthest advanced, a chimeric dengue vaccine based on an attenuated yellow fever vaccine, is currently in phase III testing, though phase II testing raised questions over the vaccine's ability to show efficacy against all DENV

serotypes (Sabchareon et al., 2012). In the absence of clear efficacy against all four serotypes, and particularly in light of the existence of a fifth serotype, the possibility of antibody-dependent enhancement and the question of whether a vaccine could affect this process remains an important safety issue; it is one which will need to be addressed before any vaccine is used widely.

Several candidate vaccines against chikungunya have been developed, one of which, developed by the US army, showed promise in phase II studies (Edelman, 2007). However, no vaccine is currently available, and it remains uncertain whether market incentives are sufficient to support commercial development of such a vaccine (Weaver et al., 2012).

1.1.3 EPIDEMIOLOGY

In recent years, the global incidence of dengue fever has increased rapidly. The World Health Organisation reports that the disease is endemic in over 100 countries, and it has been estimated that up to 2.5 billion people, or around 40% of the global population, may be at risk (WHO, 2014) (see Figure 1-2). More recent modelling suggests that WHO figures of 50-100 million cases each year may significantly underestimate the global disease burden of dengue, and that global annual cases may in fact exceed 300 million (Bhatt et al., 2013). Moreover, the incidence of severe disease also appears to be on the increase: the number of countries reporting cases of severe dengue is rising (Mackenzie et al., 2004), possibly associated with increasing prevalence of multiple dengue serotypes, and hyper-endemicity in many areas (Messina et al., 2014). Severe dengue results in an estimated 20,000 deaths each year (Gubler, 2002), while the number of Disability Adjusted Life Years (DALYs) lost each year has been estimated at 700,000 (Hotez et al., 2009).

Chikungunya, too, is a disease increasing in global prevalence. In 2005, a major epidemic infected an estimated 40% of the population of La Reunion, resulting in over 200 deaths (Boutin, 2006). In India, the economic cost of the same epidemic has been estimated at over 25,000 DALYs (Krishnamoorthy et al., 2009). In 2007, more than 200 people became infected in the Emilia-Romagna region of Northern Italy, in the first example of a major chikungunya outbreak in Europe (Bonilauri et al.,

2008). Over the last year, the disease has swept through the Caribbean, with the latest case estimates approaching 500,000 suspected or confirmed (PAHO, 2014). With cases also occurring in El Salvador, Venezuela and in the United States, large parts of the Americas now appear to be at risk from the disease.

The rapid increase in both incidence and severity of dengue fever and chikungunya has largely been coincident with the expanding distribution and population of the mosquito vectors *Ae. aegypti* and *Ae. albopictus*. In the absence of a vaccine, effective vector control strategies are key to preventing the spread of disease.

1.2 THE YELLOW FEVER MOSQUITO, *AE. AEGYPTI*, AND THE ASIAN TIGER MOSQUITO, *AE. ALBOPICTUS*, ARE THREATS TO PUBLIC HEALTH

Ae. aegypti (Linnaeus) is the primary vector of dengue fever world-wide, whilst *Ae. albopictus* (Skuse) can also act as a vector of dengue as well as a host of other arboviruses (Figure 1-1). While the two species differ in key aspects of their behaviour and habitat, they share an invasive capacity and an expanding global distribution which has resulted in both species being regarded as growing threats to global health.

1.2.1 BIOLOGY AND HABITAT

The life cycle of both *Ae. aegypti* and *Ae. albopictus* involves four distinct stages, and takes around

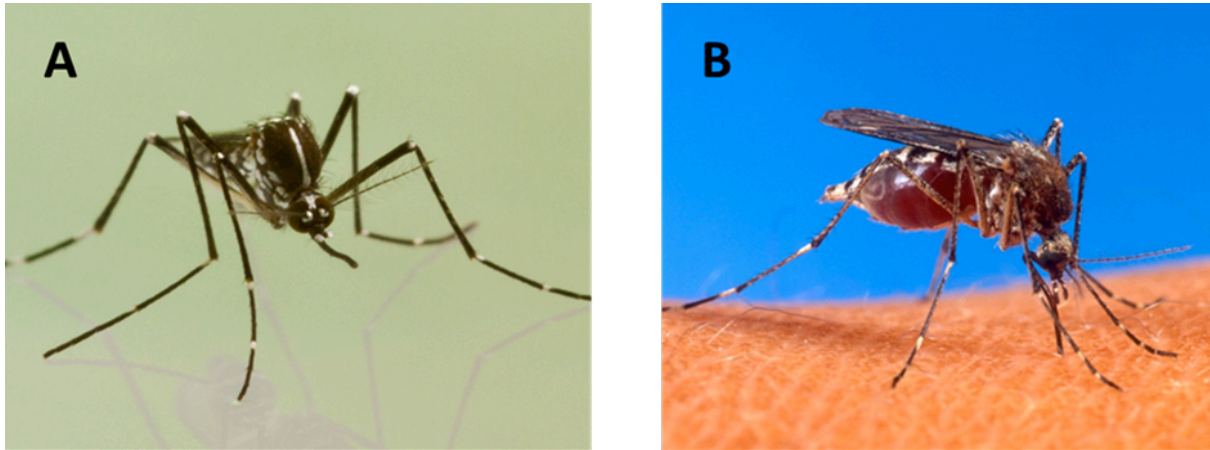


Figure 1-1: Adult *Aedes aegypti* (A) and *Aedes albopictus* (B)

Aedes albopictus is easily distinguished from *Aedes aegypti* due to the distinctive white stripe which bisects its thorax, in contrast to the 'lyre' like markings of *Aedes aegypti*

Photographs courtesy of Jake Galson (Jake Galson Photography) and Derric Nimmo (Oxitec Ltd).

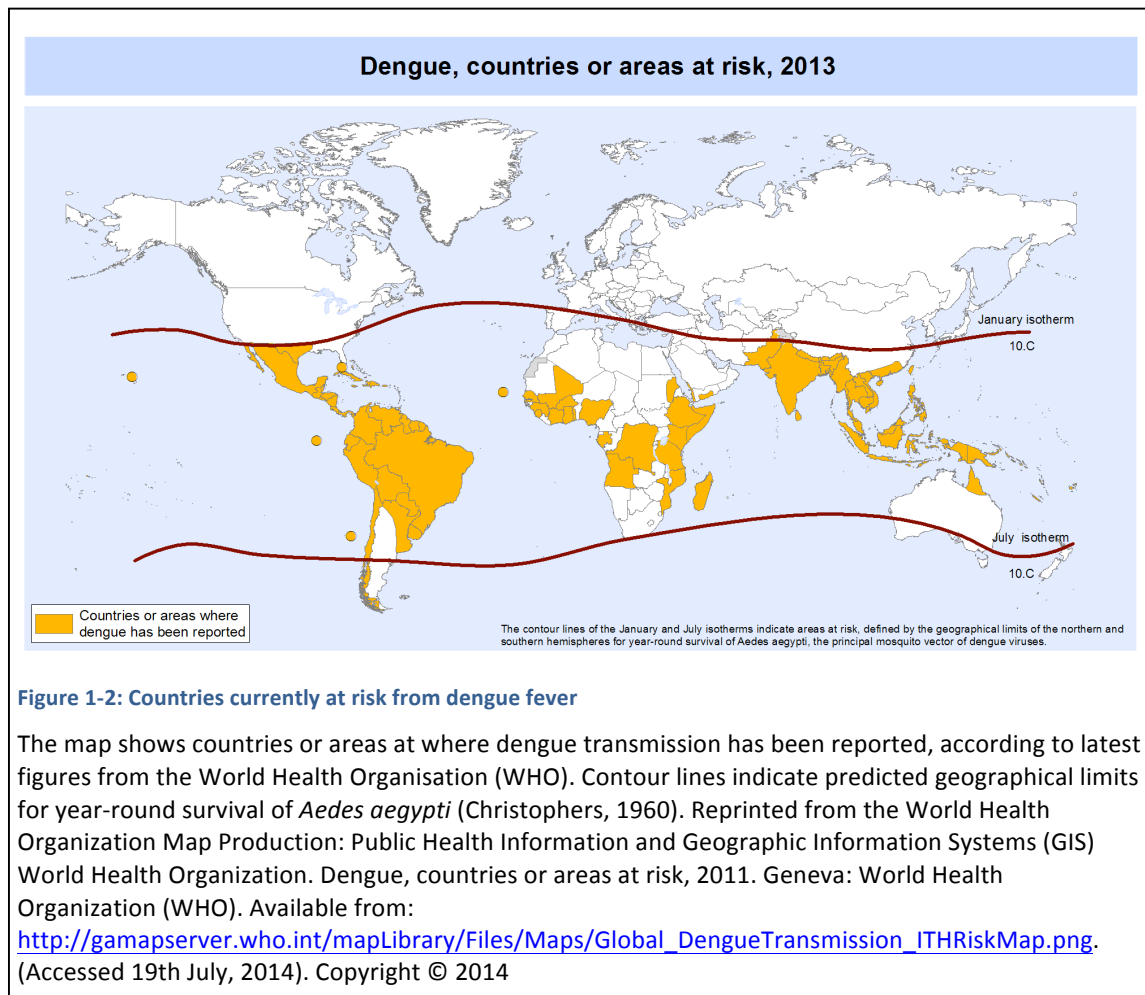
10-14 days to progress from egg to adult mosquito, depending on temperature. Eggs are laid by female mosquitoes on damp surfaces close to standing water. Upon hatching, larvae develop over a period of 7-10 days before pupation. Adult mosquitoes emerge around 48 hrs following pupation.

Ae. aegypti is primarily a tropical species (Christophers, 1960). Originating in Africa, its distribution is at present largely confined to tropical regions and is thought to have been bounded historically by the northern January and southern July 10°C isotherms (Figure 1-2). However, *Ae. aegypti* is a peri-domestic species, preferentially laying its eggs in man-made containers in and around human habitation (Christophers, 1960, Chan K. L. et al., 1971) The close association of *Ae. aegypti* with human habitation and the peri-domestic environment has enabled it to elide what might otherwise have been strict climatic restrictions upon its habitat. Hence, for example, human activity such as cultivation of house plants has enabled the vector to survive in very hot, arid climates such as Arizona (Fink et al.), whilst exploiting underground water storage tanks has enabled it to survive and

vector dengue fever in Rajasthan, Northern India, where summer temperatures regularly exceed 40°C (Tyagi and Hiriyan, 2004). Conversely, *Ae. aegypti* has also been found in colder climates where winter temperatures fall well below its theoretical tolerance limit of 10°C (Reiter, 2001).

While the remarkable hardiness of this mosquito means that it is difficult to establish a clear relationship between climate and potential habitat (Jansen and Beebe, 2010), it is nonetheless possible that a changing climate, introducing warmer, wetter conditions to many areas, will present opportunities for *Ae. aegypti* to expand its range (Naish et al., 2014). Perhaps more than any other factor, however, increasing urbanisation in tropical regions has contributed to the increase in global populations of *Ae. aegypti* (reviewed in (Gubler, 2011a). Unplanned urban expansion in particular, with associated poor sanitation and waste management, has resulting in a burgeoning of potential habitats for the mosquito. Finally, the advent of global trade and travel has presented another means for *Ae. aegypti* to expand its global distribution, in particular via international cargo shipments (Murray et al., 2013).

Ae. aegypti is strongly anthropophilic in its blood-feeding habits, with alternative blood sources forming only a minor part of its diet (Christophers, 1960, Harrington et al., 2001, Ponlawat and Harrington, 2005). Furthermore, it is easily disturbed whilst feeding, and as a result may take several



blood-meals during the same gonotrophic cycle (Platt et al., 1997). These feeding habits, combined with its peri-domestic habitat, contribute to the efficiency of *Ae. aegypti* as a disease vector.

Ae. albopictus, commonly called the Asian tiger mosquito due to its distinct striped scales, is a species endemic to many tropical and sub-tropical regions around the world. Originating in South-East Asia, it is thought to have migrated alongside humans to Madagascar and islands in the Indian Ocean (Smith, 1956). *Ae. albopictus* has also shown itself to be a highly adaptive species; indeed, it is classed as one of the world's top 100 most invasive species by the invasive species specialist group (Lowe et al., 2000). Its global distribution has expanded rapidly in recent years, with populations

being reported in the Middle East, Africa, Australia, the US and Europe (Gratz, 2004, Schaffner, 2001, Sabatini et al., 1990, Sprenger and Wuithiranyagool, 1986, Linthicum et al., 2003).

Like *Ae. aegypti*, the advent of global trade and travel has accelerated the range expansion of *Ae. albopictus*. In particular, the growing international trade in used tyres and ‘lucky bamboo’, which provide ideal breeding sites, has contributed to its rapid global spread (Reiter and Sprenger, 1987, Linthicum et al., 2003, Medlock et al., 2012).

Ae. albopictus is capable of thriving in more temperate conditions than *Ae. aegypti*. The eggs of both species are extremely resistant to desiccation, and once embryonated can survive desiccation for long periods (Russell et al., 2001). However, *Ae. albopictus* is also able to produce diapausing eggs in response to short day lengths, which exhibit higher resistance to desiccation and increased cold tolerance in comparison to non-diapausing embryos (Urbanski et al., 2010, Hanson and Craig, 1994). These characteristics may have contributed to its rapid spread through the milder climes of much of Europe, where it has now been reported in 20 countries (Medlock et al., 2012).

In contrast to the peri-domestic habitat of *Ae. aegypti*, *Ae. albopictus* usually lives on the edges of forests and lays its eggs in natural reservoirs such as tree hollows or coconut shells (Rao et al., 2011). It is a more opportunistic feeder than *Ae. aegypti* and has been found to feed on birds and small reptiles, though its preference appears to be for mammals (Niebylski et al., 1994, Richards et al., 2006).

1.2.2 *AE. AEGYPTI* AND *AE. ALBOPICTUS* AS DISEASE VECTORS

Ae. aegypti is the principal vector of dengue and Dengue Haemorrhagic fever (DHF), considered responsible for the global re-emergence of this disease during the latter decades of the last century (Gubler, 1998). *Ae. aegypti* can also serve as a vector for a number of other flaviviruses, including chikungunya virus (Islam et al., 2012), and yellow fever. *Ae. albopictus* is also a competent vector of all four dengue serotypes, as well as at least 22 different arboviruses (Moore CG, 1997).

An analysis of global dengue epidemics recently confirmed *Ae. aegypti* as the primary vector in the majority of past outbreaks, with *Ae. albopictus* playing a minor role (Lambrechts et al., 2010); moreover, the authors note that *Ae. albopictus* appeared not to be associated with the more explosive epidemics. Studies differ as to whether vector competence for dengue differs significantly between the two species (Vazeille et al., 2001, Vazeille et al., 2003, Richards et al., 2012) and vector competence in any case varies between different populations (Black et al., 2002). The more catholic blood-feeding preferences of *Ae. albopictus*, together with its tendency to feed on just one host per gonotrophic cycle, may explain why dengue outbreaks where it is the principal vector have tended to be milder and less explosive than those involving *Ae. aegypti*. However, *Ae. albopictus* has been responsible for large dengue outbreaks, such as that on La Reunion in 1977 (Coulanges P, 1979); moreover, its wide range of potential habitats mean that it may be of increasing importance as a dengue vector in areas with no *Ae. aegypti* population (Peng et al., 2012).

Ae. albopictus may also be of significance as a maintenance and bridge vector of dengue fever. It is capable of transmitting the virus sexually, and vertical transmission has been reported to be more efficient in *Ae. albopictus* compared with *Ae. Aegypti* (Vazeille et al., 2003). It has also been suggested that dengue fever may be maintained in sylvatic cycles, vectored by *Ae. albopictus* (Scanlon, 1965).

Ae. aegypti and *Ae. albopictus* are the principal vectors of the chikungunya virus. In 2005, *Ae. albopictus* was responsible for a very large chikungunya epidemic in the Indian Ocean territories, which was particularly severe on La Réunion (Boutin, 2006). The subsequent discovery of a single point mutation in the viral genome which is thought to have enhanced its competency to be vectored by *Ae. albopictus* is of particular concern: the same mutation was also present in the 2007 outbreak in Northern Italy (Bonilauri et al., 2008), though not in recent cases of autochthonous transmission in France (Grandadam et al., 2011), both of which were vectored by *Ae. albopictus*. The current explosive epidemic of chikungunya in the Caribbean appears to involve an Asian chikungunya strain, rather than the East/Central/South African strain responsible for the La Réunion

epidemic, though it is not yet clear which of the two aedine species may be primarily responsible for vectoring the outbreak (Van Bortel et al., 2014).

1.3 CURRENT VECTOR CONTROL STRATEGIES FOR *Aedes* MOSQUITOES

As principal vectors of dengue fever and chikungunya, with rapidly expanding global distribution, developing effective control strategies for *Ae. aegypti* and *Ae. albopictus* is increasingly a public health priority. The WHO global strategy for dengue prevention and control aims to reduce dengue morbidity by 50% by 2020 (WHO, 2012). It advocates the principles of Integrated Vector Management (IVM), which in the case of dengue vectors involves the judicious use of insecticides in combination with synergistic approaches, in particular breeding site management. Key approaches currently in common usage are summarised in the paragraphs below.

1.3.1 CHEMICAL CONTROL

The mainstay of control mechanisms against *Ae. aegypti* and *Ae. albopictus* has been a combination of environmental management and chemical control methods. DDT was applied successfully against *Ae. aegypti* and *Ae. albopictus* populations prior to the 1970s, eradicating *Ae. aegypti* in many areas. A continent-wide campaign using DDT in addition to breeding site control succeeded in eliminating *Ae. aegypti* from most of the Americas by the 1960s (Camargo, 1967). The damaging environmental impact of DDT led to its use being banned by the WHO in 1979. Since then, *Ae. aegypti* and dengue fever have re-emerged in all 19 Latin American countries previously certified as *Ae. aegypti* free (Tapia-Conyer et al., 2009). Alternative chemicals have been employed, principally pyrethroids as adulticide sprays, and the organophosphate temephos as a larvicide added to drinking water (WHO, 2009). Unfortunately, the development of resistance to these chemicals has reduced their efficacy whilst at the same time requiring that their use be limited to avoid the further spread of resistance. Furthermore, community members often object to the addition of chemicals to drinking water, or to

the spraying of chemicals inside their homes, with a consequent risk of non-compliance with chemical control methods (Knudsen, 1992).

However, an interesting novel approach shows some promise for enhancing the efficacy of chemical control approaches. Here, adult resting sites are contaminated with a juvenile hormone analogue (JHA). The JHA is then transferred by the adults to larval breeding sites, where it interferes with pupal metamorphosis and adult development. Extremely low levels of JHA can be effective in preventing adult emergence and/or severely compromising the fitness of resulting adults (Hirano et al., 1998), and harnessing adult mosquitoes is an effective means of targeting larval breeding sites. Known as auto-dissemination, the technique has shown some early promise, but field experiments indicate that efficacy can be highly variable between sites and is not fully penetrant (Caputo et al., 2012).

1.3.2 ENVIRONMENTAL MANAGEMENT

Environmental management, in particular monitoring and removing potential breeding sites, is a key component of most dengue prevention and control strategies (Paupy et al., 2009). However, such methods can involve significant investment in manpower and infrastructure, as well as requiring a high level of community engagement. The recent implementation of a successful control programme in Vietnam, involving breeding site elimination and control using biological predators, along with a strong component of community engagement, suggests an important role for the latter in successful environmental management and control (Kay and Vu, 2005).

However, wider evidence for the impact of community-based projects is weak (Heintze et al., 2007), and thus far programmes relying on environmental management have failed to achieve sustainable control of dengue fever or of dengue vectors (Gubler, 2011a). Furthermore, while the removal of artificial breeding sites through community participation can be an effective measure against *Ae. aegypti*, the preference of *Ae. albopictus* for natural breeding sites such as tree hollows means that such interventions may be of limited efficacy in controlling the latter species.

Following the observation that the rapid spread of *Ae. albopictus* had been facilitated in part by trade in used tyres, a number of countries have attempted to regulate this trade in an attempt to control the spread of the mosquito. However, in most cases the economic burden imposed by such regulation has been judged to be too damaging to justify its continuance (Reiter, 1998).

1.3.3 BIOLOGICAL CONTROL

Biological control methods have also been employed against *Aedes* disease vectors with some success. Formulations of *Bacillus thuringiensis* (*var. israelensis*) (Bti) are frequently added to water containers as a larvicide. Resistance to Bti, which combines four different toxins with synergistic activity, has been recorded on one occasion in mosquitoes (Paul A., 2005). Bti resistance has not been observed in *Aedes* species, but the development of resistance pathways towards individual Bti toxins has been noted, and could therefore impact on the use of Bti formulations against *Aedes* species in the future (Boyer et al., 2007). The Copepod *Mesocyclops*, a natural predator of mosquito larvae, has also been utilised as a larvicidal agent in water storage containers in Vietnam (Vu et al., 2005). A trial programme achieved considerable success, with both mosquito numbers and dengue incidence decreasing dramatically during a three year period. However, in addition to operational difficulties involved in implementing a similar programme on a larger scale, there may be cultural barriers to adding small animals to drinking water (Cattand P, 2006).

1.4 THE STERILE INSECT TECHNIQUE (SIT) IS AN EFFECTIVE METHOD OF INSECT PEST CONTROL

The Sterile Insect Technique (SIT) was first developed in the 1950s as an alternative to traditional chemical methods of pest control (Dyck et al., 2005, Knipling, 1955). The technique is based on the release of large numbers of sterilised insects. Sterile males mate with wild female insects and thereby prevent them having offspring or reduce their reproductive potential. They thus reduce the

reproduction rate of the target population. Serial releases of sufficient numbers of sterilised insects can dramatically reduce or eliminate the target population in an area (Dyck et al., 2005).

SIT programmes have been implemented with considerable success against a wide range of insect pests. In the Americas, screwworm populations have been eradicated across the US and Central America through a series of SIT programmes (Wys J.H., 2000). The SIT has also been successfully applied to control a severe screwworm outbreak in Libya (Lindquist et al., 1992). The technique has been used successfully against fruit flies (Enkerlin, 2005), against pink bollworm in the United States (Henneberry, 1998), and Tsetse fly (Vreysen et al., 2000).

The SIT offers numerous benefits in comparison with alternative mechanisms of vector control. Unlike chemical pesticides, it is entirely species specific, thereby limiting effects on off-target species and hence reducing the potential for harmful ecological impacts. There is also less scope for the target species to develop resistance, although assortative mating, particularly where there is a marked fitness penalty associated with the sterilised males, may emerge which could limit a programme's efficacy (Koyama et al., 2004). The SIT becomes more effective as the size of the target population declines, so it offers the additional advantage of being an ideal tool to implement alongside existing programmes of control.

The application of the SIT to mosquitoes has hitherto achieved mixed success. While sterilised mosquitoes have been released in a number of different field trials, few of these studies were aimed at achieving full population suppression, and of those which were, none have shown effectiveness in achieving lasting population suppression other than in small, isolated areas. Control of a population of *Culex pipiens* was achieved in Burma in 1967 through releases of insects sterilised by cytoplasmic incompatibility: a nine week release period was sufficient to eliminate an isolated target population (Laven, 1967). Isolated populations of *C. pipiens* and *C. quinquefasciatus* in Montpellier, France, and Florida, respectively, have been eliminated through the use of SIT programmes (Laven, 1971), (Patterson et al., 1970). The SIT was also applied to *Anopheles albimanus* in El Salvador in 1974

(Lofgren et al., 1974); however, while the programme initially achieved success in eliminating a small, isolated population of the mosquito, a larger scale programme failed to achieve marked success and was finally abandoned due to the outbreak of civil war in the country (Dame et al., 2009).

Most recently, irradiation-based SIT has been used in a pilot study against an established population of *Ae. albopictus* in Northern Italy (Bellini et al., 2013). The programme has recorded some success, with sterility levels of 70-80% induced in the local population. It is too early to tell whether these results will translate into sustainable population suppression; yet with this exception, SIT programmes against mosquitoes have failed to obtain long-term control.

The reasons for the ultimate lack of success of these programmes to date have no doubt been many and various. However, Benedict and Robinson (2003) have cited as critical factors the insufficient production of sterile insects, in part due to the lack of sexing strains (i.e. strains possessing a trait permitting facile separation of males from females, thereby allowing single-sex (male-only) releases to be conducted), in combination with low fitness of released males. Immigration of fertile wild insects into release areas also compromised some programmes (Benedict and Robinson, 2003). Lack of a sexing strain was one of the key reasons for the failure of the SIT programme in Salvador, in which attempts to separate sexes by providing adults with Malathion-treated bloodmeals appeared to compromise male fitness. Indeed, the introduction of a genetic sexing strain possessing a Y-chromosome linked insecticide resistance allele enabled the programme to return to male-only pupal releases, which showed some promise prior to termination of the project (reviewed in Dame et al. (2009)). In this context, the ongoing SIT trial in Italy is insightful: rearing sufficient numbers of sterile males for release has been hindered by an inefficient manual sex-sorting system resulting in low recovery (26-29%) of total males (Bellini et al., 2013). This inefficiency may translate into excessive fixed costs, in addition to the potential costs associated with releasing the smallest ~30% of males, which may be of lower fitness, and which could potentially lead to assortative mating (Papathanos et al., 2009).

The application of the SIT to mosquito populations on a large scale would therefore benefit from a modified approach which could reduce or eliminate at least some of these complicating factors.

1.5 THE DEVELOPMENT OF GENETIC TRANSFORMATION TECHNIQUES HAS FACILITATED AN IMPROVED MECHANISM FOR APPLYING SIT TO MOSQUITO POPULATIONS

1.5.1 CLASSICAL GENETIC APPROACHES: PROVEN SUCCESS BUT LIMITED APPLICABILITY

Genetic techniques first developed during the 1960s provided a new set of tools for the creation of desirable phenotypes in insects. Of particular importance to the SIT was the advent of chromosomal translocation tools which linked selectable markers to the male determining chromosome, and allowed elimination of females from a sterile release generation. Some success in the earlier-cited SIT suppression programme against *An. albimanus* in El Salvador followed the introduction of a genetic sexing line based on translocation of an insecticide resistance gene to the male-specific Y chromosome (Seawright et al., 1978). Chromosomal translocations to the Y-chromosome which induce partial male sterility have also been exploited in sterile insect release programmes as an alternative to radiation-based sterilisation (Laven, 1971). However, these approaches suffer from translocation instability and fitness costs (discussed further below). Furthermore, a key limitation of these tools is that they are restricted to a single species, and cannot be transferred to establish the same desirable phenotype in other species (Alphey, 2002).

1.5.2 GERMLINE TRANSFORMATION: A MORE PRECISE ENGINEERING TOOL

The more recent development of germline transformation techniques in insects has provided a more effective set of tools for manipulating insect populations. The basis of the technique involves transposable elements: DNA sequences encoding a transposase gene which can excise the

transposon from its existing location and re-insert it into a new chromosomal location. Removing the transposase gene from a transposon sequence and inserting a desired DNA construct between the transposon recognition sites allows the DNA construct to be stably integrated into a target genome through the inclusion of DNA encoding the transposase, which is not integrated into the genome. Germline transformation is achieved where this process occurs in the germ cells, resulting in a heritable transgene. The technique was first successfully applied in *Drosophila* using the *P*-element transposon (Rubin and Spradling, 1982). However, while one incidence of *P*-element transformation has been reported in *An. gambiae* (Miller et al., 1987), this has yet to be repeated; hence it appears that *P*-element-based transformation cannot be applied outside of the drosophilids (O'Brochta D and Handler, 1988).

More recently, a variety of alternative transposons has been developed to facilitate germline transformation in almost any insect species (reviewed in O'Brochta (2003)). In mosquitoes, the *piggyBac* transposon has proved effective in facilitating efficient germline transformation. Successful germline transformation using the *piggyBac* transposon has been achieved in a number of mosquito species, including *Ae. Aegypti* (Kokoza et al., 2001), (Lobo et al., 2002), *Anopheles stephensi* (Nolan et al., 2002), *Anopheles albimanus* (Perera et al., 2002), and in *Ae. albopictus* (Labbe et al., 2010).

The *piggyBac* element, as originally described (Cary et al., 1989), comprises a 2.4 kb sequence with 13 bp and 19 bp inverted repeats at either end of the element. The mechanism of achieving germline transformation using *piggyBac* elements is much like that described previously, with a 'helper' plasmid or mRNA encoding the *piggyBac* transposase co-injected with a vector plasmid containing the construct sequence flanked by the *piggyBac* inverted terminal repeats. *piggyBac*-mediated integration occurs at target TTAA sites, which are duplicated following insertion of the transposon. Flanking sequencing in the examples of *piggyBac* –mediated transposition cited above confirmed the presence of these canonical insertion events in the germline transformations observed. However, multiple *piggyBac* insertions in tandem arrays have also been observed (Adelman et al., 2004).

Remobilisation of the *piggyBac* element has been observed in both *Drosophila melanogaster* (Horn et al., 2003) and *Tribolium castaneum* (Lorenzen et al., 2003). However, despite attempts to achieve remobilisation in *Ae. aegypti*, no such observation has yet been reported, suggesting that *piggyBac* insertions in the latter species are remarkably stable, even in the presence of a *piggyBac* transposase (Sethuraman et al., 2007). This is of significance for the use of *piggyBac*-mediated transformation in the creation of transgenic insects for release into wild populations, since it suggests that the risk of mutation of the transgenic strain by remobilisation of the *piggyBac* element is extremely low. In contrast, recent results show that *piggyBac* remobilisation *can* be achieved in *A. stephensi* (O'Brochta et al., 2011). While this may provide a useful tool for molecular studies in this species (such as enhancer detection), it also indicates that biosafety concerns in relation to potential transgene remobilisation may need to be considered in this species. Measures to stabilise insertions, such as by removing the terminal repeats post-insertion, may be advisable in the development of transgenic technologies in this and other *Anopheline* species.

Germline transformation using *piggyBac* elements is now a routine technique to achieve stable germline introgression of exogenous genes into the genome of both *Ae. aegypti* and *Ae. albopictus*. (See, for example (Fu et al., 2010, Labbe et al., 2012, Labbe et al., 2010)).

1.6 GENETIC MODIFICATION ALLOWS FOR THE INTRODUCTION OF A REPRESSIBLE LETHAL PHENOTYPE IN INSECT PEST SPECIES

The development of efficient tools for insect germline transformation has enabled a new approach to the SIT. By engineering insects to carry a dominant, repressible lethal allele, it is possible to render them effectively sterile (their progeny will be unable to survive), without a requirement for ionising radiation or for alternative approaches, such as chemical sterilisation, with their associated fitness costs (Thomas et al., 2000), (Heinrich and Scott, 2000). The release of such insects (a technique sometimes referred to in the literature as “Release of Insects carrying a Dominant Lethal” (RIDL)

(Thomas et al., 2000)) is becoming established as one of the most promising new approaches to mosquito population control (Alphey et al., 2007).

1.6.1 TETRACYCLINE-REGULATED SYSTEMS ENABLE A REPRESSIBLE LETHAL PHENOTYPE

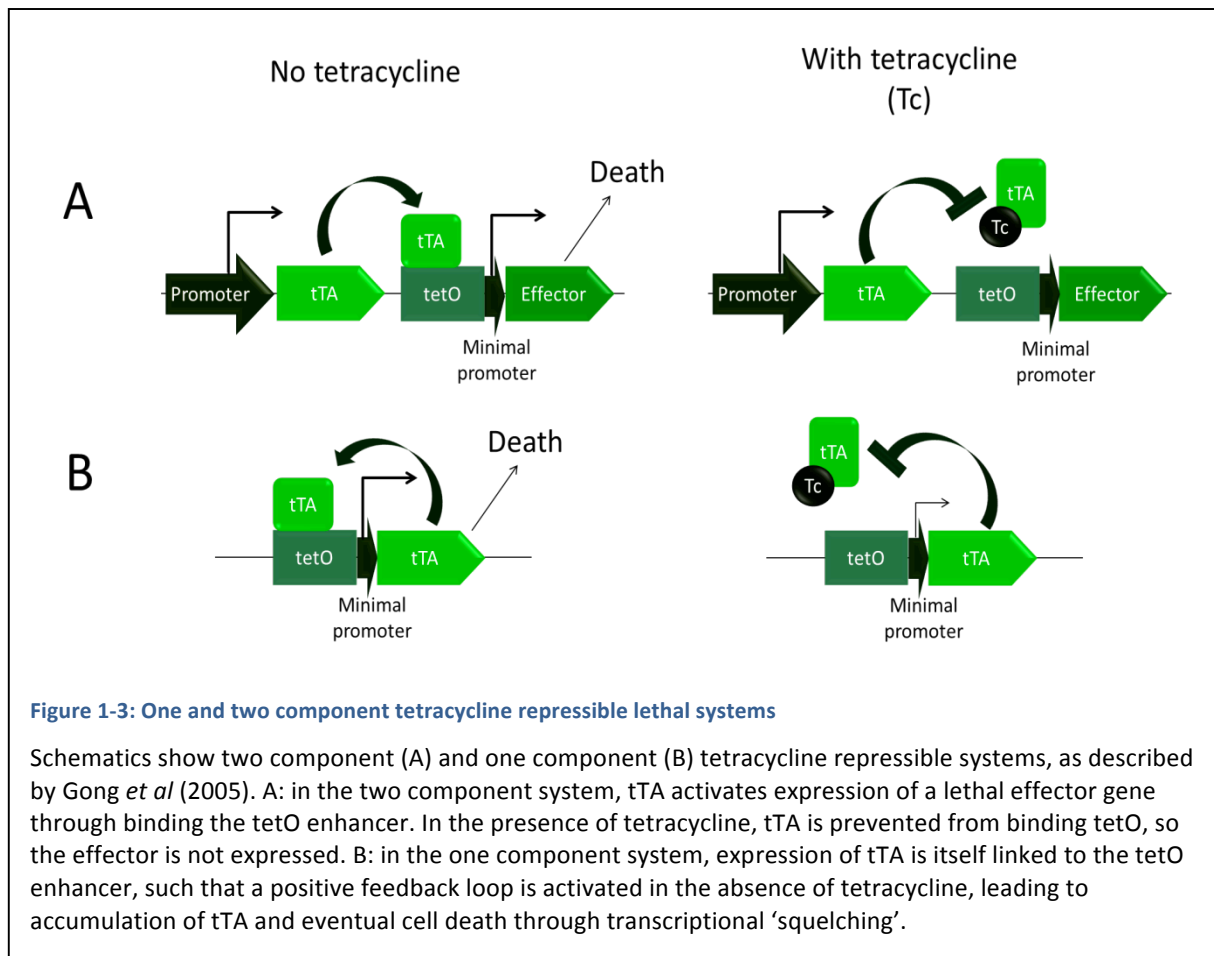
A key requirement of such a system is that the dominant lethal gene be repressible, enabling transgenic insects to be mass-reared under permissive conditions, before being released into the environment. Hence, the repressible lethal system first developed in *D. melanogaster* involves a tetracycline-repressible ('Tet-Off') system, in which suppression of the lethal gene is repressible by the antibiotic, tetracycline (Heinrich and Scott, 2000), (Thomas et al., 2000).

Tetracycline-regulated systems are well characterised genetic tools first developed in mammalian cell lines (Gossen and Bujard, 1992, Zhu et al., 2002). The first part of the system is a regulatable transcriptional activator, tTA: tTA is a fusion protein comprising the DNA-binding region of the TetR protein, derived from the tetracycline-resistance operon from *Escherichia coli*, and the transcriptional activation domain of virion protein 16 (VP16) of the Herpes Simplex virus. The second part of the system is a gene of interest under the control of a minimal promoter combined with the *E. coli* Tet operon enhancer (tetO). In its native form, TetR is prevented from binding the tetO enhancer when bound to tetracycline. However, TetR mutants have been developed that only bind DNA in the presence of tetracycline, resulting in both 'Tet-Off' and 'Tet-On' systems, depending on the effect of tetracycline binding upon the transcriptional activator. In the 'Tet-Off' system, tTA binds tetO in the absence of tetracycline, and activates transcription of the downstream gene of interest. In the presence of tetracycline, tTA binds the antibiotic and undergoes a conformational change which causes it to dissociate from the tetO enhancer, preventing transcription initiation of the downstream gene. The system therefore provides a mechanism for constitutive activation of the gene of interest which is repressible in the presence of tetracycline.

1.6.2 THE 'TET-OFF' SYSTEM HAS BEEN EXPLOITED TO ENGINEER REPRESSIBLE LETHALITY IN INSECTS

The 'Tet-Off' system has been adapted to produce a repressible, dominant lethal phenotype in insects. Gong et al. (2005) describe two variants to this approach (see Figure 4-3). The first is a two-component system: insects are transformed with a construct expressing tTA under the control of a developmental promoter (inactive in adults), alongside the tetO enhancer linked to the expression of a lethal effector gene. Hence in the absence of tetracycline, transgenic insects will express the lethal gene and die. In the second, single component system, the tTA gene is itself placed under the control of the tetO enhancer. In this case, a positive feedback loop is activated in the absence of tetracycline, leading to the accumulation of tTA protein. High levels of tTA are toxic to cells, probably due to the recruitment by VP16 of transcriptional machinery, a process termed transcriptional 'squenching' (Berger et al., 1990, Gill and Ptashne, 1988). This second system was implemented in *Ceratitis capitata* and resulted in a tetracycline-repressible lethal phenotype without the need for an additional lethal effector gene (Gong et al., 2005).

A similar, single component construct has been used to generate a tet-repressible lethal phenotype in *Ae. aegypti* (Phuc et al., 2007). In the absence of tetracycline, the majority of insects carrying the



construct were found to die during late larval or pupae stages. Insects transformed with such a system and made homozygous for the lethal construct are effectively 'sterile' without the requirement for radiation treatment: their progeny will inherit the dominant lethal construct and therefore be unable to survive in the absence of tetracycline (Thomas et al., 2000).

Following this work, a variant of this system which produces a repressible, female-specific flightless phenotype, has been developed in both *Ae. aegypti* and *Ae. albopictus* (Fu et al., 2010, Labbe et al., 2010). The same approach has also been used to engineer a female flightless phenotype in the malaria vector *An. stephensi* (Marinotti et al., 2013), while female-specific dominant lethal systems have also been developed in the agricultural pests *Ceratitidis capitata*, *Bactrocera oleae*, *Bombyx mori* and *Plutella xylostella* (Ant et al., 2012, Tan et al., 2013, Jin et al., 2013, Fu et al., 2007). These

systems are based on the positive feedback circuit of Gong *et al* with female-specificity conferred by the addition of a sex-specific alternative splicing cassette.

1.7 INSECTS EXPRESSING A DOMINANT LETHAL HAVE SUCCESSFULLY SUPPRESSED POPULATIONS OF *Ae. AEGYPTI* IN THE FIELD

1.7.1 FIELD RESULTS HAVE DEMONSTRATED EFFECTIVE SUPPRESSION

The development of a strain of *Ae. aegypti* which exhibits a tetracycline-repressible lethal phenotype has enabled the testing of a dominant-lethal based approach to mosquito population control in both semi-field and field conditions. The OX513A strain, developed by Oxitec Ltd, is a single-component tetracycline-repressible lethal system: in the absence of tetracycline more than 90% of insects heterozygous for the construct die in late larval or pupal stages (Phuc et al., 2007). Open field releases with this strain have now been carried out in the Cayman Islands and in several locations in Brazil. Serial releases of the OX513A strain in a trial area in the Cayman Islands were sufficient to achieve an 80% reduction in the target population of *Ae. aegypti* relative to the control area (Harris et al., 2012). In Brazil, field experiments in the small villages of Itaberaba and Mandacaru demonstrated suppression of 80% and 96%, respectively (Danilo O. Carvalho, 2014, McKemey, 2013). Suppression trials are currently being conducted on a larger scale in Jacobina, a town of 50,000 inhabitants.

1.7.2 REGULATORY APPROVAL AND PUBLIC OPINION HAVE NOT PROVED INSURMOUNTABLE BARRIERS

Aside from demonstrating the efficacy of a RIDL approach, these trials are also notable for their achieving two other pre-requisites for a successful mosquito control programme; namely, regulatory approval and public acceptance. Both have long been considered severe and potentially insurmountable barriers to the release of transgenic insects, yet field experiments in Brazil appear to

have widespread public support (Otis, 2014). Moreover, in addition to securing regulatory approval for the field experiments conducted to date, the Oxitec OX513A strain was recently approved for commercial release by the National Technical Commission for Biosecurity (CTNBio) – the first such approval for a genetically modified insect anywhere in the world (CTNBio, 2014).

These successes may relate to the high level of public recognition of the dengue threat in Brazil, as well as to the extensive public engagement exercise which has accompanied the field experiments. In Florida, where the threat of dengue remains in abeyance for the present, proposals for similar field experiments have met with vociferous public opposition (Mier, 2012). While public opinion surveys have indicated that this vocal opposition may misrepresent wider public opinion in the local area (Cobb M, 2012), it nonetheless highlights the necessity for future developments of this technology to be considered in terms of their likely impact on both public perception and regulatory questions, as well as upon efficacy.

1.8 ALTERNATIVE TRANSGENIC AND PARATRANSGENIC APPROACHES ARE BEING DEVELOPED IN PARALLEL WITH DOMINANT LETHAL SYSTEMS

To date, engineered dominant lethal systems remain the only transgenic approach to vector control with proven field efficacy. The work presented in this thesis focuses on strategies to improve these systems in mosquitoes. Nonetheless, a number of alternative transgenic and paratransgenic (involving the introduction and/or genetic engineering of endosymbionts) approaches to vector control and management are in development. They comprise both novel population suppression tools and population replacement (wherein a desirable trait – namely disease resistance – is spread through the population). Some of these tools are briefly considered in the following paragraphs.

1.8.1 ENGINEERING DISEASE RESISTANCE

It has been proposed that germline transformation could facilitate the introduction of a disease resistant phenotype in vector mosquitoes. For example, dengue resistance has been introduced into

Ae. aegypti by exploiting RNA interference pathways (Olson et al., 2002, Adelman et al., 2002, Franz et al., 2006), while a number of approaches have been explored for introducing parasite resistance into *Anopheles* species. These have included expression of anti-plasmodium antibodies (de Lara Capurro et al., 2000); the expression of peptides which compete with *Plasmodium* for ligand binding in mosquito midguts and salivary glands (Ghosh et al., 2001, Ito et al., 2002); boosting the expression of immune factors to inhibit viral or plasmodium infection (Kokoza et al., 2000, Corby-Harris et al., 2010); and expressing anti-parasitic toxins (Yoshida et al., 2001, Zieler et al., 2001).

However, since it is likely that such approaches will not confer any significant selective advantage, spreading the introduced desirable trait through the native population will require the resistance gene to be coupled with a form of 'gene drive' mechanism, which will tend to spread the introduced gene to fixation in a population (reviewed in Sinkins and Gould (2006)). Examples include homing endonuclease genes, which 'cut and paste' themselves into a homologous region on the adjacent allele (Windbichler et al., 2007); meiotic drive mechanisms such as the *Medea* system, in which embryonic survival in the offspring of female carriers is dependent on inheritance of the *Medea* gene (Chen et al., 2007); and engineered under-dominance systems which are based on co-inheritance of introduced alleles containing a desirable (anti-pathogen) gene, which are lethal when inherited singly (Davis et al., 2001, Magori and Gould, 2006).

1.8.2 POPULATION SUPPRESSION BY SEX-RATIO DISTORTION

An additional application of a homing endonuclease gene has recently been developed in the malaria vector *An. gambiae*. The homing endonuclease I-PpoI has been found to specifically cleave sequences in the *An. gambiae* X chromosome (Windbichler et al., 2007). By destabilising the I-PpoI transcript, researchers have been able to restrict its activity to spermatogenesis in transgenic males. This resulted in males that produced predominantly male offspring, and hence a male sex-bias in their progeny ranging from 70-97% (Galizi et al., 2014). Male progeny inheriting the I-PpoI gene would also have predominantly male progeny: hence, serial releases of transgenic males would be

expected to efficiently suppress a target population by reducing or eliminating females: indeed, laboratory-based caged suppression experiments reported in the same paper show that the tested I-Ppol strain was effective in this regard.

1.8.3 *WOLBACHIA* AS A MECHANISM FOR SPREADING DISEASE RESISTANCE

Another mechanism of spreading pathogen resistance is based on viral refractoriness conferred by the bacteria *Wolbachia*. *Wolbachia* is an endosymbiont which spreads rapidly through populations through a mechanism known as cytoplasmic incompatibility (CI) (Hoffman, 1997). CI refers to a phenomenon whereby fertilisation of eggs of uninfected females by a *Wolbachia*-infected male results in embryonic lethality; as a result, infected males can only successfully reproduce with infected females. All the offspring of infected females are born with the infection, regardless of the infection status of the father, thus providing a mechanism for rapid spread of the infection through a population. Infection with some *Wolbachia* strains, namely wMel and wMelPop, has also been shown to reduce parasite infection and transmission in *Ae. aegypti* (Moreira et al., 2009, Walker et al., 2011) and in *Ae. albopictus* (Blagrove et al., 2012). Moreover, the wMel strain has also been successfully established in an open-field population of *Ae. aegypti* (Hoffmann et al., 2011).

Trials aimed at establishing the wMelPop and wMel strains in field populations are ongoing or planned in various locations, including Vietnam, Indonesia and Brazil. However, early results suggest that the *Wolbachia* strain wMelPop, which is more effective in blocking dengue transmission, has high fitness costs and may be more difficult to establish in the field than wMel (interim reports available from the “Eliminate dengue” website (O’Neil, 2014)). In addition, while *Wolbachia* establishment in a local population may be possible, recent modelling suggests constraints on the ability to achieve spatial spread of the infection (Schraiber et al., 2012). Expanding this approach to a larger scale may therefore be costly.

1.9 THE RELEASE OF INSECTS EXPRESSING A DOMINANT LETHAL GENE OFFERS CONSIDERABLE ADVANTAGES WITH RESPECT TO TRADITIONAL SIT APPROACHES

1.9.1 ELIMINATING THE IRRADIATION STEP COULD IMPROVE FITNESS AND CREATE COST SAVINGS

Releasing insects carrying a dominant lethal gene offers considerable advantages over a traditional radiation-based approach to SIT. One such advantage is the presumed reduction of fitness penalties due to the elimination of the irradiation step. Irradiation has been shown to impart significant fitness (i.e., strictly, mating competitiveness) costs upon insects used in sterile release programmes and specifically upon mosquitoes (though radiation resistance appears to vary between species) (Lance et al., 2000, Shelly et al., 1994, Andreasen and Curtis, 2005, Helinski et al., 2006). While there may be fitness costs associated with the transgene in a RIDL approach, iterative analysis of different insertions can allow the development of strains which minimise these costs. Furthermore, regardless of these benefits, a radiation programme is costly, such that removing this step will introduce significant savings; hence a more economic production process, in combination with reduced fitness impairment of released males, means that a RIDL-based approach has the potential to offer a more cost-effective alternative to radiation-based SIT (Atkinson et al., 2007).

Chemosterilising agents have also been used in SIT programmes against mosquitoes, with some success (Patterson et al., 1970, Lofgren et al., 1974). In the latter programme, in particular, chemosterilised males released as pupae were found to compete effectively against wild males. Chemosterilisation also avoids the high costs associated with radiation-based sterilisation. Hence, while dominant lethal systems may offer important fitness benefits in comparison to radiation-based programmes, juxtaposed against chemosterilisation, it can be seen that they do not represent a paradigm shift in this regard alone. However, the discovery of toxic effects upon predators resulting

from chemosterilant residues has raised important questions surrounding the possible environmental effects of large-scale use of chemosterilisation (Bracken and Dondale, 1972).

1.9.2 A CONSTITUTIVELY ACTIVE SYSTEM OFFERS SAFETY BENEFITS

While concerns have been voiced in relation to the release of genetically modified insects (as discussed in section 1.7.2), a RIDL approach in theory offers safety benefits over traditional SIT approaches. A large-scale accidental release of non-sterilised agricultural pests or, in particular, of disease vectors from a mass rearing facility could cause significant economic and health damage in a traditional SIT programme. Indeed, in 2003, an equipment malfunction did result in the aerial release of fertile screwworm flies in Panama, though this was quickly controlled through further release of sterile flies (Steve C. Smith, 2009). In contrast, transgenic insects carrying a dominant lethal gene are constitutively 'sterile', thereby providing an in-built 'fail-safe' mechanism which would limit the impact of an accident of this nature.

1.9.3 GENETIC APPROACHES CAN BE TAILORED TO THE TARGET SPECIES

A key advantage of genetic engineering approaches is that they can be tailored to the target species in question. This is of particular relevance to SIT programmes against many mosquito species, including *Ae. aegypti*, where density-dependent competition during larval stages is known to play an important role in population dynamics (Weidhaus, 1971, Rodgers, 1984). Expressing the dominant lethal gene at a late juvenile stage (but before reaching adulthood), will maximise the competition for larval resources between wild target populations and the offspring of released males, increasing the overall efficiency of the control programme (Phuc et al., 2007, Atkinson et al., 2007).

1.9.4 GENETIC APPROACHES OFFER THE POTENTIAL TO ENGINEER GENETIC SEXING SYSTEMS, TO EFFICIENTLY DELIVER A MALE-ONLY RELEASE

Finally, engineering dominant lethal systems offers a solution to one of the most intransigent problems facing SIT programmes: that of ensuring a male-only release. Male-only releases have been shown to significantly increase the efficiency of SIT programmes (Rendon et al., 2004). Moreover, male-only releases are essential in disease vectors, such as mosquitoes, where females are responsible for disease transmission and hence release of females carries a nuisance biting and potential health risk. Sex-separation techniques based on biological and morphological differences (Fay RW, 1959, Focks, 1980) or on classical genetic approaches (Robinson, 2002a, Curtis, 1979, Seawright et al., 1978), have had some success. However, the former has often proven inefficient (Dame et al., 1974, Bellini et al., 2013), while female-killing systems based on chromosomal translocations have been dogged both by problems with long-term stability of the chromosomal translocation, and associated fitness costs (Seawright et al., 1978) – see (Black IV et al., 2011) for review. Moreover, classical genetic sexing strains are time consuming to establish and cannot be transferred to other species. However, by expressing the lethal gene in a female-specific manner, a dominant lethal approach could achieve extremely high efficiency in removing the female population (Heinrich and Scott, 2000, Thomas et al., 2000). To achieve maximum efficiency gains, such a system would act early in development: early elimination of females in this manner would effectively double the number of males which could be reared in the release generation for the same resources.

Female-specific strains have been developed in *Ae. aegypti* and *Ae. albopictus*. However, both of these strains induce a flightless phenotype in females, rather than being true female-killing strains. Moreover, the strains do not remove females from the release generation at an early stage, obviating one of the principal potential efficiency gains of a genetic sexing system. A more

comprehensive discussion of these strains, their limitations, and alternative approaches to developing genetic sexing mechanisms, is given in Chapter 5.

1.9.5 FLUORESCENT MARKERS PERMIT FACILE IDENTIFICATION OF TRANSGENIC INSECTS

In addition to a conditional lethal construct, transgenic approaches also permit the introduction of fluorescent marker genes such as Enhanced Green Fluorescent Protein (EGFP) (Higgs and Lewis, 2000), its cyan spectral variant (ECFP – or AmCyan), or DsRed (Horn et al., 2002). Expressed under the control of constitutively active promoters such as 3xP3 (Berghammer et al., 1999) or Hr5/le1 (Rodems and Friesen, 1993), these genes serve as easily-identifiable transformation markers. Moreover, they also permit facile identification of transgenic insects in the field – an essential part of successful pest management programmes (Hagler and Jackson, 2001). Non-transgene-based approaches have used of fluorescent dyes for this purpose, but loss of the marker and the potential for contact contamination of wild-type insects are both significant problems. Hence, while transgenic markers are not limited to the use of dominant lethal systems, they are a key aspect in which the latter offers a considerable improvement over traditional SIT approaches.

1.10 ‘NEXT GENERATION’ RIDL PRODUCTS SHOULD OFFER THE FULL SUITE OF ADVANTAGES WHICH A DOMINANT LETHAL GENETIC SYSTEM CAN PROVIDE

Although the OX513A *Ae. aegypti* strain has shown demonstrable efficacy in open field experiments, it does not benefit from all of the advantages which an engineered dominant lethal system can potentially offer over traditional SIT, as outlined in the previous section. In particular, it does not offer a genetic sexing mechanism: male-only releases of the strain have been achieved by manual sex-sorting at the pupal stage. This technique has enabled the release of populations comprising >99% males (Harris et al., 2011). However, some females are inevitably released, the technique may impose fitness costs upon the mosquitoes, and it imposes considerable time and human resources

costs. Additionally, it may not be possible to develop such efficient physical sex separation methods for other mosquito species, including *Ae. albopictus*.

There remains, therefore, considerable scope for developing improved dominant-lethal strains in both *Ae. aegypti* and *Ae. albopictus*. The schematic in Figure 1-4 juxtaposes key attributes of the current OX513A strain against an ideal 'next-generation' product strain. As shown in the figure, OX513A possesses two attributes of particular significance to a successful suppression programme:

- i) The lethal gene exerts its effect primarily in late larval stages, allowing density-dependent competition with wild-type larvae to be maintained in the field. However, the construct does not contain a stage-specific promoter, and the late-acting phenotype is considered largely to result from chromosomal position effects specific to this particular insertion (Phuc et al., 2007)
- ii) OX513A males appear to compete effectively with wild-type males for females (Harris et al., 2011)

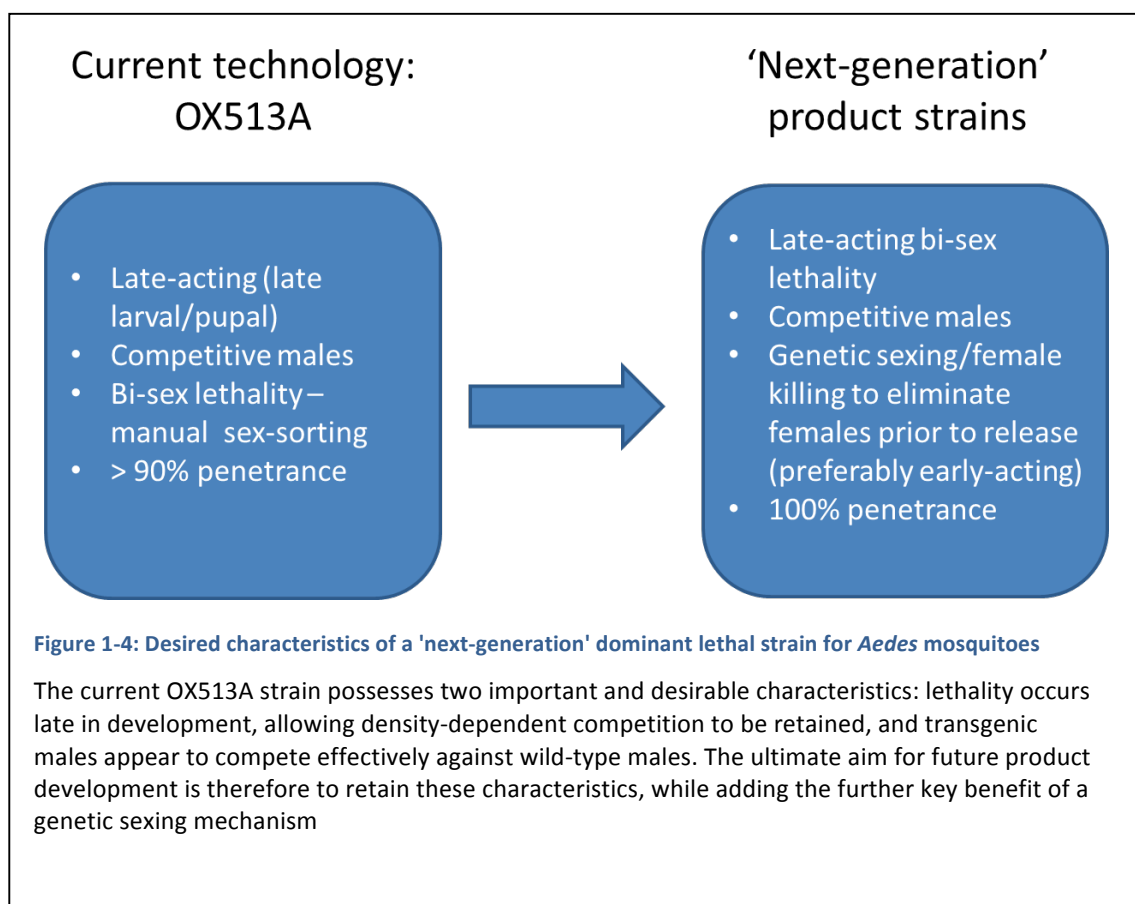
The ultimate aim for future development of dominant lethal systems in *Ae. aegypti* and *Ae. albopictus* will therefore be to introduce the key benefit of a genetic sexing system, whilst retaining a dominant lethal system which manifests itself in late immature stages. Preservation of mating competitiveness will of course be a pre-requisite for the ultimate success of such a 'next-generation' strain.

To obtain maximum efficiency, a genetic sexing mechanism would enable females to be removed from the release generation at an early stage in their development: this then introduces the additional requirement of combining both female specific lethality which acts early in development, and a late juvenile-acting, bi-sex lethal mechanism, as currently present in the OX513A strain. These considerations enable the ultimate goals for developing an ideal dominant lethal strain to be outlined as follows:

- A reliable mechanism for engineering late-acting, bi-sex lethality in new strains.

- A female-specific expression system to enable early killing of females in the release generation.
- A means of regulating the activation of these systems independently, to allow both features to be combined in a single strain.

Development of such a strain would build on the success to date of the OX513A *Ae. aegypti* strain, in establishing the release of insects carrying a dominant lethal gene as a highly promising new approach to controlling mosquito populations. The experimental work described in this thesis has therefore been conducted with the aim of developing and testing strategies in pursuit of this goal.



1.11 CONCLUSION

Mosquito-borne diseases are a severe and growing global health challenge. The disease vectors *Ae. aegypti* and *Ae. albopictus* are responsible for a massive global disease burden, in particular through their vectoring of the viral diseases dengue fever and chikungunya.

The release of insects carrying a dominant lethal gene offers a new paradigm in the control of vector-borne diseases. It has the advantages of the species-specificity and minimal environmental impact associated with the SIT, while circumventing the fitness damage and hence increased programmatic costs which can result from radiation-induced sterilisation. Releases of a strain of *Ae. aegypti*, OX513A, carrying a repressible dominant lethal construct, have demonstrated a high level of efficacy in numerous field experiments.

Nevertheless, there remains considerable room to improve the current state of dominant lethal technology in *Ae. aegypti* and *Ae. albopictus*. Strategies to realise these improvements, as outlined in section 1.10, will form the basis of research and development in pursuit of 'next generation' product strains in these species, and are the focus of the experimental work described in the following chapters.

2 GENERAL METHODS AND MATERIALS USED

2.1 STRAIN REARING AND TRANSFORMATION

2.1.1 DESCRIPTION OF STRAINS AND REARING APPROACH

The *Ae. aegypti* wild-type strain (WT) used here originated from Malaysia and was colonised by the Institute of Medical Research (Kuala Lumpur) in 1977. The *Ae. albopictus* WT strain also originated from Malaysia and was colonised from the Institute of Medical Research, Kuala Lumpur in 2006. Insects were reared in standard insectary conditions: 27°C [$\pm$ 2°C], 70% [$\pm$ 10%] relative humidity, 12h: 12h light: dark cycle. Larvae were fed on finely ground Tetramin[®] fish flakes (Tetra GmbH, Germany) and adults provided with 10% sucrose solution supplemented with 14 U/ml penicillin and 14 μ g/ml streptomycin. To obtain eggs, mated females were provided with defibrinated horse blood (TCS Biosciences Ltd., UK) and allowed access to wet Whatman[™] filter papers (GE Healthcare Life Sciences, Buckinghamshire, UK) as oviposition substrate.

2.1.2 MICROINJECTIONS AND GENERATION OF TRANSGENIC LINES

Microinjections were performed essentially as described (Morris et al., 1989). Embryos were injected with a solution containing 200 μ g/ μ l plasmid DNA, and 500 μ g/ μ l helper mRNA encoding *piggyBac* transposase; the latter was prepared by Andrea Miles, Oxitec Ltd. Injected embryos were placed in a humid environment (sealed plastic box containing wet cotton wool) for 4 days before hatching.

Surviving generation 0 (G0) adults were pooled (typically 2 G0 males or 15 G0 females per pool) and crossed to WT adults. In the next generation (G1), progeny were screened for expression of the fluorescent transformation marker. Iso-lines were established in G2 from positive single G1 males. The transgenic:wild-type ratios in resulting progeny were recorded

and further single male outcrosses were performed if these ratios indicated the presence of more than one insertion. Fluorescence was observed using a Leica M80 microscope (Leica Microsystems, U.K.) and images taken using a Canon PowerShot S5IS camera with an MM99 adaptor (Martin Microscopes, U.K.).

2.2 PREPARATION OF CDNA FOR RT-PCRS

Total RNA was extracted from whole-insect samples (or as otherwise indicated) using the RNeasy mini-kit (Qiagen, Limburg, Netherlands), according to manufacturer's instructions. First strand cDNAs were synthesised using the RevertAid first-strand synthesis system (Fermentas/Thermo Scientific, MA, USA) with polyT primers, using approximately 0.5 µg extracted RNA per sample.

2.3 ON/OFF TETRACYCLINE SURVIVAL ASSAYS

Heterozygous populations were hatched and on day 1 post-hatch 200 larvae per replicate (or as otherwise specified in text) were counted into rearing water. 'On tet' treatments were set up by adding chlortetracycline to rearing water at a concentration of 30 µg/ml. 'Off tet' treatments were maintained in rearing water without tetracycline. Larvae were at a density of 1 larvae/ml, and were fed crushed dry fish food (TetraMin flakes, Tetra GmbH, Germany)

Table 2-1: Standard feeding regime

Day	Amount Tetramin per larvae (g)
1	0
2	0.00006
3	0
4	0.00008
5	0.00016
6	0.00032
7	0.00032
8	0.00032
9	0.00016
10	0.00008
11	0
12	0

according to the standardised regime given below (Table 2-1)

Pupae were picked and screened for fluorescence Leica M80 microscope (Leica Microsystems, U.K.). Unless otherwise specified, transgenic and wild-type pupae were then placed separately into cages or hatch pots and allowed to eclose. Numbers successfully eclosing were counted two days after the last pupae were collected.

2.4 SEQUENCING OF PCR PRODUCTS

PCR products were analysed on 1% agarose gels. To sequence PCR products, fragments of the expected size were extracted and purified using the MinElute gel extraction kit (Qiagen, venlo, Netherlands), before cloning into the pJET vector using the CloneJET™ kit (Fermentas, Leicestershire, UK). pJET vectors containing the fragments of the expected size were purified and sent to GATCBiotech (www.gatc-biotech.com) for sequencing.

2.5 SUMMARY OF CONSTRUCTS USED

All constructs used in this thesis are described in detail in specific results chapters. However, for ease of reference, a summary of all constructs used, their key components, authors, and the chapter in which they are described are listed in Table 2-2:

Table 2-2: Summary of constructs used in this thesis

NB: additional constructs used solely as templates in construct building are not included

Construct name	Key components	Transformation marker	Developed by	Chapter described
OX4708	Os1pro- <i>DsRed</i>	Hr5/IE1- <i>AmCyan</i>	Author	3
OX4732	Os2pro- <i>DsRed</i>	Hr5/IE1- <i>AmCyan</i>	Author	3
OX4709	Os1pro- <i>tTAV</i>	Hr5/IE1- <i>AmCyan</i>	Author	4
OX4800	Os1pro- <i>tTAF3</i>	Hr5/IE1- <i>AmCyan</i>	Author	4
OX4811	Os2pro- <i>tTAF3</i>	Hr5/IE1- <i>AmCyan</i>	Author	4
OX4489	tetO- <i>AaeDsx-tTAV</i>	3xP3- <i>DsRed</i>	D'afaalla, T (Oxitec Ltd)	5
OX3978	tetO- <i>AmCyan</i>	Hr5/IE1- <i>DsRed</i>	D'afaalla, T (Oxitec Ltd)	5
OX4786	Hsp83- <i>AID-Gal80-TIR-Gal4</i> , UAS- <i>AmCyan</i>	3xP3- <i>DsRed</i>	D'afaalla, T (Oxitec Ltd)	6
OX4787	Hsp83- <i>Gal4DBD-TIRΔ-AID-VP16</i> , UAS- <i>rpr</i>	3xP3- <i>DsRed</i>	D'afaalla, T (Oxitec Ltd)	6
OX4797	Hsp83- <i>Gal4DBD-TIRΔ-AID-VP16</i> , UAS- <i>rpr</i>	3xP3- <i>DsRed</i>	D'afaalla, T (Oxitec Ltd)	6
OX4499	tetO- <i>micelob_x</i>	Hr5/IE1- <i>DsRed</i>	Phillips, C (Oxitec Ltd)	5
OX5055	Os2pro- <i>tTAF3</i> , tetO- <i>micelob_x</i>	Hr5/IE1- <i>AmCyan</i>	Scaife, S (Oxitec Ltd)	4
OX4601	Hex1γ- <i>Act4exons-tTAV</i> , tetO- <i>VP16</i>	Hr5/IE1- <i>DsRed</i>	Scaife, S (Oxitec Ltd)	5
OX5024	tetO- <i>AalDsx-tTAV</i>	3xP3- <i>DsRed</i>	Scaife, S (Oxitec Ltd)	5
OX5048	tetO- <i>AalDsx-tTAV</i>	3xP3- <i>DsRed</i>	Scaife, S (Oxitec Ltd)	5
OX3582	tetO- <i>micelob_x</i>	3xP3- <i>DsRed</i>	Scaife, S (Oxitec Ltd)	4

2.6 STATISTICAL ANALYSIS

All statistical analysis presented in this thesis was performed using the “R” software for statistical computing (R Development Core Team, 2010).

Note on methods used: Since survival assays involve bound data where the assumptions of normality cannot be met, Generalised Linear Models (GLM) have generally been used to analyse this data (discussed in (Crawley, 2005)). Models were fitted by stepwise removal of factors followed by ANOVA testing of revised against original models. Reported *p* values are

those obtained from ANOVA tests of revised against original models, or t-tests for the z statistic of individual model parameters against the benchmark value, as described for specific assays.

Other statistical tests used are described within text in relation to specific assays.

3 IDENTIFYING AND TESTING NOVEL BI-SEX LETHAL PROMOTERS IN *Aedes Aegypti*

3.1 INTRODUCTION

The ability to create a repressible lethal phenotype in insect species has offered the potential for an improved approach to applying the sterile insect technique to mosquito populations (Heinrich and Scott, 2000, Thomas et al., 2000).

A key advantage offered by a dominant lethal genetic system is the potential to avoid the attenuation of population reduction through sterile insect release due to density-dependent competition effects during larval development. Density-dependent competition among larvae for nutritional resources is considered a key driver of population dynamics among *Aedes* species in the wild (Dye, 1984), though more recent studies have questioned some of the assumptions underpinning the canonical model (Legros et al., 2009). The efficacy of a given control strategy may therefore be highly dependent on its interaction with density-dependent effects, and a control measure may indeed be counter-productive if density dependent effects are over-compensatory for the effects of the measure in question (Juliano, 2010, Washburn, 1995). Radiation-based sterilisation will generally result in embryonic lethality in progeny resulting from mating between irradiated males and wild females (reviewed in Robinson (2002b)). While this reduces the population of the subsequent generation of mosquitoes, the reduced competition for nutritional resources during larval development may be an undesirable compensatory effect, resulting in an increased rate of survival to adulthood among the remaining wild population.

Engineering a late-acting lethal phenotype, in which lethality occurs at late larval or pupal stages, can overcome this problem, thereby increasing the efficiency of a sterile insect release (Phuc et al.,

2007). Late-acting expression of the lethal effector is therefore a desirable component of any application of a repressible, dominant lethal system to mosquito population control.

3.1.1 THE NEED FOR NOVEL, LATE-ACTING PROMOTERS IN *AEDES AEGYPTI* AND *AEDES ALBOPICTUS*

A strain of *Aedes aegypti*, named OX513A, has been created which exhibits such a late-acting phenotype, and releases of this strain have successfully reduced target mosquito populations in both closed and open field testing (Harris et al., 2011, Lee, 2008, Harris et al., 2012). However, the OX513A construct was not designed with a stage-specific promoter, and the late-acting lethal phenotype in this case is thought to be due largely to a combination of a cumulative effect of tTAV accumulation through larval development, together with positional effects that are specific to this particular insertion (Phuc et al., 2007). Indeed, subsequent attempts to re-create this phenotype using the same genetic construct have failed to generate late-acting lethal strains (Nimmo, 2012).

While the OX513A strain exhibits the desired late-larval/pupal lethality, there is considerable scope for further improvement of this phenotype. The discussion in Chapter 2 has outlined some of this potential, in particular, the addition of a genetic sexing mechanism, as will be discussed in Chapter 5 of this thesis. Identifying promoters capable of driving late-acting expression in a consistent, position-independent manner will allow for the development of new strains, whilst ensuring that lethality in the late juvenile phases is retained. In addition, the development of alternative dominant lethal strains exhibiting the desired late-acting lethal phenotype will be an important asset in the event of any future need to replace the existing OX513A strain.

The first germline transformation of *Aedes albopictus* (Labbe et al., 2010), followed by the development of an *Ae. albopictus* strain exhibiting a female-specific, dominant repressible

flightless phenotype (Labbe et al., 2012), has recently opened the prospect of developing a dominant lethal strain for population control in this species.

As yet, no late-acting bi-sex lethal strain has been developed in *Ae. albopictus*. While the female flightless phenotype, in providing a putative genetic sexing system, offers some advantages over a bi-sex lethal mechanism, this is partially offset by limitations, particularly potential regulatory issues, which are discussed at greater length in Chapter 5.

Successful development of flightless lines in *Ae. albopictus* using the *Ae. aegypti* *Actin4* promoter demonstrates that, in at least some cases, regulatory elements can be transferred between these species whilst retaining their native function (Labbe et al., 2012). Thus, while achieving proof-of-principle of new late-acting promoters in *Ae. aegypti* would be no guarantee that the same promoters would be functional in *Ae. albopictus*, it may prove a promising route to the development of a late acting phenotype in the latter species.

This chapter describes the identification and testing of novel late-acting promoters predicted to drive late-acting expression in *Ae. aegypti*. Promoters from two *Ae. aegypti* genes, AAEL004275 and AAEL004298 were cloned and used to drive expression of a fluorescent marker in *Ae. aegypti* and in *Ae. albopictus*. The expression profile of the promoters in transgenic insects are characterised in detail, and their potential suitability in downstream RIDL applications discussed. Chapter 4 goes on to describe the use of these promoters in tTAV/tetO based repressible lethal constructs.

3.2 METHODS

3.2.1 IDENTIFYING PROMOTER CANDIDATES

The principal resource used for this purpose was the *Ae. aegypti* Genome Browser gateway provided by UCSC/Caltech (Akbari et al., 2013) (<http://aedes.caltech.edu/cgi-bin/hgGateway>). This database provides detailed expression profiles across all developmental stages. At the time this work was conducted, raw data were not available¹, and the database itself is not searchable by expression level at particular developmental stages. In order to help identify potential candidates, the *Anopheles gambiae* genome expression profile at UC Irvine (Dissanayake et al., 2006) was also used to search for genes with high levels of expression at pupal and late larval stages in *An. gambiae* before examining *Ae. aegypti* orthologues of these genes in the UCSC/Caltech browser.

3.2.2 STRAIN REARING AND TRANSFORMATION

WT strains, rearing and transformation were as described in General Methods (Chapter 2)

3.2.3 RT-PCR

RNA extraction and preparation of cDNA were as described in General Methods (Chapter 2)

To create a wild-type developmental cDNA library, cDNA was synthesised from the following WT *Ae. aegypti* samples:

- 48 hr old embryos (pooled samples approximately equivalent in biomass to two pupae)
- 1st instar larvae (pooled samples of ~100 individuals)

¹ As noted in the Discussion to Chapter 4, this database has now been published and the raw data feeds made available. This offers the potential for more systematic searches to identify late-developmental or pupal-specific promoters.

- 2nd instar larvae (pooled samples of ~10 individuals)
- 3rd instar larvae (pooled samples of 5 individuals)
- 4th instar larvae (pooled samples of 3 individuals)
- Male and female pupae (pooled samples of 3 individuals (male) or 2 individuals (female))
- Male and female adults (pooled samples of 3 individuals)
- Blood fed females ~24 hrs after feeding (pooled samples of 3 individuals)

PCR for candidate pupal/late-larval specific genes was performed using the primers shown in Table 3-1.

Table 3-1: Primers for RT-PCR of candidate genes

Primer name	Sequence	Target	Target Length
AL4275-F	5'-CTCTCTGAACTCGATGCTCTTC-3'	AAEL004275	413 bp (gDNA)
AL4275-R	5'-GATCAGTTTCTTGATACCGATGAT-3'		331 bp (cDNA)
AL4298-F	5'-GAAGGTCGTAAGAAGATGAAGAAG-3'	AAEL004298	805 bp
AL4298-R	5'-AGAGGTTATTTGCTGTAATTTTGG-3'		
AL11037-F	5'-GAAATCTCCTCTCTCTTCACAG-3'	AAEL011037	270 bp
AL11037-R	5'-CTAGGACTGGAGGCTTAGTAGTG-3'		
AL1290-F	5'-TCAATAGTTCTATCAAGTGTTGTCC-3'	AAEL001290	706 bp (gDNA)
AL1290-R	5'-ATCATCTTCGTGCTCCTTGACC-3'		527 bp (cDNA)
AL3027-F	5'-ACCCTGCTCAGTAACGTATAACTC-3'	AAEL003027	965 bp (cDNA)
AL3027-R	5'-GCTGTTCTTCTCGTAATCGTAGT-3'		
895) AeMuAcR	5'-CCCAGGAAGGATGGCTGGAAGAG-3'	AAEL001928 (Muscle Actin)	3141 bp (gDNA)
1098) AeMuAcRTF	5'-GGCATTCTTCTAGTCCGAAACCG-3'		967 bp (cDNA)

Primers 895 and 1098 (Table 3-1) amplify a region of the *Ae. aegypti* muscle actin gene AAEL001928. This gene is expressed throughout development (Scaife, S., personal communication) and therefore served as an endogenous control. To provide a semi-quantitative assessment of expression levels, samples were removed for analysis on 1% agarose gels after 25, 30 and 35 cycles.

3.2.4 RAPID AMPLIFICATION OF CDNA ENDS (5'RACE)

Sequencing of the 5' end of the AAEL004275, AAEL004298 and AAEL011037 mRNAs was performed by 5' RACE using the Smarter RACE amplification kit (Clontech, CA, USA). Reverse transcription was performed on RNA samples from WT female pupae, obtained as described in section 3.2.3, according to supplier's instructions. Outer and nested PCR reactions were then performed using the reaction conditions recommended by the supplier, with the primers shown (Table 3-2):

Table 3-2: Gene-specific primers for 5' RACE reactions

Primer name	Sequence	Target
Ae4275-5'OuterRev	5'-CAGGGTGTGGGTTTGAAGAAG-3'	AAEL004275 (outer RACE primer)
Ae4275-5'InnerRev	5'-ATTGGGCTTCGCTCTTGACTG-3'	AAEL004275 (nested RACE primer)
Ae4298-5'OuterRev	5'-GGTACAGCAGTTCATCGATCTTGG-3'	AAEL004298 (outer RACE primer)
Ae4298-5'InnerRev	5'-CGTTTAGCGAGGGTTCGTTTCAG-3'	AAEL004298 (nested RACE primer)
Ae11037-5'OuterRev	5'-TAGTGGTCGGCACTGTGGTGAT-3'	AAEL011037 (outer RACE primer)
Ae11037-5'InnerRev	5'-GTTGAACCCGCTGTGCTTGTC-3'	AAEL011037 (nested RACE primer)

Amplification products were purified and sequenced as described in General Methods (Chapter 2)

3.2.5 AMPLIFICATION OF PROMOTERS AND CONSTRUCT SYNTHESIS

Putative promoter regions of AAEL004275, AAEL004298 and AAEL011037 were amplified by PCR using primers 4275prom-FAvr -4275psRAsc-b; 4298p-FNheAsi-4298psRAsc-a (Table 3-3).

Table 3-3: Primers for promoter amplification

Primer name	Sequence	Target
4275prom-FAvr	5'-AGCGCCTAGGTTTGATTAATTTGGCTAGGACTCCG-3'	AAEL004275 promoter region (with AvrII and AscI restriction sites)
4275psRAsc-b	5'-ACGCGGCGCGCCAAGCAAATTAATAAACTCTAGGTTCTTTC-3'	
4298p-FNheAsi	5'-AGCGGCTAGCGCGATCGCTGCACTGCTCTGAATCTTTGACTAAGG-3'	AAEL004298 promoter region (with NheI, AsiSI and AscI restriction sites)
4298psRAsc-a	5'-ACGCGGCGCGCCCATCGACGGTACATCTTGAGACAC-3'	
Dsred-FAvr	5'-AGCGCCTAGGACCCAAGAAGAAGCGCAAAC-3'	DsRed coding region, with AvrII and NotI restriction sites
Dsred-RNot2	5'-AGCGGCGGCCGCCAGGTTCAAGGGGAGGTG-3'	
DsRed-FAsi	5'-AGCGGCGATCGCACCCAAGAAGAAGCGCAAAC-3'	DsRed coding region with AsiSI and NotI restriction sites
Dsred-RNot2	5'-AGCGGCGGCCGCCAGGTTCAAGGGGAGGTG-3'	

Bands of the expected size were extracted, purified and cloned into the pJET vector system before being sequenced, as described in General Methods (Chapter 2). Sequences were aligned with the predicted upstream region for each promoter, as annotated in the VectorBase database (www.vectorbase.org/Aedes_aegypti/), using the AlignX software (Invitrogen, CA, USA).

Constructs OX4708 (AAEL004275pro-*dsRed*) and OX4709 (AAEL004275pro-*tTAV2*): Construct OX4531 (Hex1 α pro-*tTAV2* – Oxitec Ltd) was digested with AvrII and AscI to remove the Hex1 α promoter. The PCR product of primers *4275prom-FAvr* and *4275psRAsc-b* (AAEL4275 promoter) was then digested from the pJET vector using AvrII and AscI. Digests were analysed on a 1% Agarose gel before extracting bands of the expected size and purifying using the MinElute Gel Extraction kit (Qiagen, Venlo, Netherlands). The OX4531 backbone and AAEL004275 promoter were ligated using the T4 Rapid Ligation kit (Roche, Basel, Switzerland) to make construct OX4709. Construct OX4708 was made by removing an AvrII-NotI cassette, containing the TetR-VP16 (*tTAV*) coding sequence, and replacing this with a DsRed coding sequence amplified from plasmid OX3645 (Oxitec Ltd) using primers Dsred-FAvr and Dsred-RNot2 (Table 3-3).

Construct OX4732 (AAEL004298pro-*dsRed*): Construct OX4531 (Hex1 α pro-*tTAV2*) was digested with AvrII and AscI as described in the previous paragraph. The AAEL004298 promoter fragment was amplified with primers *4298p-FNheAsi* and *4298psRAsc-a*. It was then digested with NheI and AscI, before being ligated into the OX4531 backbone (NheI and AvrII produce compatible sticky ends). Construct OX4732 was then made by removing an AsiSI-NotI cassette, containing the TetR-VP16 (*tTAV*) coding sequence, and replacing with a DsRed coding sequence amplified from plasmid OX3645 using primers DsRed-FAsi and Dsred-RNot2 (Table 3-3).

3.2.6 REAL-TIME RT-PCR

RNA extraction and first-strand cDNA synthesis was performed as described in General Methods (Chapter 2). The dsDNA dye SYBR green was used for amplicon detection. Primers 4275p-DiagF1–825)diag4DsRed and 4298p-DiagF1–825)diag4DsRed (Table 3-4) were used to amplify DsRed; primers 1381) AeAlbS17F and 1382)AeAlbS17R (Table 3-4) amplify a 300 bp region of the ribosomal S17 subunit and were used as an endogenous control. Three technical replicates were performed with each sample, and three separate biological samples were analysed from each developmental stage.

Table 3-4: Primers for Real-time RT-PCR

Primer name	Sequence	Target
4275p-DiagF1	5'-GAGAGTGAGGAGTTCCGTGAAGCG-3'	AAEL004275 5'UTR (forward)
4298p-DiagF1	5'-TACCTTAGTCAAAGATTCAGAGCAGTGCA-3'	AAEL004298 5'UTR (forward)
825) Diag4-DsRed	5'-CTCGATCTCGAACTCGTGGC-3'	DsRed coding region (reverse)
1381) AeAlbS17F	5'-AAGCCCCTGCGTAACAAGAT-3'	Ribosomal S17 subunit (forward)
1382)AeAlbS17R	5'-GTTATCTCTGCGCTCACGTTC-3'	Ribosomal S17 subunit (reverse)

3.3 RESULTS AND DISCUSSION

3.3.1 IDENTIFICATION OF CANDIDATE GENES

An initial set of 25 genes with promising expression profiles was identified using the *Ae. aegypti* Genome Browser gateway provided by UCSC/Caltech (Akbari et al., 2013), and from these, five genes were selected for more detailed characterisation (Figure 3-1). The principal selection parameter was the relative strength of expression at later developmental stages (4th instar larvae and pupae) versus earlier stages. Adults released into the environment in a RIDL programme will be unable to access tetracycline, hence expression of a lethal effector during adult stages is

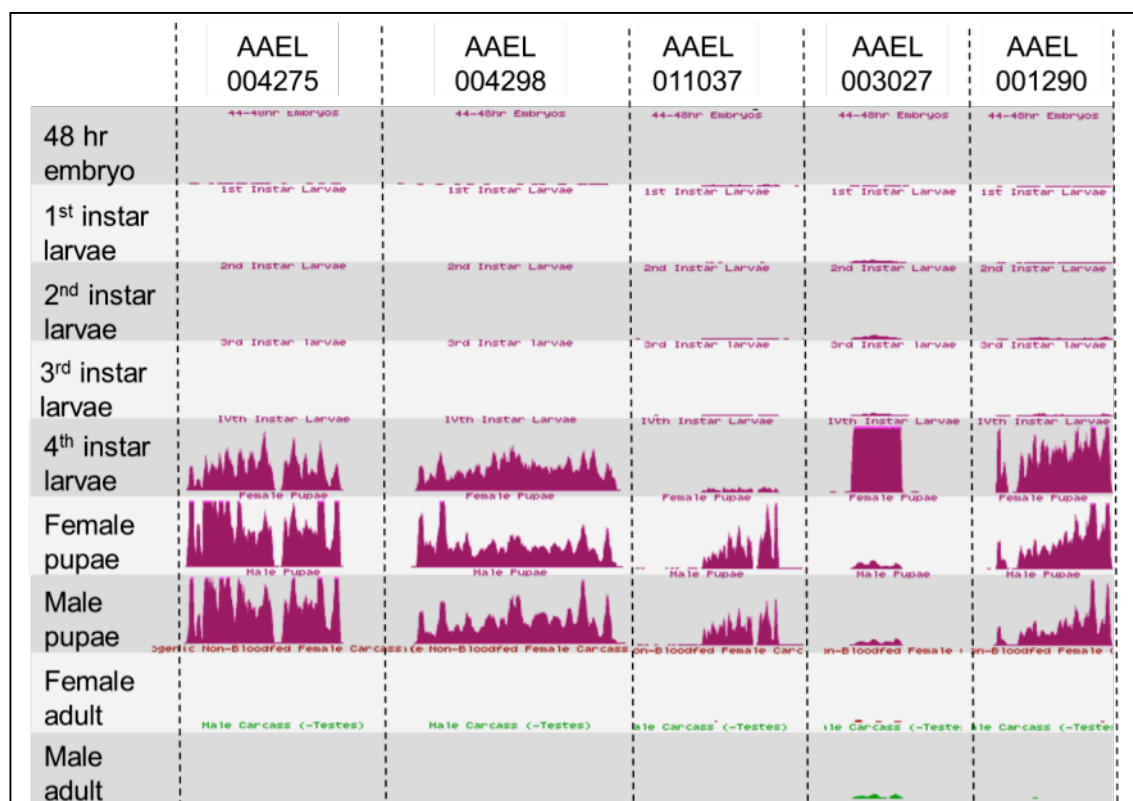


Figure 3-1: expression profiles of 5 candidate genes as reported by UCSC genome browser.

Selected developmental stages are shown as indicated. Purple bars indicate total number of reads mapped to the region shown.

considered undesirable. Candidates found to be closely clustered with other genes showing a similar expression profile were avoided due to the potential for a shared regulatory region and consequent difficulties in isolating the promoter region (for a review of the importance of microsynteny in genomic regulatory blocks with shared regulatory elements, see Dyck et al. (2005)).

3.3.2 RT-PCR OF CANDIDATE GENE EXPRESSION

Semi-quantitative RT-PCR was used to confirm the published expression profiles of the five candidate genes.

Three of the five genes, AAEL004275, AAEL004298 and AAEL011037, showed strong expression in pupal and/or 4th instar larvae, and low expression in other developmental stages (Figure 3-2). The output of the *Ae. aegypti* developmental transcriptome suggested that low levels of expression of AAEL004275 and AAEL004298 may occur in 48 hour old embryos (Figure 3-1). However, additional RT-PCRs which included samples from 48 hour old embryos showed no evidence of this (data not shown). AAEL003027 and AAEL001290 appeared to show either low levels of transcript, in the case of AAEL003097, or less differentiated expression between developmental stages, as for AAEL001290, and were therefore not included in further investigations (data not shown).

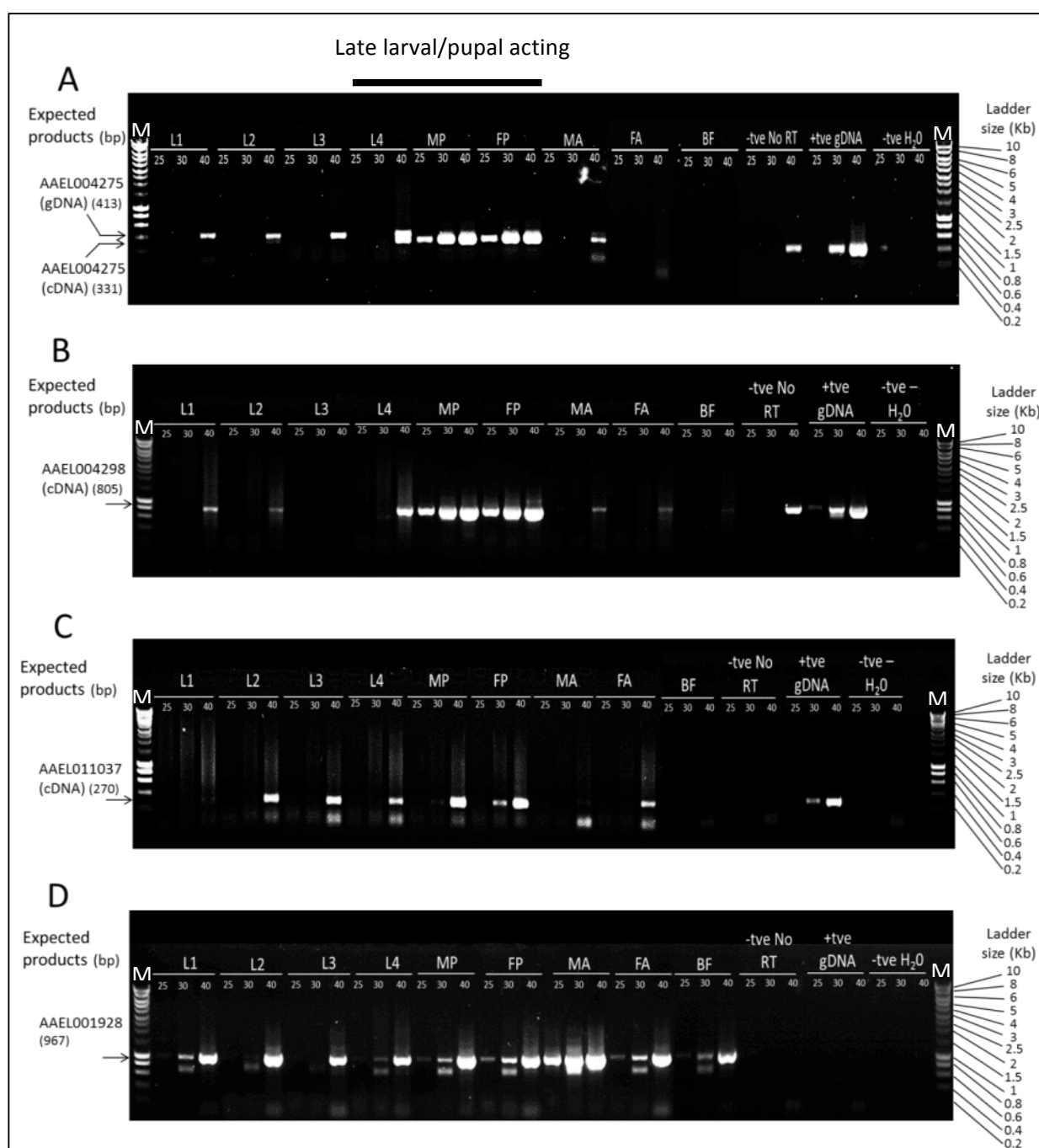


Figure 3-2: Stage-specific expression of AAEL004275, AAEL004298 and AAEL011037 genes

RT-PCRs were performed on 1st – 4th instar larvae (L1-L4 respectively); male and female pupae (MP and FP); male and female adults (MA and FA) and blood fed females 24hrs post feed (BF). (A) PCR for AAEL004275 transcript; (B) PCR for AAEL004298 transcript; (C) PCR for AAEL011037 transcript; (D) PCR for AAEL001928 (Muscle Actin) transcript (endogenous control). Primers and expected product sizes are shown in table 3.1 (Methods), expected amplification products are indicated at left of gels. Samples from the PCR reaction were taken after 25, 30 and 40 cycles, as indicated above lanes.

-tve-NoRT = Male pupae sample without reverse transcriptase; +tve-gDNA = genomic DNA; -tve-H₂O = water. M = SmartLadder molecular weight marker (Eurogene): sizes of markers are indicated at right of gels.

AAEL011037 appears from its orthologues in *A. gambiae* and other species to be a member of the CPR family of cuticular genes, which are required for the formation of adult cuticle and many of which show pupal-specific expression in diverse arthropod species (Dotson et al., 1998). Although expression appears to be higher in pupae than earlier larval stages, the differential is less marked than that seen for either AAEL004298 or AAEL004275.

AAEL004275 and AAEL004298 have been characterised as putative *Osiris* genes (Dorer et al., 2003). AAEL004275 is orthologous to the *Osiris 8* gene in *D. melanogaster*, and AAEL004298 is orthologous to *Osiris 11* (Shah et al., 2012). Little is known about the function of the *Osiris* family, which is conserved across a number of arthropod species, beyond their role as a cluster of twenty four genes (as characterised in *D. melanogaster*) which apparently are responsible for the unique triplo- and haplo- dosage sensitivity of the triplo-lethal (*tpl*) locus identified in *D. melanogaster* (Shah et al., 2012) (see below and in Conclusions for further consideration of possible function and biological significance of these genes). A search for *Ae. aegypti* paralogues of these genes using the VectorBase genome browser (Megy et al., 2011) indicated seven related genes, also identified as putative *Osiris* genes. These were not closely clustered, although one, AAEL004280, was within 200 bp of the AAEL004298 and AAEL004275 genes. A subsequent examination of the USCS/Caltech transcriptome database revealed that none of these genes appeared to exhibit the clear pupal-specific expression shown by AAEL004275 and AAEL004298.

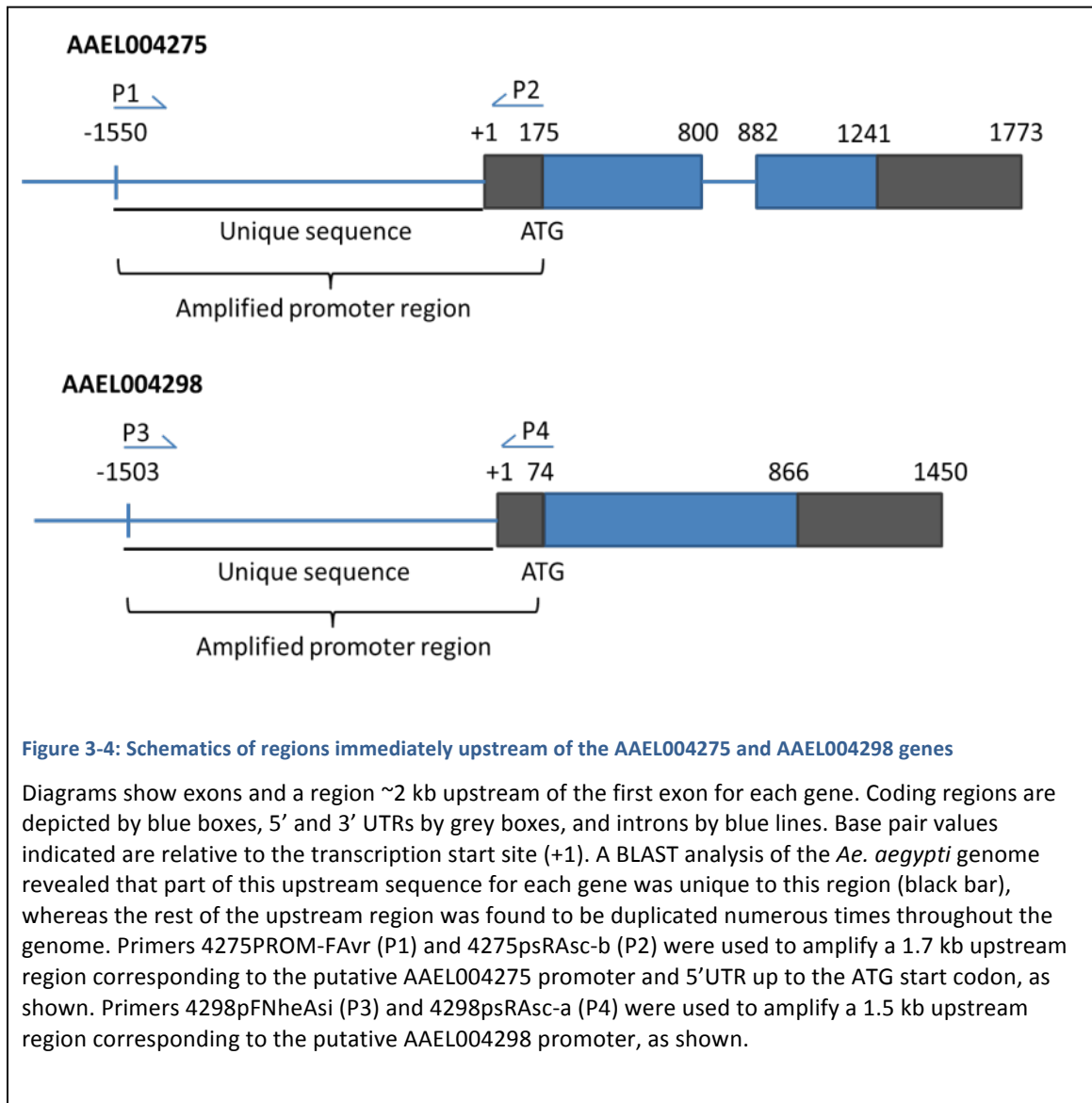
3.3.3 5'RACE OF CANDIDATE GENES

In order to isolate the promoter regions of the three candidate genes selected, it was important to determine the exact location of the transcription start site. While putative transcription start sites were annotated in the *Ae. aegypti* genome browser, additional assays were required to confirm precisely the 5' terminus of transcribed regions and thus the beginning of the upstream regulatory sequences. 5'RACE was therefore used to sequence the 5' terminus of the mRNAs. Sequences were found largely to match the transcripts for each gene predicted in the published *Ae. aegypti* genome database (Figure 3-3). However, in each case, it was found that the actual transcription start site was 5' of the transcription start site predicted; i.e. that the 5'UTR extended somewhat further upstream than indicated in the annotated sequence.

Aside from this discrepancy, the transcribed region for each gene was located as predicted, such that the sequence immediately upstream of this region could be characterised as putative promoter.

3.3.4 AMPLIFICATION OF PUTATIVE PROMOTER REGIONS

Putative promoter regions from AAEL004275 and AAEL004298 were amplified from *Ae. aegypti* genomic DNA using primers designed to amplify a ~1.5 kb region immediately upstream from the start codon in each gene (Figure 3-4) - see Table 3-3, Methods, for description of primers used.



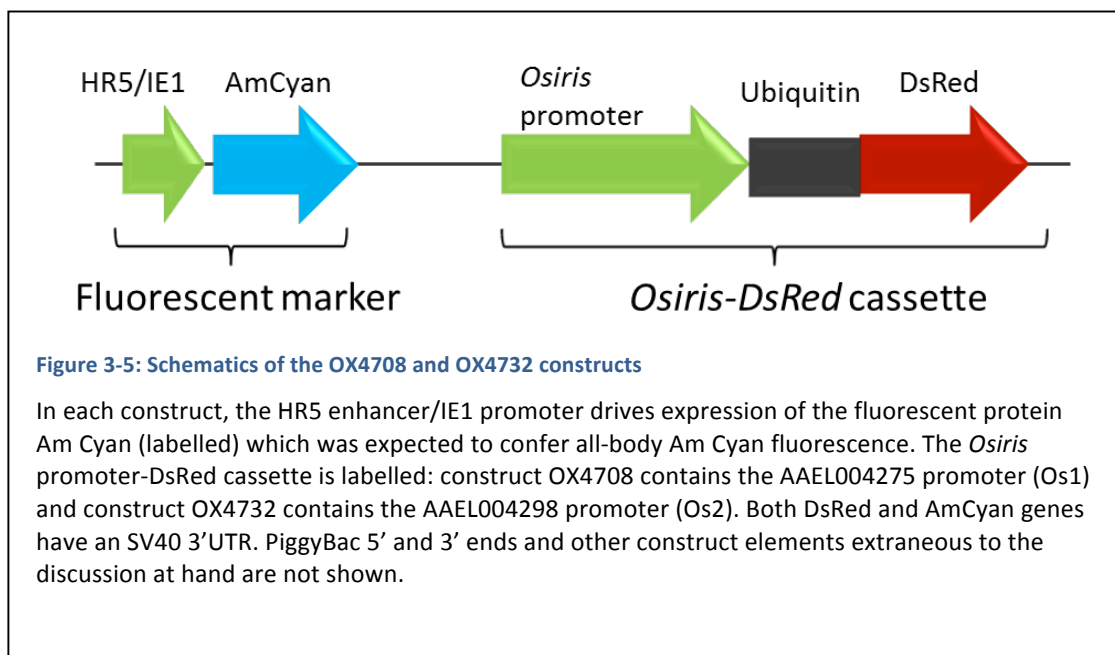
The identity of amplified PCR fragments was confirmed by sequencing and alignment with published sequence data (not shown). The putative promoter regions were determined following

a BLAST analysis, conducted in VectorBase (Megy et al., 2011), for a larger region encompassing three kilobases upstream of the two *Osiris* transcripts. The analysis revealed that, in each case, a region of ~1.5 Kb immediately upstream of the *Osiris* transcript was unique to that genomic location. However, for the region immediately upstream of this ~1.5 Kb region, high-identity matches (> 90% identity) were found in numerous other locations throughout the *Ae. aegypti* genome. Much of this upstream sequence may therefore consist of repetitive elements, such as transposons, which are thought to constitute over 50% of the *Ae. aegypti* genome (Nene et al., 2007). This result suggested that these upstream regions lie outside of the core promoter elements of the two *Osiris* genes, and that the critical promoter regions were likely to reside in the unique ~1.5 kb region amplified.

Attempts to amplify an equivalent region upstream of the coding sequence of AAEL011037 were unsuccessful. It is possible that this was due to the presence of repetitive DNA elements in the sequence upstream of the AAEL011037 exons, and consequently the potential for non-specific binding of the chosen primers. In that eventuality, re-designing primers, perhaps such as to amplify a smaller region of upstream sequence, may have solved this problem. However, two promising candidate promoters were now isolated, and in view of the fact that the AAEL011037 gene had not shown such a promising expression profile, work on this promoter was not pursued.

3.3.5 TRANSFORMATION OF *AE. AEGYPTI* WITH MARKER CONSTRUCTS EXPRESSING FLUORESCENT PROTEINS UNDER THE CONTROL OF AAEL004275 AND AAEL004298 PROMOTERS

To assess the *in vivo* activity of the isolated promoter regions, marker constructs were created in which each respective promoter was placed upstream of a *DsRed* fluorescent gene. Constructs were made by modifying an existing plasmid, OX4513 (HR5IE1-*AmCyan*-Hex1.2pro-*tTAV* – Oxitec



Ltd); the Hex1.2-*tTAV* cassette of OX4513 was removed and replaced with the AAEL004275promoter-*DsRed* (*Os1-DsRed*) or AAEL004298promoter-*DsRed* (*Os2-DsRed*) cassette, to create plasmids OX4708 and OX4732 (Figure 3-5), as described in Methods.

3.3.6 PHENOTYPE OF OX4708 AND OX4732 LINES

Injection data for constructs OX4708 and OX4732 is summarised in table Table 3-5.

Table 3-5: Injection data for constructs OX4708 and OX4732

Construct	Eggs injected	G0 survival (to adult)	Transgenic G1 pools
OX4708	1040	86	15
OX4732	1250	133	10

15 transgenic OX4708 (*Os1-DsRed*) families were isolated from separate pooled G0 crosses. From these, four unique heterozygous lines were established, designated OX4708 H, K, L and N.

10 G0 males resulting from injections with construct OX4732 (*Os2-DsRed*) were crossed individually to WT females. Four separate lines were established from these crosses for initial characterisation of fluorescence phenotype, designated OX4732 E, I, J and L.

Fluorescence phenotypes of OX4708 and OX4732 lines were examined during each developmental stage using a fluorescent microscope. All OX4708 (*Os1-DsRed*) lines showed strong multi-tissue fluorescence from the AmCyan marker, driven by the *HR5/IE1* promoter, which showed little variation from early instar larvae through to adults (Figure 3-6 A – upper panels). In contrast, *DsRed* expression, driven by the AAEL004275 promoter, varied considerably by developmental stage. All lines were characterised by very weak or undetectable *DsRed* fluorescence during larval and adult stages, but strong fluorescence was observed during pupal development, being strongest in late (> 1 day-old) pupae (Figure 3-6 A – lower panels). The apparent presence of *DsRed* fluorescence in both transgenic and wild-type early-stage larvae can be attributed to auto-fluorescence due to food present in the insect gut. This can be readily distinguished from the bright fluorescence resulting from the fluorescent marker.

A more systematic observational study using age-synchronised pupae was carried out for line OX4708N (*Os1-DsRed*). This revealed that *DsRed* fluorescence could be observed most strongly in

24 -32 hour old pupae, and was virtually undetectable in pupae up to eight hours old (Figure 3-6 B). Expression appeared to occur most strongly in the developing legs and antennae.

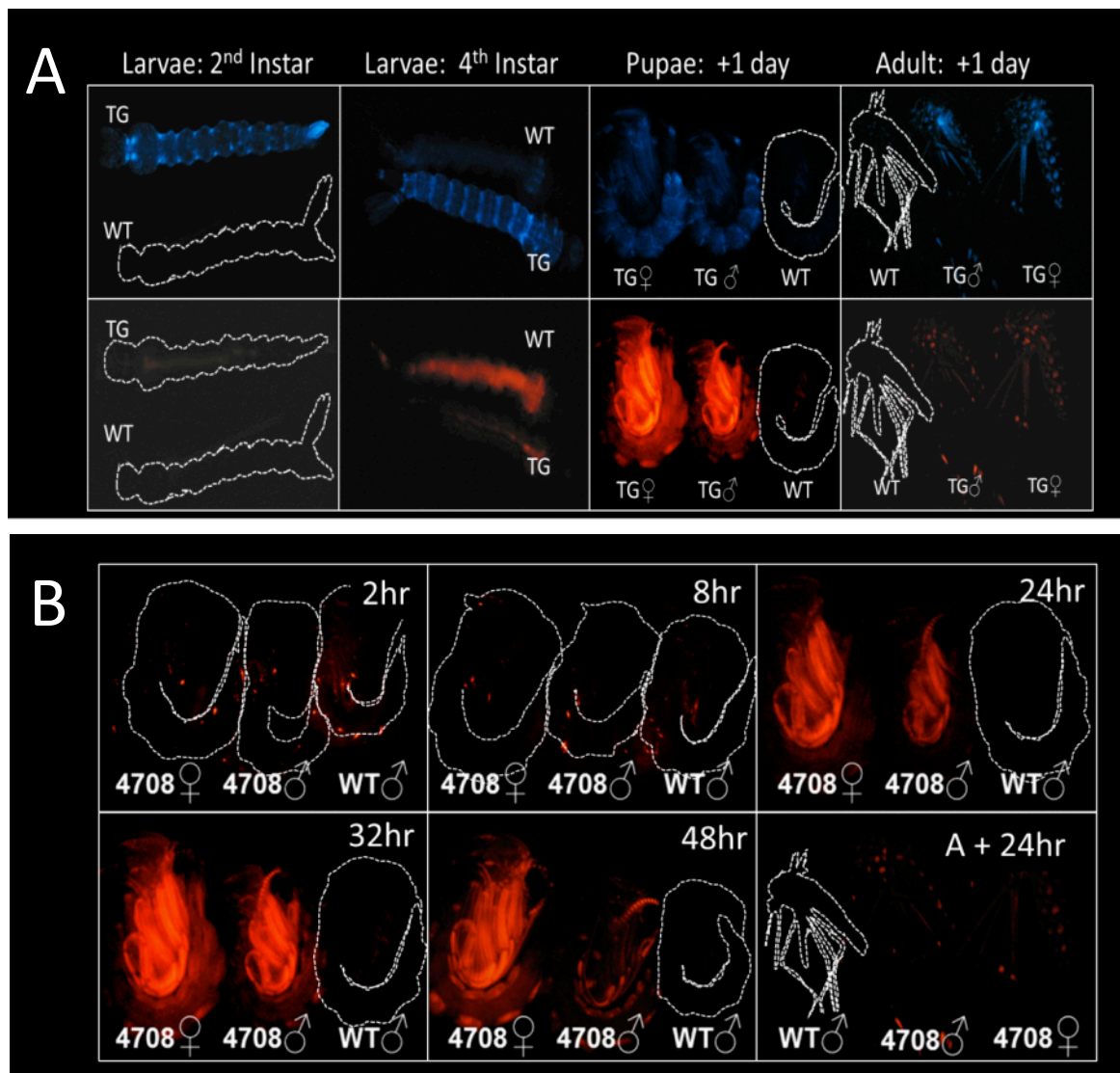
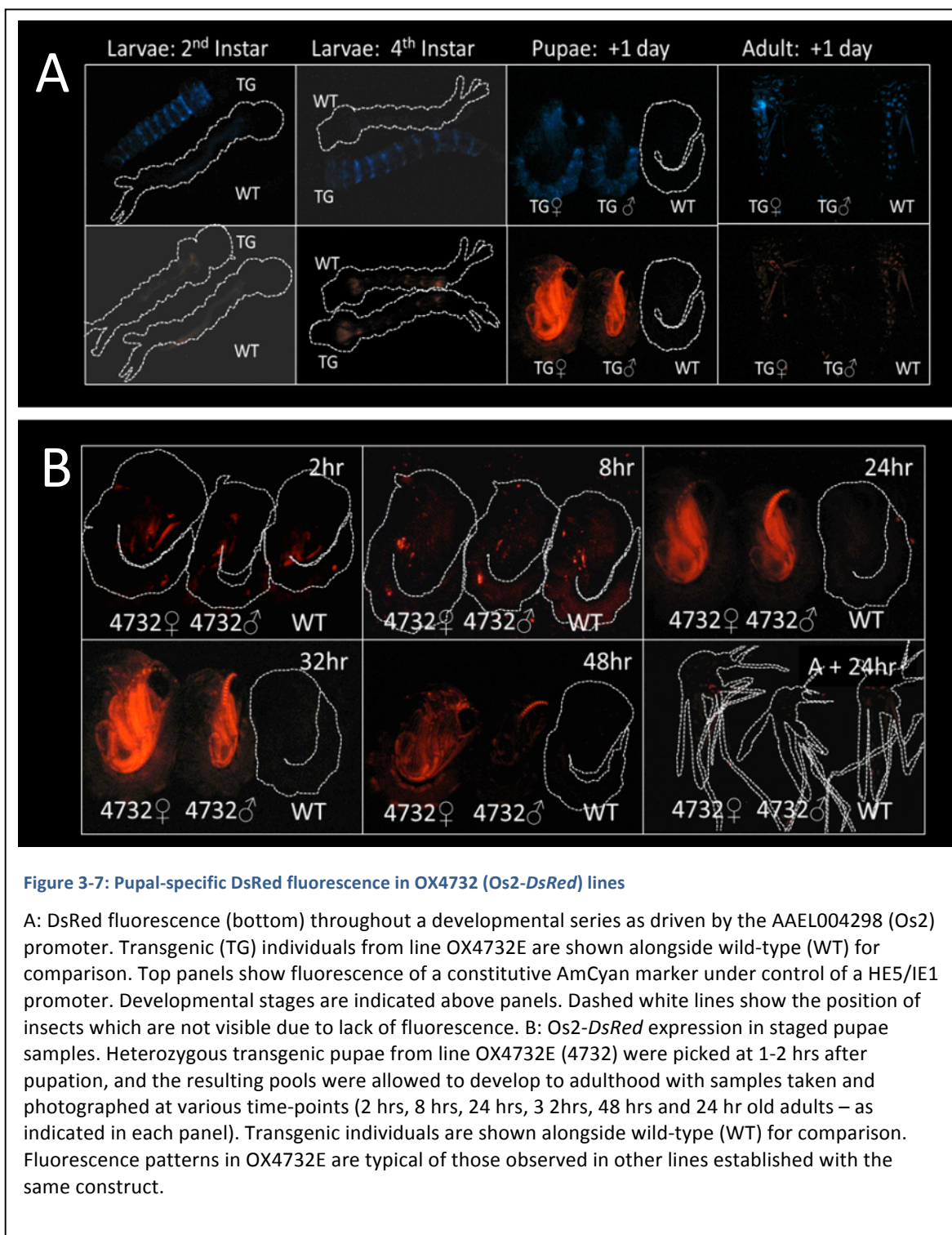


Figure 3-6: Stage-specific expression of the *Osiris-DsRed* cassette in *Ae. aegypti* transgenic lines

A: DsRed fluorescence (bottom) throughout a developmental series as driven by the AAEL004275 (*Os1*) promoter. Transgenic (TG) individuals from line OX4708N are shown alongside wild-type (WT) for comparison. Top panels show expression of a constitutive AmCyan marker under control of a HE5/IE1 promoter. Developmental stages are indicated above panels. Dashed white lines show the position of insects which are not visible due to lack of fluorescence. B: *Os1-DsRed* expression in staged pupae samples. Heterozygous transgenic pupae from line 4708N (4708) were picked at 1-2hrs after pupation, and the resulting pools were allowed to develop to adulthood with samples taken and photographed at various time-points (2hrs, 8hrs, 24hrs, 32hrs, 48hrs and 24hr old adults – as indicated in each panel). Transgenic individuals are shown alongside wild-type (WT) for comparison. Fluorescence patterns in OX4708N are typical of those observed in other lines established with the same construct.

A similar observational study was conducted for transgenic insects expressing the OX4732 (Os2-*DsRed*) construct. The pattern of DsRed fluorescence, driven by the AAEL004298 (Os2) promoter, was found to be similar to that seen in Os1 lines – both in respect of its stage-specificity and tissue distribution (Figure 3-7). Again, expression of the DsRed marker was seen to be specific to late pupal stages, underlining the potential suitability of these promoters for engineering late-acting lethal expression systems.

Little has been reported regarding the possible *in natura* function of the *Osiris* genes. However, the fact that they have been identified as being responsible for the triplo-lethal phenotype of the *Drosophila Tpl* locus is evidence of their critical function (Dorer et al., 2003), and the high degree of sequence conservation and synteny among orthologues in numerous insect species is further evidence of a conserved role for these genes among the diptera (Shah et al., 2012). Certainly the highly similar expression profile of the two promoters observed here is suggestive of a shared function for the native genes. Moreover, the *D. melanogaster* orthologues of these genes, *Osiris 8* and *Osiris 11*, both show a similar expression profile, with peaks in 2-3 day old pupae (Gelbart and Emmert, 2013). Hence, function may be conserved across species. The sequence of *Osiris* proteins, which includes a conserved hydrophobic domain near the N-terminus, suggests a role as trans-membrane proteins, and Dorer et al. (2003) speculate a role in extra-cellular signalling. The pattern of DsRed fluorescence as driven by the *Osiris* promoters suggests high levels of expression in many structures within the developing pupa. Strong expression in developing adult structures during later-stage pupae may indicate an important role for the native genes in tissue-remodelling during metamorphosis. However, expression appears to be concentrated in the developing adult legs, antennae, and to a degree in other parts of the developing adult cuticle.



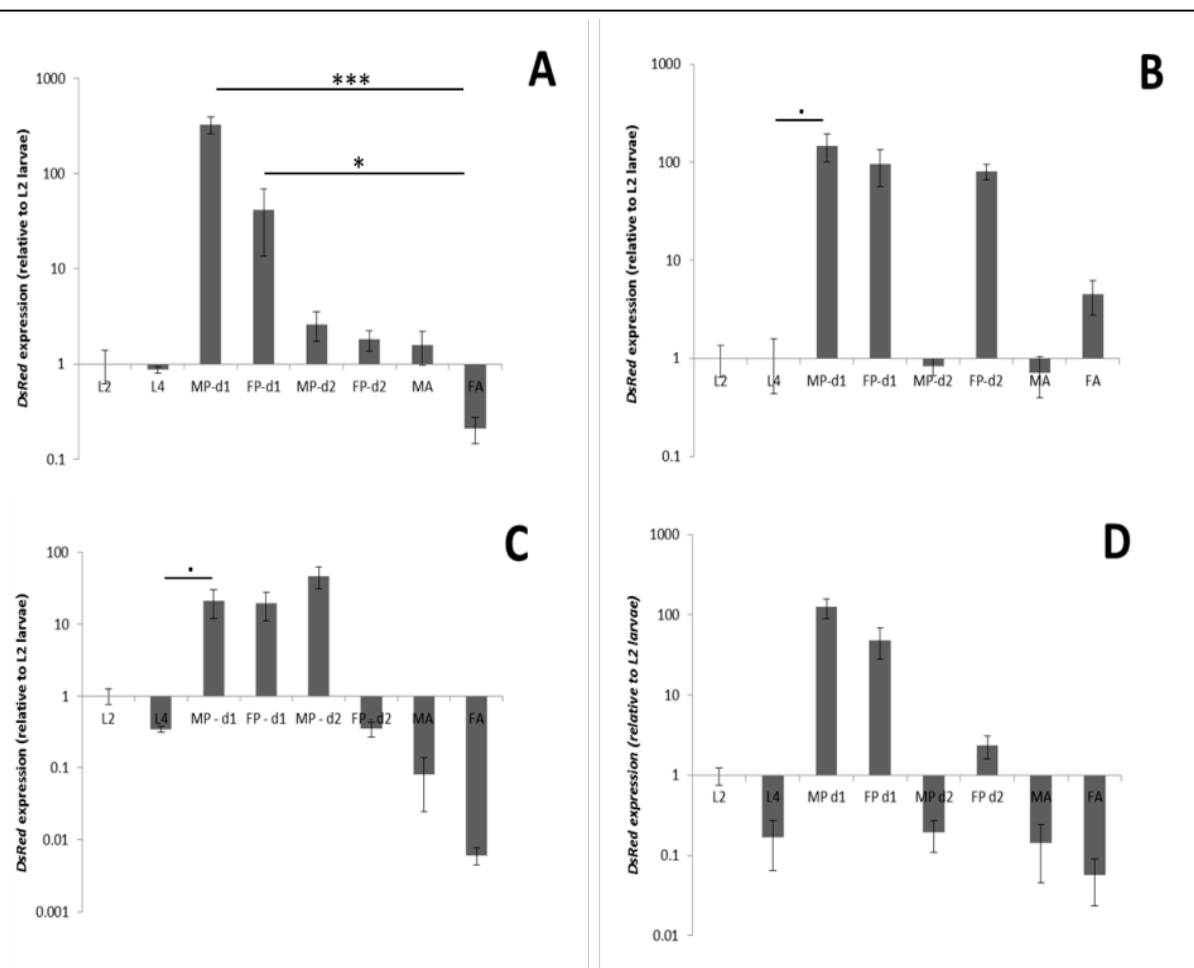


Figure 3-8: Induction of DsRed expression at different developmental stages in 4708 and 4732 transgenic lines

DsRed expression in two OX4708 (Os1-DsRed) and two OX4732 (Os2-DsRed) lines. A: OX4708[N]; B: OX4708[H]; C: OX4732[E]; D: OX4732[I]. Expression was measured using Real Time PCR with the SYBR green probe, using cDNA samples prepared from different developmental stages: L2 = 2nd instar larvae; L4 = 4th instar larvae; MP-d1/d2 = male pupae, day 1/day2; FP-d1/d2 = female pupae, day1/day2; MA = male adult (1 day post-emergence); FA = female adult (1 day post-emergence). Expression was normalised to expression of the s17 ribosomal RNA using the standard curve approach. Fold expression is shown relative to 2nd instar larvae samples. Error bars show SEM of three biological replicates, each containing ~15 individuals for L2 samples or 3 individuals for L4, pupae and adult samples. The value of each biological replicate represents the average value of three technical replicates. Black diamonds indicate expression values of individual samples where an outlying value was observed. Horizontal bars indicate statistically significant differences between means (Kruskal-Wallis rank sum test followed by Nemenyi-Damico-Wolfe-Dunn post-hoc test: $p = 0$ '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 '' 1). Initial Kruskal-Wallis tests showed statistically significant differences between groups for all lines except 4732I.

3.3.7 QUANTIFICATION OF DSRED EXPRESSION IN 4708 AND 4732 TRANSGENIC LINES

Quantitative RT-PCRs were used to further assess the level and developmental stage of *DsRed* induction in OX4708 and OX4732 transgenic lines. Consistent with the fluorescence imagery, *DsRed* was found to be strongly induced in pupal stages in each of four lines tested carrying either the OX4708 (Os1-*DsRed*) or OX4732 (Os2-*DsRed*) constructs (Figure 3-8).

Induction appeared to be concentrated in earlier pupal stages, with strongest induction seen in day 1 pupae samples for lines OX4708N, OX4708H and OX4732I. For females, line OX4732E showed peak expression in day 2 samples. Considerable variation was observed between samples from the same developmental stages – particularly around the peak expression levels seen in pupae. This likely explains the lack of statistically significant differences between groups reported by the Nemenyi-Damico-Wolfe-Dunn test, for all lines except OX4732E. This may reflect genuinely large variation in the expression level of the same transgene between individuals – for example, in response to environmental stimuli. However, since pupae were not assayed at a particular time point, the samples do not represent precise and directly correlated stages of development. A more probable explanation for the observed variation is therefore that peak expression occurs over a very discrete period during pupal development; this would explain the large differences in measured expression observed between samples of similar, but not identical, age, making it difficult to draw firm conclusions as to the timing and level of peak expression.

To probe the timing of peak induction in pupae further, Real Time PCR was performed on staged pupae samples of similar age (within a 1-2 hour time window). Results shown in Figure 3-9 indicate that peak expression likely occurs between 8 and 24 hours. Again, apparently large differences in mean expression levels masks a considerable amount of variation between individual samples at

each time point assayed. This explains the lack of significant differences in expression levels at different time points, for all but one of the lines studied. Nonetheless, in view of the expression peaks seen in Figure 3-8, together with the expression peaks seen through the fluorescence assay, it is unlikely that this apparent lack of significant difference represents a true picture. Rather, these results provide further evidence that peak expression occurs over a very specific but short period in pupal development. As a consequence, pupal samples of very similar, but not identical, ages might show considerably different levels of fluorescence.

Although these results do not pinpoint the time of peak *Osiris* expression precisely, they do show clearly that, for both promoters, there is a strong peak in expression during pupal development. They therefore give further support for the use of these promoters to engineer late-acting lethal systems.

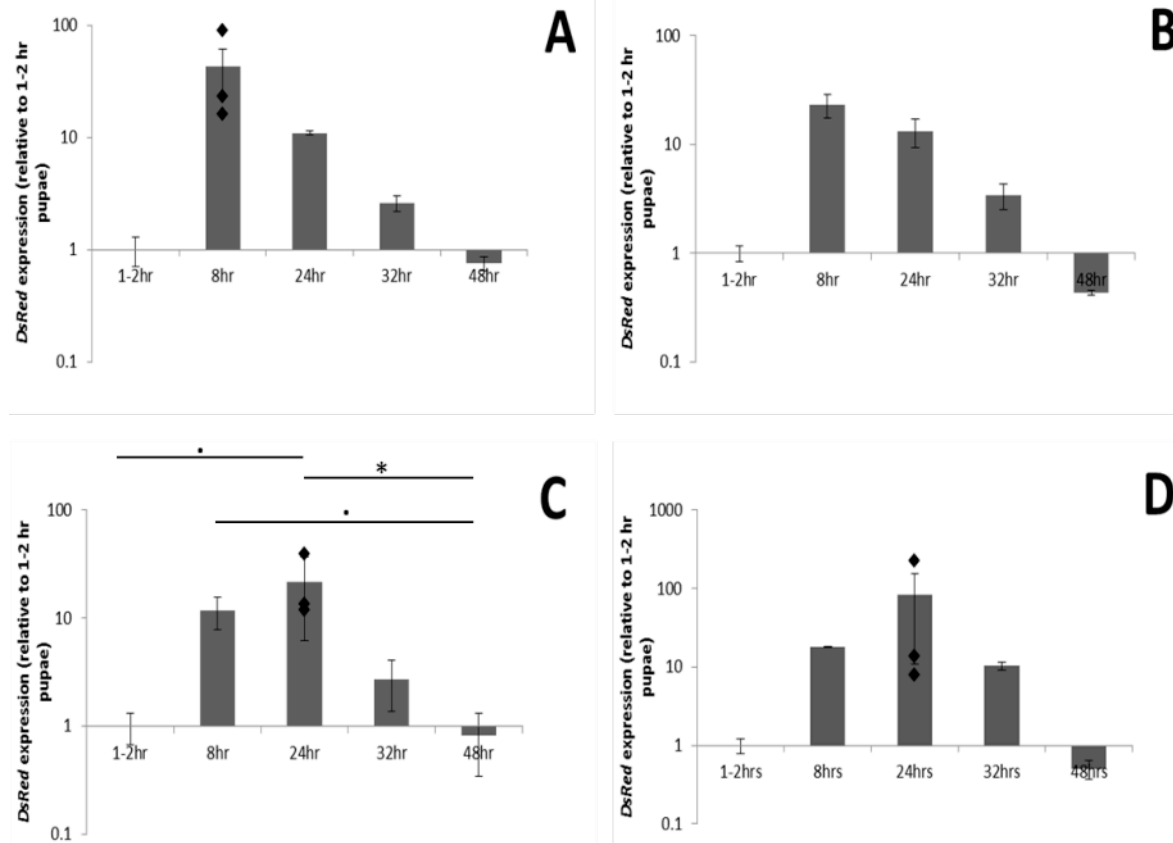


Figure 3-9: Induction of DsRed expression in staged pupae samples in 4708 and 4732 transgenic lines

DsRed expression at specific time-points in pupal development, in two OX4708 (Os1-DsRed) and two OX4732 (Os2-DsRed) lines. A: OX4708[N]; B: OX4708[H]; C: OX4732[E]; D: OX4732[I]. DsRed expression was measured using Real Time PCR with the SYBR green probe. Expression was normalised to expression of the s17 ribosomal RNA using the standard curve approach. Fold expression is shown relative to 1-2 hr old pupae. Error bars show SEM of three biological replicates, each containing two male pupae or 1 female pupae (C). The value of each biological replicate represents the average value of three technical replicates. Black diamonds indicate expression values of individual samples where an outlying value was observed. cDNA was prepared from pupae aged 1-2 hrs, 8 hrs, 24 hrs, 32 hrs and 48 hrs, as indicated. All pupae were picked within a 1-2 hr time window to ensure like samples were of the same or similar age. Horizontal bars indicate statistically significant differences between means (Kruskal-Wallis rank sum test followed by Nemenyi-Damico-Wolfe-Dunn post-hoc test: $p = 0$ '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 '' 1).

3.3.8 THE AAEL004298 (OS2) PROMOTER CONFERS A NEARLY IDENTICAL EXPRESSION PATTERN IN *AE. ALBOPICTUS*

Given the highly conserved nature of the *Osiris* genes, and the conserved expression pattern between the AAEL004275 and AAEL004298 *Osiris* genes and their orthologues in *D. melanogaster*, it was thought possible that the *Ae. aegypti* promoters could also induce late-acting expression in the related species, *Ae. albopictus*. To test this hypothesis, the construct OX4732 (*Os2-DsRed*), which contained the fluorescent marker DsRed expressed under the control of the AAEL004298 (*Os2*) promoter, was used to transform wild-type *Ae. albopictus* mosquitoes.

Injection data were not recorded, in light of the facile transformation recorded with the same construct in *Aedes aegypti*. A total of four transgenic lines were established, all of which showed a nearly identical pattern of DsRed fluorescence to that observed in OX4732 lines in *Ae. aegypti*: expression was seen to peak during late (> 1 day old) pupae, with little or no DsRed fluorescence being detectable in other developmental stages (Figure 3-10). Moreover, the tissue-specific pattern of fluorescence observed in *Ae. aegypti* was also conserved, with expression appearing to be highest in legs and antennae of developing pupae.

It therefore appears that the *Ae. aegypti* AAEL004298 (*Os2*) promoter acts in a near-identical manner in both *Ae. aegypti* and *Ae. albopictus*. Since both *Os1* and *Os2* promoters appear to have highly similar expression patterns, it seems likely that the utility of either of these promoters for engineering late-acting expression systems in *Ae. aegypti* will be equally manifest in *Ae. albopictus*.

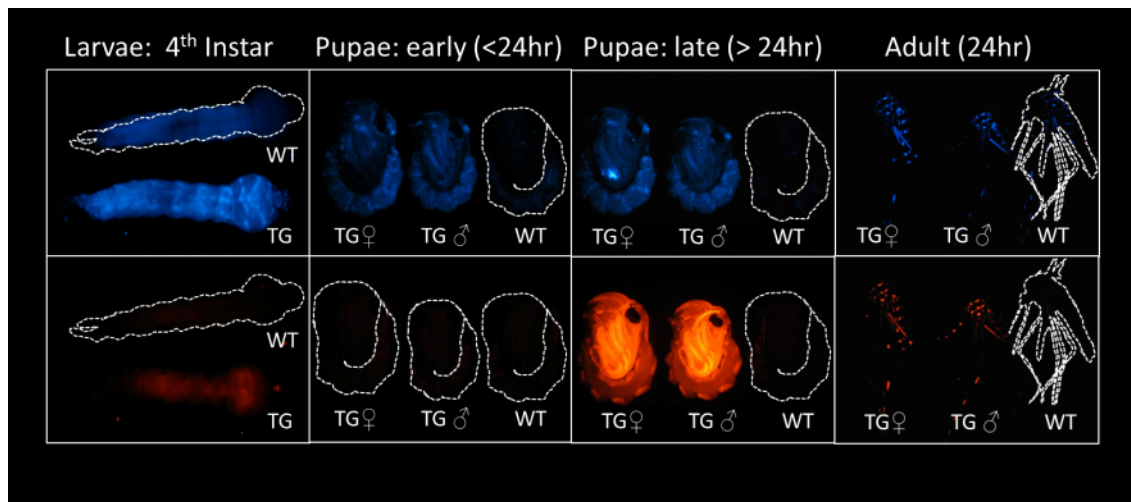


Figure 3-10: Os2-*DsRed* expression in OX4732 *Ae. albopictus* transgenic insects

DsRed fluorescence (bottom) throughout a developmental series in *Ae. albopictus* insects containing the Os2-*DsRed* cassette. Transgenic (TG) individuals from line OX4732F are shown alongside wild-type (WT) for comparison. Top panels show fluorescence of a constitutive AmCyan marker under control of a HE5/IE1 promoter. Developmental stages are indicated above panels. Dashed white lines show the position of insects which are not visible due to lack of fluorescence. Fluorescence patterns in OX4732F are typical of those observed in 3 other *Ae. albopictus* lines established with the same construct

3.4 CONCLUSIONS: *OSIRIS* PROMOTERS AND THEIR POTENTIAL USE IN DESIGNING LATE-ACTING EXPRESSION SYSTEMS IN *Aedes* MOSQUITOES

Suppressing insect populations through the release of insects carrying a dominant lethal gene has been shown to be an effective approach for mosquito population control (Harris et al., 2012). The efficiency of this approach is achieved, in part, due to the presence of a lethal phenotype which acts in the late immature stages of the mosquito, and hence maintains density-dependent competition among wild-type and transgenic heterozygous larvae in the field (Atkinson et al., 2007). However, there is currently a lack of promoters known to be capable of driving late-stage expression consistently in transgenic *Ae. aegypti* or *Ae. albopictus* mosquitoes, while avoiding significant expression in earlier stages.

The *Ae. aegypti* genes AAEL004275 and AAEL004298 are of unknown function, and have been identified as coding for putative *Osiris* proteins. RT-PCR results describing native expression of these genes, together with data gathered from the *Ae. aegypti* Transcriptome Browser Gateway provided by UCSC/Caltech (Akbari et al., 2013) (<http://aedes.caltech.edu/cgi-bin/hgGateway>) have demonstrated that they are strongly up-regulated during both male and female pupal stages, identifying them as promising candidates for the isolation of promoter regions to drive a late-stage bi-sex lethal system.

Osiris genes have been identified as responsible for conferring the triplo-lethal phenotype of the *Drosophila Tpl* locus (Dorer et al., 2003). This, and the high degree of sequence conservation and synteny among orthologues in numerous insect species, is suggestive of a conserved role for these genes among the diptera (Shah et al., 2012). The expression profile of all *Osiris* genes is reported to be characterised by expression peaks in embryos, 2nd Instar larvae, and in pupae (Shah et al., 2012). Expression of some genes peaks during all three of these developmental stages, while

others – as has been found to be the case for AAEL004275 and AAEL004298 – are highly expressed during only a single developmental stage. Following the work described here, bioinformatics analysis on the putative promoter regions of several *Ae. aegypti* *Osiris* genes, including AAEL004275 and AAEL004298, revealed conserved binding sites for the transcription factors broad (br) and E74 (Molloy, 2014). Both of these transcription factors have been identified as having roles in specifying pupal development and metamorphosis, including pupal cuticle formation and appendage elongation (Zhou and Riddiford, 2002, Fletcher and Thummel, 1995). The fact that these genes appear to be highly conserved among dipterans may also mean that their promoter elements may have utility in dominant lethal approaches outside the originating species.

This chapter further reports on the use of putative promoter regions isolated from these genes to drive the expression of a DsRed fluorescent marker in transgenic *Ae. aegypti*. The fluorescent phenotype of transgenic individuals indicates that, as expected, the level of *DsRed* expression as driven by these promoters was consistently highest during pupal stages across multiple independent insertions of the constructs, suggesting that there is indeed potential to use these promoter regions to induce a late-acting lethal phenotype in the context of a dominant lethal system.

Further evidence for this is provided in quantitative RT-PCRs which provide a direct measure of *DsRed* expression throughout the development of transgenic individuals. In all lines tested, *DsRed* expression clearly peaked during pupation, with peak expression levels up to 10^3 -fold higher than that observed in either larvae or adult insects. Further testing on staged pupae samples indicates that expression peaks in pupae between 8 hrs and 24 hrs old, but the large degree of variation of expression among similarly-aged individuals suggests that peak expression may occur over a very short time-window. Alternatively, it is possible that there is a large amount of genuine variation in expression between individuals, perhaps in response to environmental cues. However, earlier

observations of fluorescence phenotype suggested a pattern of transgene induction over time which was highly consistent across different individuals (and indeed different insertion lines).

The phenotype of DsRed fluorescence as driven by the promoters from AAEL004275 and AAEL004298 suggests that expression is widely distributed throughout the insect body; however, it appears strongest in developing legs and antennae. This may indicate high levels of expression in developing cuticle, or expression associated with developing appendages – particularly in view of a possible role for the transcription factors *broad* and *e74* (as discussed above). Confirming these observations through tissue-specific RT-PCRs was considered an unnecessary step in view of the fact that these questions, while interesting, are subsumed under the more general one of whether the *Osiris* promoters are capable of driving a late-acting dominant lethal expression system. Clearly, if these promoters restrict both expression and locus of activity of a downstream effector to structures which are non-essential for adult viability, this may impact on the ability of the promoters to induce a fully penetrant lethal phenotype when coupled to a toxic effector. However, such a question can only be properly elucidated once the promoters are used as part of an engineered repressible lethal system, as will be described in the subsequent chapter. Overall, the results reported in this chapter suggest that these promoters could be promising candidates for incorporation into a late-stage, repressible lethal system.

Finally, results reported here also indicate that the *Ae. aegypti*-derived *Osiris* promoters retain their characteristic expression pattern in the closely-related species, *Ae. albopictus*. Hence, it is likely that success in developing late-acting lethal systems in *Ae. aegypti* using these promoters will also provide a promising route to engineering equivalent systems in *Ae. albopictus*.

4 DEVELOPMENT OF A LATE-ACTING LETHAL PHENOTYPE DRIVEN BY 'OSIRIS' PROMOTERS

4.1 INTRODUCTION

The previous chapter described the identification of two novel promoter elements from the putative *Osiris* genes AAEL004275 and AAEL004298. Both of these genes demonstrated the desired pupal-specific expression profile necessary for engineering late acting expression systems. Isolation of the promoters from these genes and their use in transgenic constructs demonstrated their ability to drive late-acting expression of engineered systems *in vivo*. Further characterisation of the expression of a fluorescent transgene under the control of the two promoters demonstrated that expression peaks in pupae of around 24 hours old. However, while this would be an ideal life stage at which to induce lethality in the context of a RIDL system, it remained unclear whether the strength or tissue-specificity of expression would be appropriate for an engineered late-acting lethal system.

This chapter describes the use of the promoters from AAEL004275 and AAEL004298 to drive late-acting, tetracycline-repressible lethal systems in *Ae. aegypti*. A construct based on the AAEL004298 promoter has been shown to induce a repressible late-acting lethal phenotype in transgenic lines, which shows considerable promise as a basis for the development of future product lines in *Ae. aegypti* and *Ae. albopictus*.

4.2 METHODS

4.2.1 STRAIN REARING AND TRANSFORMATION

All procedures were carried out as described in General Methods (Chapter 2)

4.2.2 RT-PCR

Total RNA extraction and cDNA synthesis was performed as described in General Methods (Chapter 2). RT-PCR of OX4709 samples was performed using the primers shown in Table 4-1:

Table 4-1: Primers for RT-PCR of tTAV expression in OX4709

Primer name	Sequence	Target	Target length
1015)Seq2-tTAV2	5'-AGCGGCGATCGCACCCAAGAAGAAGCGCAAAC-3'	tTAV	390 bp
1903)Diag2-VP16new	5'-AGCGGCGGCCGCCAGGTTGAGGGGGAGGTG-3'		

To provide a semi-quantitative assessment of expression levels, samples were taken for analysis on 1% agarose gels after 25, 30 and 35 cycles.

4.2.3 CONSTRUCT SYNTHESIS

Construct OX4709 (AAEL004275pro-tTAV2): Construct OX4709 was synthesised by modifying construct OX4531 (Hex1 α -tTAV2), as described in Chapter 3 (Methods).

Construct OX4800 (AAEL004275pro-tTAF3-Dmhsp70 3'UTR): the fragment tTAF3 was amplified from construct OX4427 (Hex1 α pro-tTAF3-3xP3pro-dsRed – Oxitec Ltd) using primers tTAF3-F-Avr and tTAF3-R-Bbs (Table 4-2). A 246 bp fragment corresponding to the *Hsp70* 3'UTR from *D. melanogaster* was amplified from construct OX4774 (hsp83pro-Nipp1-DmHsp703'UTR – Oxitec Ltd) using primers Hsp70-F-Bbs and Hsp70SmaR (Table 4-2). The tTAF3 PCR fragment was digested with AvrII and BbsI. The *Hsp70* fragment was digested with BbsI and XmaI. Construct OX4709 was digested with XmaI and AvrII and this vector was then

ligated with the tTAF3 and *Hsp70* PCR fragments using T4 quick ligase (New England Biolabs, Hitchin, UK).

Construct OX4811 (AAEL004298pro-tTAF3-Dmhsp70 3'UTR): the fragment tTAF3 was amplified from construct OX4427 (Hex1αpro-tTAF3-3xP3pro-dsRed) using primers tTAF3-F-Asi and tTAF3-R-Bbs (Table 4-2). This fragment was digested with BbsI and ligated with the *Hsp70* fragment described above, before the tTAF3-hsp70 cassette was amplified using primers tTAF3-F-Asi and Hsp70SmaR. Construct OX4811 was made by digesting construct OX4732 with XmaI and AsiSI, before ligating this vector with the tTAF3-hsp70 cassette using T4 quick ligase (NEB, MA, USA).

Construct OX5055 (AAEL004298pro-tTAF3-Dmhsp70 3'UTR-tetO-MichX): Construct OX5055 was designed and built by Sarah Scaife, Oxitec Ltd.

Table 4-2: Primers for construct synthesis

Primer name	Sequence	Target
tTAF3-F-Avr	5'-CGGCCTAGGATGGGCAGCCGCCTGGATAAGTCC-3'	<i>tTAF3</i> (forward)
tTAF3-R-Bbs	5'- GCGCGAAGACTAGTCGTCCAGCATGTCGAGATCAAAGTCGTCC-3'	<i>tTAF3</i> (reverse)
tTAF3-F-Asi	5'-CGGTGCGATCGCATGGGCAGCCGCCTGGATAAGTCC-3'	<i>tTAF3</i> (forward)
Hsp70-F-Bbs	5'-GCGCGAAGACGTCGACTAAAGCCAAATAGAAAATTATTCAG-3'	<i>Hsp70</i> 3'UTR (forward)
Hsp70SmaR	5'-GGTGTGCCCCGGGGATCTAAACGAGTTTTTAAGCAAACCTCACTC- 3'	<i>Hsp70</i> 3'UTR (reverse)
1668)tetOF	5'- CGTCAGTCGCGGAGCGCGACTAGATCTCCCCGGCCATCGAGAAA GAGAGAGAG-3'	tetO (forward)
1669)michx R	5'- CGAACAAAATGTTATTGAACAACCAGGTTCTTCATTGGCTTCTAAC AGAATCTTGAC-3'	<i>Michelob_x</i> (reverse)

4.2.4 ON/OFF TETRACYCLINE ASSAYS

On/Off tetracycline survival assays were performed as described in General Methods (Chapter 2), with the exception that 300 individuals were used per replicate.

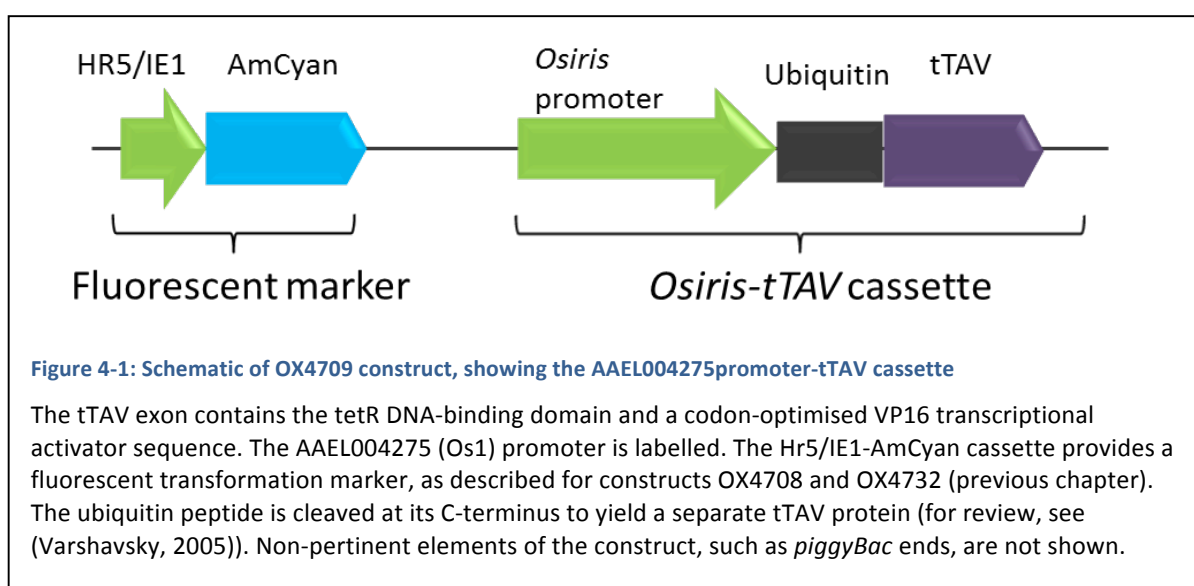
4.2.5 STATISTICAL ANALYSIS

All statistical analysis was performed using the “R” software for statistical computing (R Development Core Team, 2010), as described in General Methods (Chapter 2)

4.3 RESULTS AND DISCUSSION

To assess the ability of the AAEL004275 and AAEL004298 promoters (*Osiris* promoters) to drive late-stage specific lethality, constructs were made in which the AAEL004275 and AAEL004298 promoters were used to drive tTAV expression, with the intention of creating lines which could be crossed to tetO-lethal effector lines.

A first round of constructs was made in which the *Osiris* promoters were used to drive expression of tTAV.



OX4709 (Figure 4-1) contained the AAEL004275 promoter (Os1). Injection data for construct OX4709, along with constructs OX4800, OX4811 and OX5055 (described below) is provided in Table 4-3.

Table 4-3: Injection data for *Osiris*-tTAV lines

Construct	Eggs injected	G0 survival (to adult)	Transgenic G1 pools
OX4709	780	90	15
OX4800	1259	137	6
OX4811	2150	200	14
OX5055	1625	66	7

Testing of transgenic lines derived with this construct showed a high level of toxicity in transgenic individuals. 15 Generation 0 (G0) pools initially yielded transgenic progeny in the subsequent generation (G1); however, the majority of transgenic individuals were observed to die at late pupae stage or during adult emergence. Eggs from 6 G0 pools were re-hatched, and again a high level of mortality was observed in all OX4709 families (Table 4-4). In addition to high mortality rates, surviving adults were in many cases observed to have deformations in both legs and antennae. In some cases, the hindmost legs of adults were missing entirely, although flight ability was not obviously impaired. Single insertion lines OX4709J and OX4709M were established from the corresponding G0 pools M and J. Expression of *tTAV* mRNA in transgenic individuals was initially examined by semi-quantitative RT-PCR using cDNA samples

Table 4-4: mortality in OX4709 (Os1-tTAV) G1 populations

G1 eggs obtained from 6 G0 pools were hatched and reared to pupation. Larvae were fed *ad libitum*. Total transgenic pupae derived from each pool were selected using a fluorescence microscope and placed in cages to eclose. Total pupae numbers and adult eclosion rates were recorded. Wild-type pupae were pooled and allowed to eclose in a separate cage.

Line	Count		
	Dead Pupae	Adults dead on water	Flying Adults
B1	83	4	5
B2	23	12	21
E	179	20	0
H	115	21	6
J	80	16	18
M	0	3	6
N1	109	13	1
N2	8	14	2
Wild-type	1	2	241

from a single line, OX4709J. As expected, tTAV expression appears to be up-regulated in pupal and L4 larval stages relative to early larval stages and adults (Figure 4-2). Although there was some variation in expression of the endogenous muscle actin control gene detected between samples, nonetheless relative expression levels clearly peak in 1-day old pupae.

4.3.1 tTAV UNDER THE CONTROL OF THE AAEL004275 PROMOTER CAUSES PUPAL-STAGE LETHALITY WHEN CROSSED TO A tetO-MICHELOB_X EFFECTOR LINE

Heterozygous transgenic males from OX4709 (Os1-*tTAV*2) lines J and M were crossed to transgenic females heterozygous for the construct OX3582 (tetO-*hsp70minpro-mx*). OX3582 contained a tetO sequence linked to *micelob_x* (*mx*) (Zhou et al., 2005) – a pro-apoptotic gene – under the control of a minimal promoter derived from the *D. melanogaster hsp70* gene. It was expected that tTAV expression driven by the AAEL004275 promoter would drive pupal-stage expression of *mx* and hence pupal-stage lethality in progeny heterozygous for both OX4709 and OX3582 constructs.

Testing in the presence and absence of tetracycline showed the expected late-acting, tetracycline-repressible lethality in individuals expressing both Os1-*tTAV* and tetO-*mx* cassettes (Figure 4-4). It also provided further evidence of the non-repressible toxicity of the OX4709 construct (Os1-*tTAV*) alone. For F1 progeny derived from line OX4709J, insects carrying both constructs exhibited pupation rates less than 7% that of wild-type insects (Figure 4-4). However, death was observed late in development, primarily at the transition between L4 larvae and pupae. This effect was abrogated by the presence of tetracycline (pupation rates 10% higher than wild-type); however, individuals carrying the Os1-*tTAV* construct alone exhibited a lower pupation rate relative to other genotypes, including individuals carrying both constructs (GLM with Poisson error for ‘on-tet’ pupation rates, followed by stepwise removal of factors. Differences in variance between the saturated model and a model lacking a genotype term were analysed by Anova: $p(\chi^2) = 0.012$).

Pupation rates for F1 progeny derived from OX4709 line M were not significantly affected by genotype, regardless of tetracycline treatment (GLM with quasi-Poisson error for pupation

rates, followed by stepwise removal of factors. Differences in variance between the saturated and null models were analysed by Anova: $p(F) = 0.16$).

In the absence of tetracycline, F1 progeny derived from OX4709 line J exhibited a fully penetrant lethal phenotype in the presence of both *Os1-tTAV* and *tetO-mx* constructs, with no individuals eclosing. F1 individuals derived from OX4709 line M also had greatly reduced eclosion (38% of wild-type eclosion) where both constructs were present, in the absence of tetracycline. However, lethality clearly was not fully penetrant.

In contrast, statistical modelling for adult survival indicated that, in the presence of tetracycline, genotype did not have a significant effect on eclosion rates for either line (GLM for 'on-tet' viable adults, with quasi-Poisson error, followed by stepwise removal of factors. Differences in variance between saturated and null models were analysed by Anova: $p(F) = 0.254$ and 0.735 for line J and M respectively). However, effects here may be masked by high levels of variation between repeats. Certainly, for F1 progeny derived from OX4709 line J, high levels of mortality were observed among individuals carrying both constructs when reared on tetracycline, and among individuals carrying the *Os1-tTAV* construct alone both on and off tetracycline: eclosion rates were 77% and 61% of the wild-type level, respectively. Similar to earlier observations with OX4709 lines, surviving adults in many cases had missing legs and/or deformed antennae.

The *tetO-mx* construct alone was not observed to have a significant effect on pupation or emergence rates in any of the statistical models described, indicating that, as expected, there was no significant toxicity associated with this construct alone.

Overall, these results indicated that an *Os1-tTAV* cassette was capable of driving a late-acting lethal phenotype that was largely repressible by tetracycline. However, this phenotype was observed in only one of two lines tested. Moreover, the high mortality which appears to be

associated with the Os1-*tTAV* cassette would render constructs based on this design unsuitable for use in future product lines.

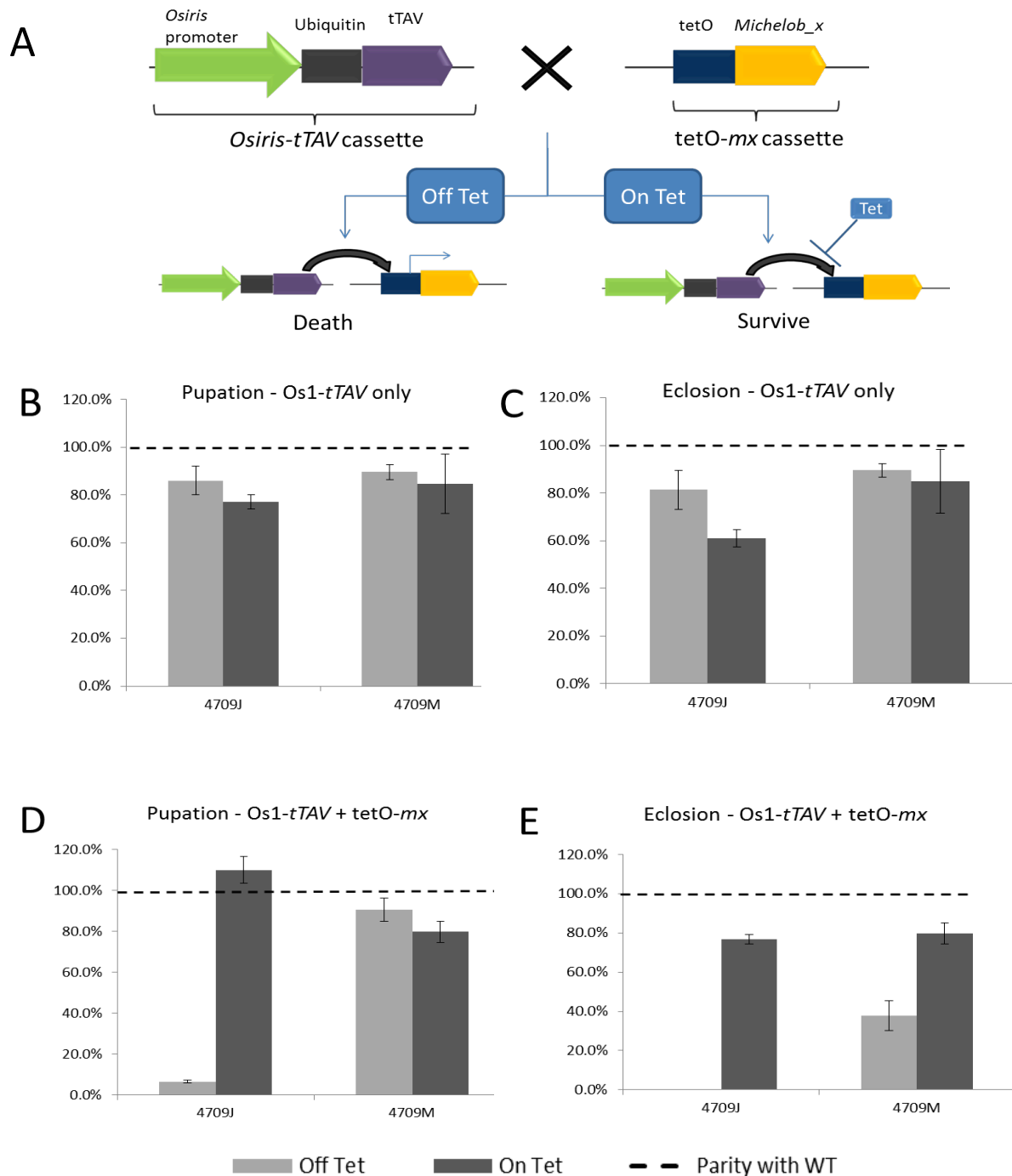
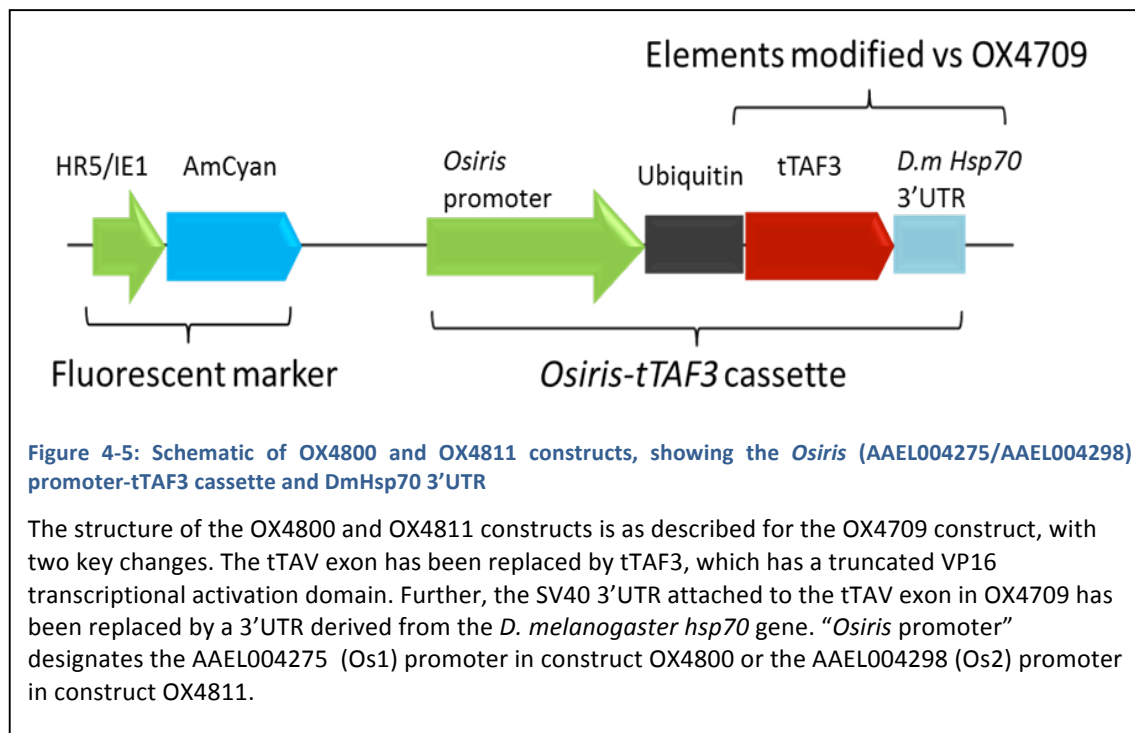


Figure 4-4: Late stage lethality in OX4709-OX3582 double heterozygotes

A: schematic of a cross between transgenic insects carrying the OX4709 (Os1-tTAV) construct and those carrying the OX3582 (tetO-mx) construct, showing expected outcomes for F1 progeny inheriting both transgenes. B-E: Pupation and eclosion rates of F1 progeny resulting from OX4709 x OX3582 crosses. 300 larvae resulting from crosses between heterozygous OX4800 transgenic individuals and heterozygous OX3582 transgenic individuals were reared in the presence ('On Tet' – dark grey bars) or absence ('Off Tet' – light grey bars) of tetracycline. Individuals were screened as pupae and designated as wild-type (WT), heterozygous for OX3582 only, heterozygous for OX4800 only, or carrying both constructs OX3582 and OX4800 according to their fluorescence phenotype. B: pupation rate of transgenic insects carrying the OX4709 (Os1-tTAV) construct only. Bars show the mean pupation rate (as a proportion of starting population) of three replicates, normalised to the wild-type pupation rate (100% = wild-type pupation rate – indicated by dashed line). Error bars denote standard error of the mean. C: adult eclosion rate of insects carrying the OX4709 construct only. D: pupation rate of insects carrying both OX4709 and OX3582 (tetO-mx) constructs. E: eclosion rate of insects carrying both constructs. 'Eclosed' adults here includes all viable adults; individuals which died during eclosion, or on the surface water immediately following eclosion, are not included. The effect of genotype upon eclosion rate in the presence and absence of tetracycline was analysed by GLM models. Results of these are reported in the main text.

4.3.2 SECOND-GENERATION OSIRIS CONSTRUCTS SHOWED IMPROVED ON-OFF TET PHENOTYPE

The high mortality evident in Os1-tTAV transgenic insects may have been due to intolerably high levels of tTAV expression when under the control of the *Osiris* promoter. Indeed, this is



probable, in view of the fact that the phenotype of affected adults, manifesting in deformations of legs and antennae in adults expressing the Os1-tTAV cassette, appears to correlate closely to the high levels of *DsRed* expression driven by *Osiris* promoters that were observed in Chapter 3. Two further constructs, OX4800 and OX4811, were therefore made in order to reduce the toxicity of tTAV expression (Figure 4-5).

Constructs OX4800 and OX4811 contain a tTAF3 exon in place of the tTAV exon of OX4709. tTAF3 contains a truncated VP16 activation domain, and has been shown to have lower toxicity in transgenic insects than tTAV (Labbe, 2011). In addition, the SV40 3'UTR at the 3' terminus of tTAV in OX4709 has been replaced with a 3'UTR sequence derived from the *D. melanogaster hsp70* gene. It was expected that the *hsp70* 3'UTR would destabilise the tTAF3 transcript,

reducing mRNA accumulation and hence levels of the tTAF3 protein in transgenic insects (Bonisch et al., 2007). Injection data is shown in Table 3-5.

Three independent transgenic lines were established from injections with the OX4800 (Os1-*tTAF3*) construct. However, lethality was again observed in positive G1 pools, resulting in the loss of three further putative transgenic lines. G3 heterozygous males from lines OX4800H, OX4800S and OX4800T were crossed with heterozygous females of the tetO-*mx* effector line OX3582. Progeny from these crosses were then reared in the presence or absence of tetracycline to test for a repressible lethal phenotype.

15 independent transgenic lines were obtained following injections with the OX4811 (AAEL004298 (Os2)-*tTAF3*) construct. Five of these lines were selected for further testing; however, it was noted that none of the lines obtained appeared to show the high level of lethality at G1 stage which had previously been observed with OX4709 and OX4800 lines, containing the Os1 promoter. G3 males from OX4811C, N, P, Q and R were crossed with heterozygous females of the tetO-*mx* effector line, OX3582. Progeny from these crosses were then reared in the presence or absence of tetracycline to test for a repressible lethal phenotype.

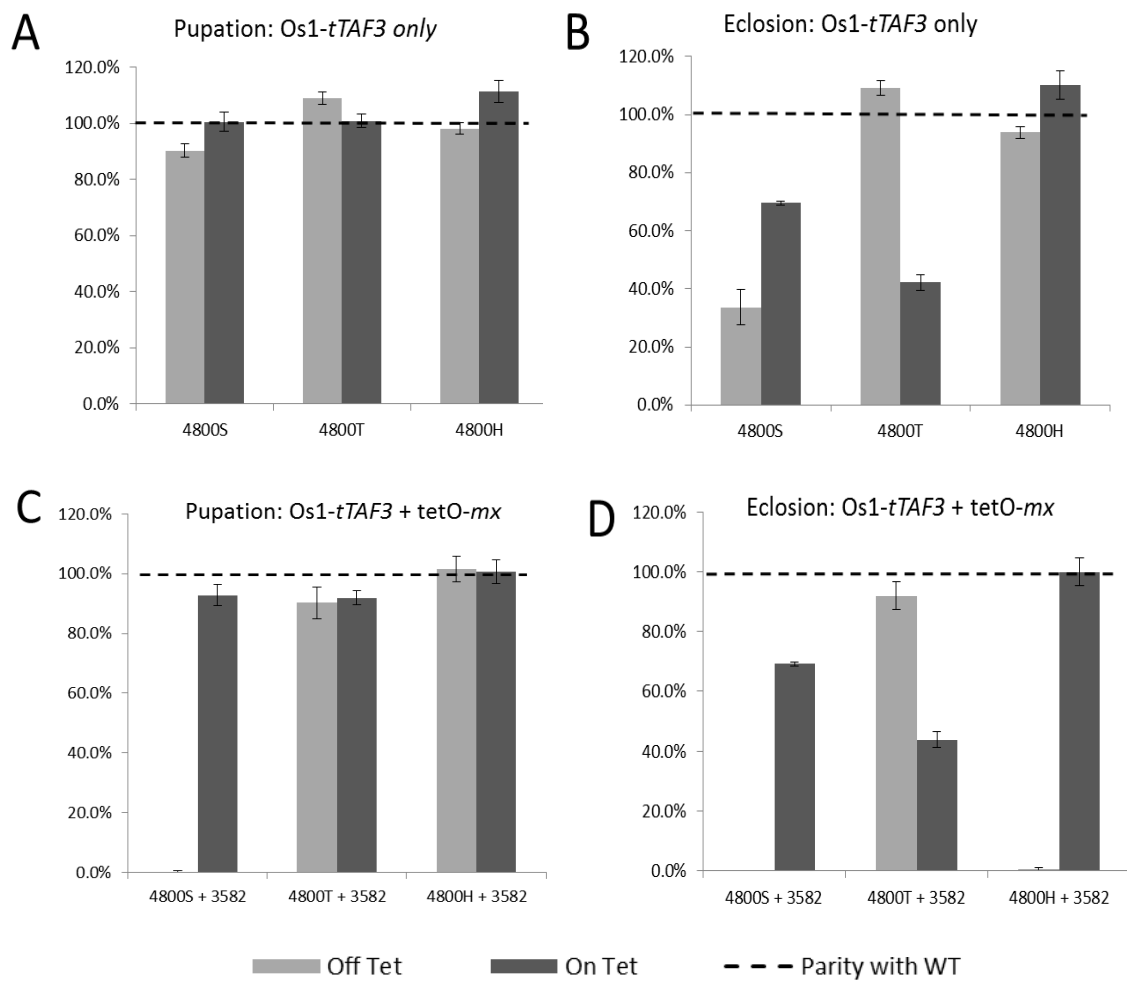


Figure 4-6: Survival of transgenic insects carrying one or both OX4800 and OX3582 constructs, reared in the presence or absence of tetracycline

300 larvae resulting from crosses between heterozygous OX4800 (*Os1-tTAF3*) transgenic individuals and heterozygous OX3582 (*tetO-mx*) transgenic individuals were reared in the presence ('On Tet' – dark grey bars) or absence ('Off Tet' – light grey bars) of tetracycline. Individuals were screened as pupae and designated as wild-type (WT), heterozygous for OX3582 only, heterozygous for OX4800 only, or carrying both constructs OX3582 and OX4800 (4800 + 3582) according to their fluorescence phenotype. A: pupation rate of transgenic insects carrying the OX4800 (*Os1-tTAF3*) construct only. Bars show the mean pupation rate (as a proportion of starting population) of three replicates, normalised to the wild-type pupation rate (100% = wild-type pupation rate – indicated by dashed line). Error bars denote standard error of the mean. B: adult eclosion of insects carrying the OX4800 construct only. C: pupation rate of insects carrying both OX4800 and OX3582 (*tetO-mx*) constructs. D: adult eclosion of insects carrying both constructs. 'Eclosion' here includes all viable adults; individuals which died during eclosion, or on the surface water immediately following eclosion, are not included.

Statistical analysis was performed by interrogating GLM models (detailed results are reported in the main text). Where both constructs were present, no pupation was observed for line S in the absence of tetracycline, whereas pupation was not significantly different from wild-type in the presence of tetracycline (Anova of GLM models following stepwise removal of factors, $p=0.3$). No effect of genotype or tetracycline treatment was observed on pupation rates for lines T and H ($p=0.2$, 0.94 respectively). Eclosion for line H was near zero in the absence of tetracycline, but not significantly different from wild-type in its presence ($p=0.68$). Eclosion rates for line S were significantly different to wild type even in the presence of tetracycline ($p=3 \times 10^{-13}$). Line T did not show any tetracycline repressible lethality and was not further analysed.

Considerable variation was observed in the phenotypes of individuals derived from each of the three OX4800 (*Os1-tTAF3*) lines (Figure 4-6). Line OX4800S showed a high degree of toxicity even in the absence of the *tetO-mx* cassette: eclosion rates were 34% and 70% of the wild-type level in the absence and presence, respectively, of tetracycline. Deformed legs and antennae were again observed in surviving adults carrying the OX4800 construct alone, and in individuals heterozygous for both constructs reared in the presence of tetracycline. For line S, insects carrying both the *Os2-tTAV* construct and *tetO-mx* construct exhibited near fully penetrant lethality prior to pupation in the absence of tetracycline. This was rescued in the presence of tetracycline, with pupation rates not significantly different to wild-type (GLM with Poisson error for 'On Tet' pupation rates, followed by stepwise removal of factors. Differences in variance between saturated and null models were analysed by Anova: $p(\chi^2) = 0.304$). In contrast, pupation rates for lines T and H did not differ significantly from wild-type, regardless of the presence of tetracycline and both transgenes (GLM with Poisson error for pupation rates, followed by stepwise removal of factors. Differences in variance between saturated and null models were analysed by Anova: $p(\chi^2) = 0.21$ and 0.94 for lines T and H respectively).

Line OX4800T failed to demonstrate the expected repressible lethality when crossed to the effector, as well as unexpectedly exhibiting some mortality in the presence of tetracycline. In contrast, individuals expressing both the *tetO-mx* cassette and the *Os1-tTAF3* cassette derived from line OX4800H displayed the expected late-acting lethal phenotype, with near fully penetrant lethality (bar a single individual) at pupal stage among insects reared in the absence of tetracycline. Moreover, lethality appeared to be fully repressible, with survival among tetracycline-reared individuals not significantly different from wild-type (GLM with binomial error for 'On Tet' eclosion rates, followed by stepwise removal of factors. Differences in variance between saturated and null models were analysed by Anova: $p(\chi^2) = 0.682$).

In summary, these results again show that the *Os1* promoter is capable of inducing a late-acting lethal phenotype when coupled to the *tetO-mx* effector. Of three lines tested, one (Line

H) had the desired late-acting lethality which was fully repressible in the presence of tetracycline. However, the non-repressible lethality observed in line S and the loss of other lines at the G1 stage, suggests that the second-generation OX4800 construct does not appear to have fully resolved the issue of toxicity associated with the Os1-transactivator cassette alone.

For the OX4811 (Os2-*tTAF3*) construct, pupation rates of insects carrying one or both transgenes were the same or similar to that of wild-type insects (Figure 4-7 – A & C). For lines R, C, Q and P, the presence of either the Os2-*tTAF3* construct alone or both Os2-*tTAF3* and tetO-*mx* constructs had no significant effect on pupation rates, (GLM with Poisson/quasi-Poisson error for pupation rates, followed by stepwise removal of factors. Differences in variance between saturated and null models for lines R, C and P were analysed by Anova: p (F) = 0.304, 0.313 for lines R and C, respectively; p (χ^2) = 0.738 for line P. Differences in variance between the saturated and a single factor model (tetracycline treatment) was analysed for line Q: p (χ^2) = 0.971). For line N, individuals carrying the Os2-*tTAF3* construct alone demonstrated a higher-than expected pupation rate (117% of wild-type pupation from the same population). This effect was reduced in the presence of tetracycline, though the interaction of tetracycline and transgene presence was found to be of only marginal significance at $p < 0.05$ (GLM with Poisson/quasi-Poisson error for pupation rates, followed by stepwise removal of factors: difference in variance between the saturated model and a model lacking a transgene:tetracycline treatment interaction term was analysed by Anova: p (F) = 0.049). Overall, these data indicate no clear effect of one or both transgenes on pupation rates, regardless of tetracycline presence, as expected for a pupal-specific effect.

Insects which expressed a tetO-*mx* cassette, in addition to the Os2-*tTAF3* cassette, showed the expected late-acting lethality when reared in the absence of tetracycline: three of five lines tested showed pupal lethality which was fully penetrant, while a fourth line exhibited average survival to adulthood of just 2.2% (Figure 4-7 - D). The fifth line (OX4811P) did not show the

expected lethality. Importantly, the lethal phenotype was repressed in the presence of tetracycline. Insects from lines C, N and Q carrying either the *Os2-tTAF3* construct or both *Os2-tTAF3* + *tetO-mx* constructs had 'On-Tet' eclosion rates which were nearly identical with that of wild-type insects, though differences were of marginal statistical significance at $p < 0.05$ in the case of lines C and Q (GLM for On-tet eclosion rates with binomial/quasi-binomial error. Differences in variance between saturated and null models were analysed by Anova: p (F) = 0.03 for line C, p (χ^2) = 0.401 and 0.031 for lines N and Q respectively). In contrast, F1 progeny derived from *Os2-tTAF3* line R did show reduced eclosion rates relative to wild-type insects (Anova on contrasted GLM models as previously: p (χ^2) = 1.63×10^{-7}). However, it is noteworthy that this effect appeared to be similar for insects carrying the *Os2-tTAF3* alone compared with those carrying both constructs, as indicated by the model coefficients (p value (z) for both transgenes vs *Os2-tTAF3* construct alone = 0.177). Hence, it appears that there is some toxicity associated with the *Os2-tTAF3* construct in line R, manifesting at the point of adult eclosion.

Overall, however, these results show that the *Os2-tTAF3* construct is capable of conferring a highly penetrant late-acting lethal phenotype, which is largely, if not fully, repressed in the presence of tetracycline. Moreover, this phenotype is achieved in a more consistent manner than that observed with the *Os1-tTAF3* construct.

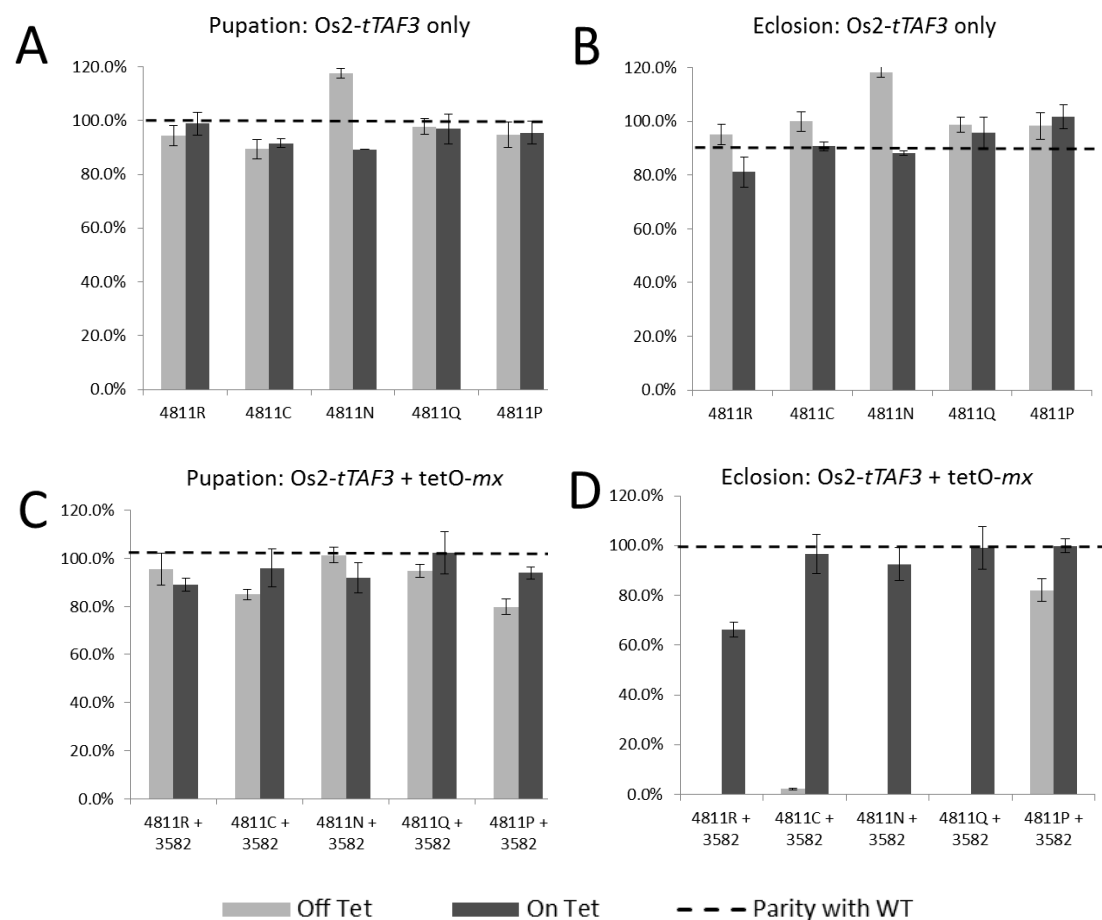


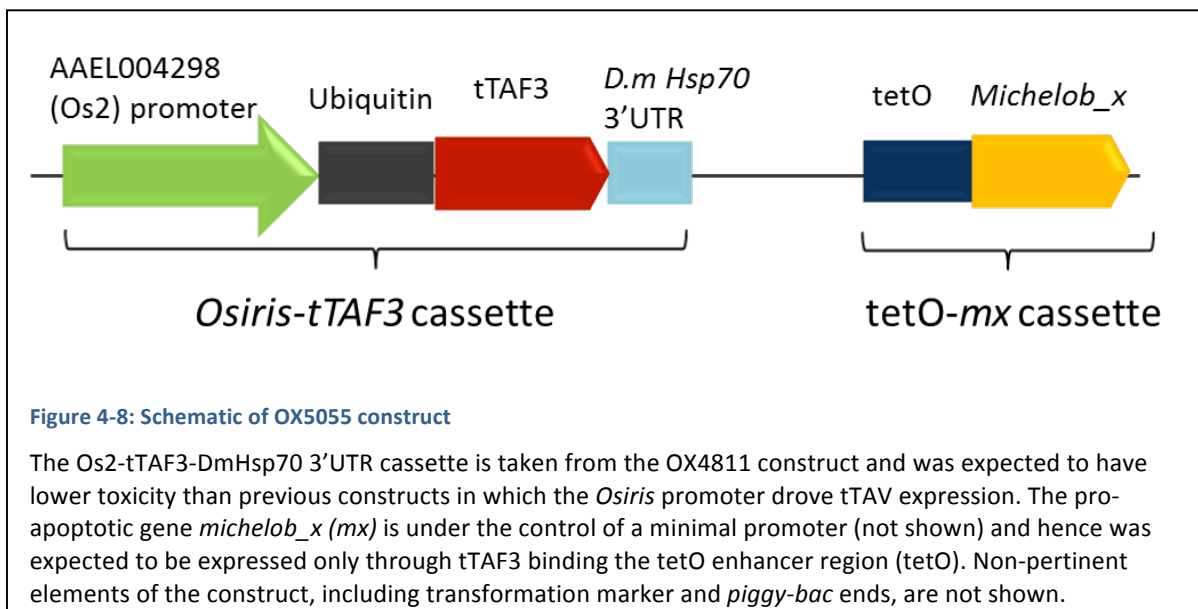
Figure 4-7: Survival of transgenic insects carrying one or both OX4811 and OX3582 constructs, reared in the presence or absence of tetracycline

A: pupation rate of transgenic insects carrying the OX4811 (*Os2-tTAF3*) construct only. 300 larvae resulting from crosses between heterozygous OX4811 transgenic insects and heterozygous OX3582 (*tetO-mx*) transgenic insects were reared in the presence ('On Tet' – dark grey bars) or absence ('Off Tet' – light grey bars) of tetracycline. Individuals were screened for the presence of the transgene as described for Figure 4-6. Bars show the mean pupation rate (as a proportion of starting population) of three replicates, normalised to the wild-type pupation rate (100% = wild-type pupation rate – indicated by dashed line). Error bars denote standard error of the mean. Statistical analysis was performed by interrogating GLM models (see main text for detailed results). No significant effect of genotype on pupation rate was recorded for lines R, C, P & Q (Anova of GLM models following stepwise removal of factors; $p=0.3$, 0.3 , 0.7 , 0.9 respectively). Line N showed marginally increased pupation for the OX4811 construct only in the absence of tetracycline ($p=0.049$).

B: adult eclosion rate (as a proportion of starting population) of insects carrying the OX4811 (*Os2-tTAF3*) construct only, normalised to wild-type rate. C: pupation rate of insects carrying both OX4811 and OX3582 (*tetO-mx*) constructs. D: adult eclosion of insects carrying both constructs. 'Eclosion' here includes all viable adults; individuals which died during eclosion, or on the surface water immediately following, eclosion are not included. On Tet eclosion rates were not significantly worse, or only marginally less than wild-type for lines C, N & Q (Anova of GLM models following stepwise removal of factors; $p=0.03$, 0.4 , 0.03 respectively) and significantly reduced for line R ($p=1.63 \times 10^{-7}$). Line P did not show selective lethality and was not subjected to this analysis.

4.4 AN *OSIRIS* PROMOTER CAN DRIVE A REPRESSIBLE LATE-ACTING LETHAL PHENOTYPE IN TRANSGENIC INSECTS WHEN COUPLED WITH A LETHAL EFFECTOR CASSETTE

Results obtained using the OX4811 (*Os2-tTAF3*) construct have shown that the *Osiris* promoter derived from the AAEL004298 gene was capable of driving repressible, late-acting lethality when crossed to transgenic insects expressing a lethal effector (*tetO-mx*) cassette. To assess whether the same promoter could induce a similar phenotype when linked directly to the *tetO*-effector cassette, a new self-contained or ‘all in one’ conditional lethal construct was synthesised. Construct OX5055 contained the AAEL004298pro (*Os2*)-*tTAF3*-hsp70 3’UTR cassette of construct OX4811 (Figure 4-8). In addition, construct OX5055 contained a *tetO-mx* cassette, such that *tTAF3* expressed under the control of the *Osiris* promoter would induce *michelob_x* expression in the absence of tetracycline, but not when tetracycline was present. This was expected to result in a tetracycline-repressible, late-acting lethal phenotype.



The OX5055 (*Os2-tTAF3*, *tetO-mx*) construct was injected into an *Ae. aegypti* wild-type background. Injection data are shown in Table 3-5. G0 crosses produced a total of five positive G1 pools, of which two, K and N, contained two distinct fluorescence phenotypes. These were

separated to yield four putatively separate lines (K1, K2 and N1, N2 respectively), resulting in a total of 7 separate transgenic lines.

To assay for the presence of a repressible lethal phenotype, G3 eggs from each OX5055 line were hatched and then reared either in the presence or absence of tetracycline, as described in Methods. Counts of pupation and emergence rates showed that all seven lines exhibited late-acting lethality, which was largely repressed in the absence of tetracycline (Figure 4-9). Nonetheless, a considerable degree of variation was noted between the seven lines, both in the degree of penetrance and developmental stage-specificity of the lethal phenotype in the absence of tetracycline, and in the extent to which lethality was repressed in the presence of tetracycline.

Line I exhibited pupal-specific lethality which was fully penetrant, with no individuals eclosing in the absence of tetracycline. Lines C, K2 and N1 exhibit near fully penetrant lethality in the absence of tetracycline.

Line K1 also exhibited near-fully penetrant lethality in the absence of tetracycline; however, lethality was earlier-acting than expected, with the majority of transgenic individuals reared in the absence of tetracycline failing to pupate. Moreover, lethality appeared not to be fully repressed by tetracycline, with an adult eclosion rate just under half that exhibited by wild-type individuals in the same heterozygous pool. In contrast, many transgenic individuals in line N2 were able to eclose even when reared in the absence of tetracycline.

As predicted from earlier results, the lethal phenotype manifests itself primarily at the pupal stage, with the majority of transgenic individuals reaching pupation even in the absence of tetracycline. This outcome was analysed further by constructing GLM models with Poisson or quasi-Poisson error for pupation rates in each line, followed by stepwise removal of factors from the saturated model. Differences between saturated and simplified models were analysed by Anovas. Pupation rates for line C were found not to significantly deviate from the

null model, indicating that neither the presence of the transgene nor tetracycline treatment significantly influenced pupation rates in this assay (Anova, null model vs saturated, $p(F) = 0.45$). For lines I, K2 and N2, tetracycline treatment was found not to be a significant factor, indicating that pupation rates were not depressed in the absence of tetracycline, though in each case the presence of the transgene did have a significant negative effect on pupation rates (Anova, Null model vs single factor (transgenic vs wild-type) model; $p(\chi^2) = 8.36 \times 10^{-11}$ and 8.6×10^{-3} for lines I and K2, respectively; $p(F) = 6.4 \times 10^{-3}$ for line N2). In contrast, the presence of the transgene was not a significant factor in the model for line N1, though tetracycline treatment did significantly influence pupation rate regardless of genotype (Anova, Null model vs single factor (tetracycline treatment) model; $p(F) = 5.2 \times 10^{-3}$). Finally, the interaction term between tetracycline treatment and transgene presence was found to be a significant factor in the models for lines D and K1, indicating that pupation rates for transgenic individuals were significantly lower than that of wild-type, and that this effect was increased in the absence vs the presence of tetracycline (Anova, two factor model vs saturated model, $p(F) = 1.05 \times 10^{-4}$ and 8.75×10^{-8} for lines D and K1 respectively). Overall, these analyses help to confirm that while pupation rates of transgenic insects were generally lower than those for wild-type, the effect of the transgene was not influenced by the presence of tetracycline, other than in lines D and K1. Hence, tTAV-dependent toxicity did not manifest itself prior to the pupal stage.

Furthermore, lethality in transgenic insects is largely rescued in the presence of tetracycline, though eclosion rates were somewhat depressed relative to wild-type (Figure 4-9): For tetracycline treated populations, eclosion rates for transgenic insects were significantly less than those for wild-type insects, with the exception of lines K2 and N2 (GLM models with binomial or quasi-binomial error were established for each line, followed by step-wise removal of factors. Differences in fit between the null model and saturated model were analysed by

Anovas: p (F)= 2.98×10^{-5} , 0.026, 0.34, 8.33×10^{-4} and 0.13 for lines D, I, K2, N1 and N2, respectively; p (χ^2) = 1.64×10^{-4} and 2.2×10^{-16} for lines C and K1 respectively).

Although all OX5055 lines with the exception of line I exhibited an eclosion rate which was above zero in the absence of tetracycline, 'eclosion' here is a stringent measure of penetrance which includes any individuals that succeeded in emerging from the pupal case. Some of these individuals were observed to have died on the surface water immediately after or shortly following eclosion; hence, the effective number of viable transgenic adults observed in the absence of tetracycline for lines K1, K2 and N1 was zero, and amounted to only two individuals in line C.

In all lines with the exception of K1 and D, lethality occurred largely between the points of pupation and eclosion. Nonetheless, variation of phenotype between the lines was again observed in relation to the precise point of death. In line N1, many individuals appeared to have completed metamorphosis, with death occurring at the point of emergence from the pupal carapace – possibly due to an inability on the part of the insect to prize itself free of the pupal case. In contrast, death in line I was observed to occur earlier, with only two individuals reaching the point at which the pupal carapace was split (Figure 4-10).

Another notable observation was the presence of deformities in the legs of some surviving transgenic adults, mirroring those earlier noted to be associated with the AAEL004275 (Os1) promoter in constructs OX4709 and OX4800. This phenotype was observed in surviving adults reared on tetracycline in lines D and K1, and also among adult survivors from the off-tetracycline rear in line N2. Taken together with the fluorescence phenotype of AAEL004298 (Os2)-*DsRed* lines reported in Chapter 3, the results are further suggestive of the AAEL004298 (Os2) promoter driving high levels of expression in developing adult legs. This expression pattern may explain the inability of individuals on reaching adulthood to free themselves from

the pupal case, and hence be at least a contributory factor in the very late-acting lethality observed in some OX5055 (*Os2-tTAF3/tetO-mx*) lines.

Overall, these results show that the AAEL004298 (*Os2*) promoter can consistently induce a tetracycline-repressible, late-acting lethal phenotype when coupled to a *tTAV/tetO*-effector system.

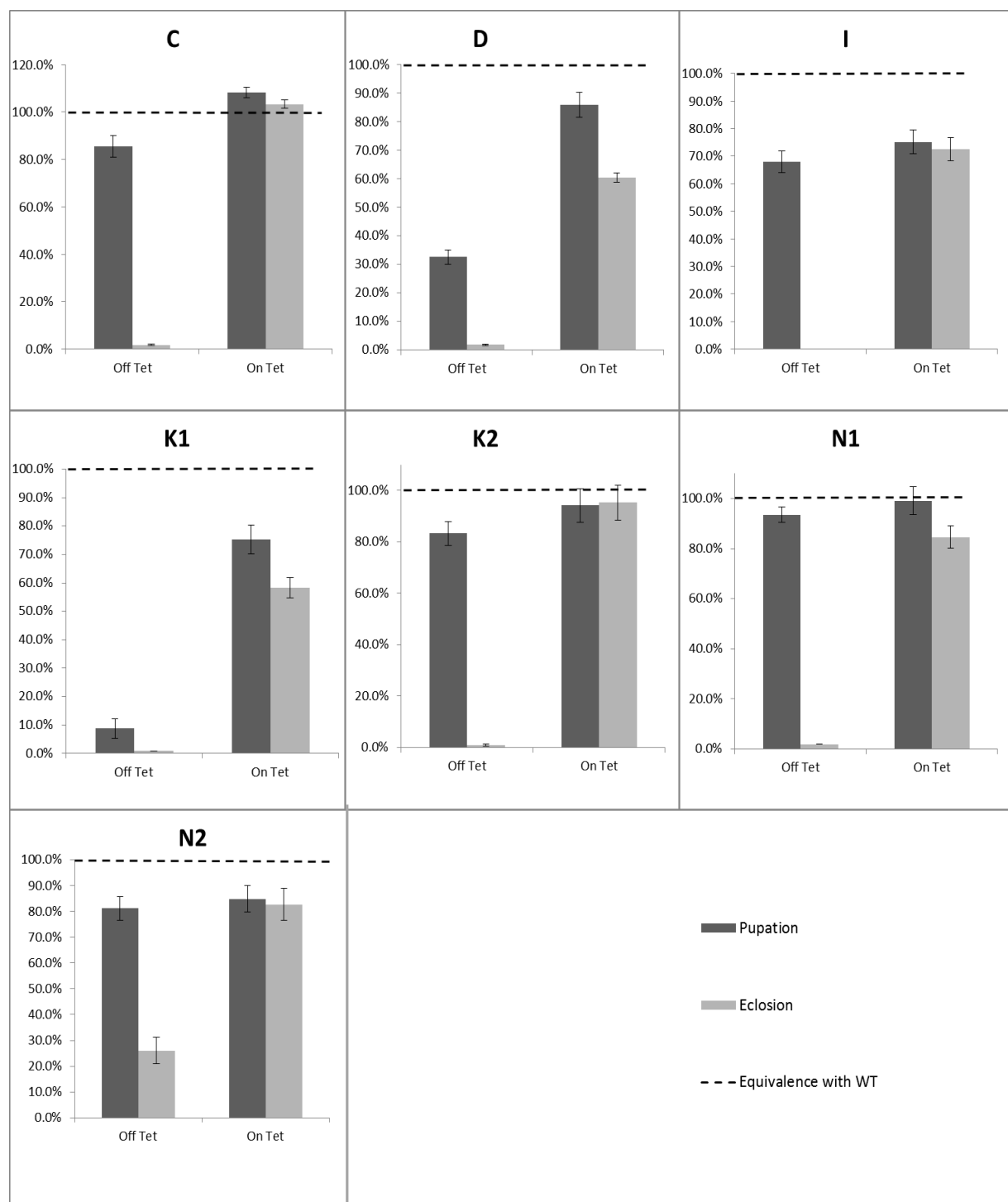


Figure 4-9: Phenotype of OX5055 (*Os2-tTAF3/tetO-mx*) lines reared in the presence and absence of tetracycline

Charts show average pupation and eclosion rates of 7 OX5055 lines (C, D, I, K1, K2, N1, N2) reared in the presence ('On Tet') or absence ('Off Tet') of tetracycline. Rates shown are normalised by wild-type pupation and eclosion rates in a heterozygous population (100% = wild-type pupation/eclosion rate – indicated by dashed lines). Error bars show standard error of five repeats, each comprising a heterozygous population of 200 individuals. 'Eclosion' here includes all individuals which fully emerged from the pupae case, regardless of subsequent survival. Statistical analysis was performed by interrogating GLM models: detailed results are reported in main text. All lines showed significantly reduced eclosion rates in the presence of tetracycline in comparison with wild-type (Anova of GLM models following stepwise removal of factors; $p = 1.64 \times 10^{-4}$, 2.98×10^{-5} , 0.026 , 2.2×10^{-16} , 0.34 , 8.33×10^{-4} , 0.13 for lines C, D, I, K1, K2, N1 and N2 respectively).



Figure 4-10: Late acting lethality in 5055 (Os2-tTAF3/tetO-mx) transgenic insects

A: Pupal death in insects heterozygous for the OX5055 (Os2-tTAF3/tetO-mx) construct, line I, after being reared in the absence of tetracycline. B: Pupal death in line OX5055 line N1. Death can be seen to occur later, with some individuals part-way emerging from the pupal case, as indicated.

4.5 A PRELIMINARY ANALYSIS SUGGESTS OX5055 LINES ARE HOMOZYGOUS-VIABLE

To test whether the OX5055 (*Os2-tTAF3-tetO-mx*) lines might be made homozygous, heterozygous males from two of the most promising OX5055 lines (I and K2) were backcrossed against heterozygous females from the same lines, to obtain an F1 population enriched for the OX5055 transgene. The F1 population was therefore expected to comprise individuals homozygous for the transgene, heterozygotes, and wild-type individuals in the ratio of 1:2:1 respectively. F1 eggs were then hatched and reared in the presence of tetracycline, as described in Methods.

Table 4-5: Raw survival (eclosion) data for OX5055xOX5055 F1 progeny

Each replicate consisted of a known population of 200 F1 larvae. Wild-type (WT) individuals were expected to comprise 25% of this population. For line K2, an additional replicate was set up as a control for the impact of handling and rearing conditions in the test replicates (K2 – without handling). Here, to avoid handling associated with counting larvae, an unspecified number of F1 individuals were reared under conditions designed to be as permissive as possible (< 1 larvae/ml; fed *ad libitum*).

Line	Eclosed adults		Eclosed adults (% of starting population)		Ratio Tg:WT*
	Transgenic (Tg)	Wild-type (WT)	Transgenic (Tg)	Wild-type (WT)	
I-1	63	55	31.5%	27.5%	1.2:1
I-2	64	33	32.0%	16.5%	1.9:1
I-3	58	53	29.0%	26.5%	1.1:1
K2-1	85	45	42.5%	22.5%	1.9:1
K2-2	37	20	18.5%	10.0%	1.9:1
K2-3	103	51	51.5%	25.5%	2.0:1
K2-4	25	13	12.5%	6.5%	1.9:1
K2-5	97	40	48.5%	20.0%	2.4:1
K2-Without handling	148	55	NA	NA	2.7:1

* Expected ratio, 100% homozygous viable: 3:1. Expected ratio, 100% homozygous inviable, 2:1

Considerable variation was observed in the outcomes of different replicates from each line, with very high levels of mortality observed in some replicates. This made it difficult to interpret the data with confidence, or to derive robust statistics on homozygous viability. Nonetheless, some useful observations can be made on the basis of the raw data shown in Table 4-5.

There appears to be mortality associated with the homozygous state which resulted in complete loss of the homozygous population for all replicates with line I, and replicates 1-4 from line K2 (i.e., a transgenic:wild-type ratio of 2:1 or less was observed). Lethality appeared to manifest itself early in development, with no significant late larval or pupal stage lethality being observed. This is not a phenotype expected to result from the action of the transgene, which has previously been shown to act predominantly at a late stage in development. Insertion of the transgene into an essential gene might result in a recessive lethal condition; however, it is unlikely that this would be observed in two separate insertion events. It is possible that this early population loss may have led to overfeeding in the affected replicates, which in turn resulted in the high levels of mortality also observed among wild-type populations in some replicates – particularly in replicates K2 and K4, in which wild-type recovery was less than half that expected.

However, it may also be observed that this homozygous mortality was not universal. For replicate K5, a transgenic:wild-type ratio of 2.4:1 was observed, indicating that at least some homozygous individuals survived and successfully eclosed. Moreover, a control replicate in which insects were not handled during early larval stages, and were reared under more permissive conditions (K2 – without handling), resulted in a transgenic:wild type ratio of 2.7:1. This is closer to the 3:1 ratio expected with 100% survival of homozygotes.

Hence, it appears that while there is clearly toxicity associated with two copies of the OX5055 transgene, at least for line K2, this does not result in complete homozygous inviability. Clearly, additional testing will be required in order to obtain more robust data on the effects of the

transgene; however, it is not unexpected that at an early stage in establishing transgenic lines (3rd generation following initial introgression), a considerable degree of toxicity might be associated with the homozygous state. Rearing through additional generations will tend to attenuate this effect by selecting for individuals more resistant to any non-repressible toxicity associated with the transgene and/or the specific insertion (though this effect may carry a concomitant risk of loss of penetrance of the transgene under restrictive conditions). In summary, prospects for establishing lines homozygous for the OX5055 (*Os2-tTAF3-tetO-mx*) construct remain open at this early stage.

4.6 CONCLUSIONS: PROSPECTS FOR USING *OSIRIS* PROMOTERS TO ENGINEER LATE-ACTING LETHALITY

Establishing approaches by which to reliably induce a late-acting repressible lethal phenotype in transgenic insects will be an essential component of developing improved manifestations of engineered dominant lethal systems in the mosquito species *Ae. aegypti* and *Ae. albopictus*.

Results presented in this chapter have evinced the ability of promoters from the putative *Osiris* genes AAEL004275 and AAEL004298 to induce such a phenotype when used as part of an engineered repressible lethal system in *Ae. aegypti*. Specifically, a construct based on the AAEL004298 (Os2) promoter has resulted in a number of transgenic lines that exhibit a near fully penetrant, repressible lethal phenotype manifesting at the pupal stage. As such, this construct, or similar constructs based on the same promoter, shows considerable promise as a building block for engineered late-acting lethal systems for use in future product lines for mosquito population control. Nonetheless, results reported in this chapter have also highlighted a number of caveats to this picture. These caveats are such as may constrain the utility of this promoter in future product lines, which are required to be suitable for mass rearing and to exhibit acceptable mating competitiveness relative to wild-type insects. They will clearly require further investigation before such constructs enter the product development stage.

4.6.1 tTAV EXPRESSION UNDER THE CONTROL OF *OSIRIS* PROMOTERS CAN RESULT IN NON-REPRESSIBLE TOXICITY

The reported phenotype of a number of transgenic lines expressing *Osiris*-tTAV constructs indicates that the expression of the transactivator tTAV when driven by the AAEL004275 (Os1) and AAEL004298 (Os2) promoters can in itself be toxic, and hence result in a degree of non-repressible lethality. This was particularly noticeable in transgenic insects carrying the first

Osiris construct, OX4709 (Os1-*tTAV*), for which a large number of transgenic lines were found to be non-viable.

High levels of *tTAV* expression are known to be toxic, and sufficient to induce lethality through the poorly-understood mechanism of transcriptional ‘squenching’ (Gill and Ptashne, 1988) (indeed, this is thought to be the cause of lethality in Oxitec’s current *Ae. aegypti* product line, OX513A). It is therefore likely that the observed lethality in the OX4709 lines resulted from toxic high expression of *tTAV* when driven by the AAEL004275 (Os1) promoter.

The success of attempts to attenuate this effect, as described in this chapter, was unclear. Both constructs OX4800 (Os1-*tTAF3*) and OX4811 (Os2-*tTAF3*) were designed to have reduced toxicity relative to OX4709. They contain a truncated form of *tTAV*, *tTAF3*, which was expected to have reduced potency relative to *tTAV* (Labbe, 2011). Adding a 3’UTR derived from the *D. melanogaster hsp70* gene to *tTAF3* was expected to increase the rate of mRNA degradation (Bonisch et al., 2007), and hence reduce overall *tTAF3* mRNA levels. Construct OX4811 (Os2-*tTAF3*) did indeed show reduced toxicity relative to OX4709, with no lethality observed in any of the transgenic lines established with this construct. However, OX4811 contained the AAEL004298 (Os2) promoter, rather than AAEL004275 (Os1), which had not been tested in a construct expressing *tTAV*. In contrast, construct OX4800 (Os1-*tTAF3*), which contained the original AAEL004275 (Os1) promoter, did not show any marked reduction in toxicity relative to OX4709. Testing an additional construct containing the Os2 promoter with un-modified *tTAV* might have shed further light on whether the *tTAF3* and *hsp70* components had any tangible effect on toxicity. However, in the context of the present study, addressing this question was secondary to the primary goal of developing a functional late-acting lethal construct.

4.6.2 NON-REPRESSIBLE LETHALITY IS ALSO OBSERVED IN TRANSGENIC INSECTS EXPRESSING AN 'ALL IN ONE' CONSTRUCT

Construct OX4811 (Os2-*tTAF3*) exhibited a markedly reduced toxicity relative to the original *Osiris*-*tTAV* construct, OX4709 (Os1-*tTAV*). Moreover, this reduced toxicity did not appear to impair the ability of the *Osiris*-*tTAF3* cassette to drive expression of a lethal effector, since crosses to transgenic lines expressing a *tetO-mx* cassette resulted in the expected late-acting lethality in insects reared in the absence of tetracycline.

It was therefore expected that the self-contained, or 'all in one' conditional lethal construct OX5055 (Os2-*tTAF3*,*tetO-mx*), which combined both Os2-*tTAF3* and *tetO-mx* cassettes, would result in a similar repressible lethal phenotype. This phenotype was indeed observed in transgenic lines expressing OX5055. However, a degree of non-repressible lethality was again observed in some lines when reared in the presence of tetracycline – most markedly in lines D and K1. Although such macro effects appeared absent from other lines, a degree of non-repressible toxicity might nonetheless manifest itself in more subtle fitness costs, and would thus be of concern from the perspective of developing future product lines based on the OX5055 construct.

Such a phenomenon may have a number of origins, which are difficult to disentangle in the context of an 'all in one' construct:

- i) **There may be 'leaky' – i.e. non-induced – expression of the toxic effector *micelob_x* from the *hsp70* minimal promoter.** This may arise from the presence of enhancer elements in the surrounding genome proximal to the transgene insertion site. It is thus likely to be position-effect dependent, and may partially explain the variation in phenotype observed between OX5055 lines – particularly the early lethality observed in lines K1, D and to some extent I, which was not previously observed with constructs containing either *Osiris* promoter. Although difficult to address

directly, this problem may be resolved simply by screening more independent insertion sites to isolate lines wherein the phenomenon is not present or is present to a less problematic degree. It is also possible that 'leaky' expression may result from enhancers present in the *Osiris* promoter region acting upon the minimal promoter upstream of the *mx* gene. This eventuality could be addressed by re-engineering the construct, for example by inserting insulator elements (reviewed in Kuhn and Geyer (2003)) either side of the tetO-*mx* cassette.

ii) The presence of tetracycline may not fully repress activation of the tetO-*mx* cassette

by tTAF3. While tetracycline-based on-off expression systems have now been widely and successfully used in engineered dominant lethal systems, it is possible that the expression levels of *tTAF3* as driven by the *Osiris* promoter are such that tTAF3-tetO binding is not completely suppressed in individuals reared in the presence of tetracycline. Increasing the concentration of tetracycline added to rearing water could be one simple solution to this problem. Recently, the tetracycline analogue doxycycline has been shown to be effective at lower concentrations and hence to give a better 'on:off' activity ratio than that associated with chlortetracycline, which is used throughout the experiments reported in this thesis (Matzen, 2014). Hence, rearing transgenic individuals with doxycycline may help to reduce some of the toxicity here observed to persist in the presence of chlortetracycline.

iii) Toxicity may be linked to over-expression of tTAF3 alone.

This phenomenon has already been observed in previous constructs containing the AAEL004275 (Os1) promoter, as discussed above. While toxicity was not noted in OX4811 lines (containing the AAEL004298pro-tTAF3 cassette alone), this cannot rule out the possibility of some toxic accumulation of tTAF3 in OX5055 lines due to over-expression from the AAEL004298 (Os2) promoter.

Of the three possible causes of non-repressible toxicity listed above, toxicity linked to over-expression of tTAF3 is perhaps the most difficult to resolve. It would be particularly problematic in view of the fact that all bar one of the OX5055 (Os2-tTAF3, tetO-mx) lines tested failed to show 100% penetrance of the lethal phenotype. Hence, it is possible that the AAEL004298 (Os2) promoter drives near toxic levels of tTAF3 expression, yet does not guarantee 100% lethality when coupled with a lethal tetO-effector cassette; i.e. that the 'on:off' activity ratio of expression systems based on this promoter is poor.

This outcome is perhaps not unexpected, in view of the apparent expression profile associated with both *Osiris* promoters. Results reported both in this chapter and in Chapter 3 of this thesis indicate that the *Osiris* promoters drive high levels of expression in legs, antennae, and/or other tissues containing cuticle. Lethality appears to occur post-metamorphosis, and may indeed result from the inability of insects with undeveloped or deformed legs to emerge from the pupal casing, rather than a more catastrophic and widespread effect during pupal development. One option for addressing this issue may be to use a different toxic effector, the effects of which would be less specific to the tissue in which it is concentrated. Secreted toxins, for example, may help to elide the tissue-specific expression of a given promoter. However, such an approach has not proved successful in expression systems based on the Actin 4 promoter (Sulston, 2013); Moreover, transgenic expression of secreted toxins may raise more questions amongst regulators than intra-cellular effectors. Alternatively, promoter deletion analysis, followed by testing of constructs through site-specific integration, might elucidate a truncated AAEL004298 (Os2) promoter fragment capable of driving pupal expression in a more ubiquitous fashion.

4.6.3 THE AAEL004298 (OS2) PROMOTER MAY BE A USEFUL COMPONENT OF FUTURE RIDL PRODUCT LINES

Despite the caveats discussed above, results presented in this chapter suggest that the promoter element isolated from the putative *Osiris* gene AAEL004298 (Os2) represents a promising avenue by which to develop late-acting repressible lethal systems. It exhibits the required late-acting expression profile, and has been shown to induce tetracycline repressible pupal-stage lethality in an engineered dominant lethal system. Moreover, it does so consistently; although phenotype variation across insertion lines was observed, across all transgenic lines with all *Osiris*-tTAV, tTAF3 constructs tested, transgene expression and associated lethality was found to predominate at the pupal stage.

Further testing will clearly be required in order to determine whether transgenic lines based on the OX5055 (Os2-tTAF3, tetO-*mx*) construct show real promise for use in future product lines. Preliminary homozygous viability assays indicate that there may be a high level of mortality associated with two copies of the transgene. However, they have also shown that, for at least one OX5055 line, this does not result in complete homozygous inviability. The possibility of creating lines homozygous for the OX5055 construct – a pre-requisite for establishing a usable product line – therefore remains open at this stage.

Finally, the results presented here vindicate the strategy pursued in Chapters 3 and 4 in order to elucidate improved mechanisms of engineering late-acting expression systems. A complete developmental transcriptome set for *Ae. aegypti* has now been made available in a format which permits users to search for genes which are strongly expressed at particular developmental stages (Akbari et al., 2013). This would enable a much more targeted approach to be adopted were the work herein described to be repeated. The transcriptome database can now be interrogated to specifically identify genes showing relatively high expression levels at late juvenile developmental stages. Results reported here indicate that it would be a relatively facile exercise to isolate upstream regions of any genes thus identified, and to use

them in the marker constructs reported as a good indicator of their likely utility as drivers of late-acting transgene expression. Rather than focussing on pupal expression, it may be advisable to consider genes which have expression peaks in late larval stages, to maximise the chance of achieving fully penetrant lethality prior to eclosion.

Following work reported here, this approach has indeed enabled a number of other genes to be isolated which show similarly promising expression profiles peaking in pupal or late-larval stages, including one additional gene from the *Osiris* family (Molloy, 2014). Constructs based on promoters isolated from these genes are in the early stages of testing; together with the *Osiris* promoters here discussed, they represent a promising cache of molecular tools with which to engineer improved RIDL product lines in *Ae.aegypti* and *Ae. albopictus*.

5 ESTABLISHING A SEX-SPECIFIC SPLICING MECHANISM IN *AE. ALBOPICTUS*

5.1 INTRODUCTION

In outlining the characteristics of an 'ideal' transgene-based population suppression approach, it was observed in the Introduction to this thesis that a genetic sexing mechanism would be a key component of an improved dominant lethal-based pest control product.

The need to separate sexes has proved one of the major bottlenecks constraining the efficiency of 'traditional' (i.e. non-transgene based) approaches to the sterile insect technique (Benedict and Robinson, 2003). Transgenic techniques have the potential to provide an effective solution to this problem, obviating the need for manual or physical mechanisms of sex separation which can be both laborious and potentially damaging to the insects.

In a dominant lethal-based system, a genetic sexing mechanism could be developed through a female-specific component to the lethal system; females in the release generation would die while males remain unharmed. There are broadly two approaches to attaining to this goal that are consistent with the requirements of an efficient system. In the first, repressible female-specific lethality is itself used as a population control mechanism. Here, late-acting lethality is desirable in order to take advantage of density-dependent competition effects in the field (as discussed in Chapters 3 & 4 of this thesis). Alternatively, a female-specific lethal system could be used purely as a sexing mechanism in the release generation. As outlined in Chapter 1, this would be established alongside a late-acting bi-sex lethal system, which would remain the population control tool. Hence, an early-acting female lethal mechanism would be specifically activated in the release generation in order to easily remove females from the population. Males released into the field would carry both the female-specific lethal and a late-acting, bi-sex lethal mechanism, such that all of their progeny would exhibit late-acting lethality. Such an

optimised system would of course require orthogonal expression control elements to allow the female-specific lethal system to be activated independently of the bi-sex lethal mechanism, which is the subject of Chapter 6 of this thesis.

The first of these approaches has already been implemented in both *Ae. aegypti* and *Ae. albopictus* (Fu et al., 2010, Labbe et al., 2010). In each case, use of the Actin 4 promoter and alternative splicing elements has enabled female-specific expression of tTAV in the indirect flight muscles. tTAV-mediated expression of VP16 via the tetO enhancer results in a flightless phenotype in females. This renders those females unable to seek hosts or oviposition sites, as well as unable to mate, as *Aedes* mosquitoes mate on the wing. Females remain healthy throughout larval development, meaning that density-dependent competition among larvae in the field is not affected.

While this approach has shown promise in early laboratory testing, it is subject to a number of issues which could limit its use in the field. In particular, despite their flightless phenotype, the release of adult female insects into the field may be problematic from a regulatory perspective (though it should be noted that a requirement to release *Wolbachia*-infected females has not proved inhibitory to open-field testing of *Wolbachia*-based control approaches (Hoffmann et al., 2011)). Should this prove to be the case, then male and female insects would still need to be separated manually in the release generation, which would negate a key advantage of a genetic sexing mechanism. A release device would also need to be devised such that flightless female insects did not interfere with the release of male adults. Furthermore, relying on a female-specific lethal system as a control approach results in short-term persistence of the transgene in the environment via healthy male progeny. This can be regarded as advantageous from the perspective of affording increased penetrance, and in any case the lethal gene would rapidly be lost from the wild population. Nonetheless, the potential for transgenes to persist in the environment may lead to regulatory authorities viewing a female-specific lethal system as offering a different control paradigm to that associated with a bi-sex lethal system, and

consequently result in a new set of regulatory challenges when developing products for open-field use..

For these reasons, a genetic sexing approach which would enable early-stage elimination of females from the release generation – or at the very least prior to adult emergence – remains a desirable goal.

5.1.1.1 ISOLATED SEX-SPECIFIC SPLICING ELEMENTS OF THE ACTIN 4 GENE MAY CONFER FEMALE-SPECIFIC EXPRESSION WITHOUT THE TISSUE SPECIFICITY OF THE ACT4 PROMOTER

One possible approach builds on the female flightless phenotype developed previously (Fu et al., 2010, Labbe et al., 2010) by joining the sex-specific splicing elements of the Actin 4 gene to a different promoter. Actin 4 is highly expressed in developing female pupae (Munoz et al., 2004). However, expression is largely limited to the indirect flight muscles of developing females; hence, use of the Actin 4 promoter to drive female-specific tTAV expression in a RIDL system results in the flightless phenotype reported by Fu *et al.* and Labbe *et al.* Fu et al. (2010) show that differential splicing occurs in the *AeAct-4* gene which results in transcription of a 244 bp male-specific exon, containing numerous start and stop codons which are thought to lead to termination of translation, and thus to reduced levels of the AeAct-4 protein in males. Combining the *Act-4* alternative splicing elements with a different promoter could therefore drive female-specific tTAV expression in a wider range of tissues, and hence generate a true female-specific lethal phenotype.

5.1.2 GENE CASSETTES COMPRISING SEX-SPECIFIC SPLICING REGIONS OF THE *DSX* GENE ARE A PROMISING ALTERNATIVE ROUTE TO DEVELOPING SEX-SPECIFIC EXPRESSION IN TRANSGENIC INSECTS.

An alternative approach is the use of alternatively spliced regions of genes comprising the sex-determining pathway to result in female-specific tTAV transcripts. Alternative splicing is known to play a key role in sex determination in insects, and therefore offers a promising locus for the development of sex-specific expression systems. In particular, the *sex-lethal-Transformer-doublesex* cascade has been well characterised in *Drosophila* (reviewed in Penalva and Sanchez (2003)). Alternative splicing of a *Ceratitis capitata* orthologue of the gene *Transformer (Tra)* has previously been exploited to achieve sex-specific expression in a dominant lethal system. Female-specific expression of the TetR-VP16 transcriptional activator tTAV was achieved through fusion with the male specific exon of the *Tra* transcript of *C. capitata*. The stop codons in the male exon result in a truncated tTAV transcript in males, thereby resulting in a female-specific, repressible lethal phenotype (Fu et al., 2007, Ant et al., 2012).

No orthologue of *Tra* has yet been identified in *Aedes*. Moreover, attempts to use the *Tra* gene derived from *C. capitata* to obtain female-specific expression in *Ae. aegypti* were not

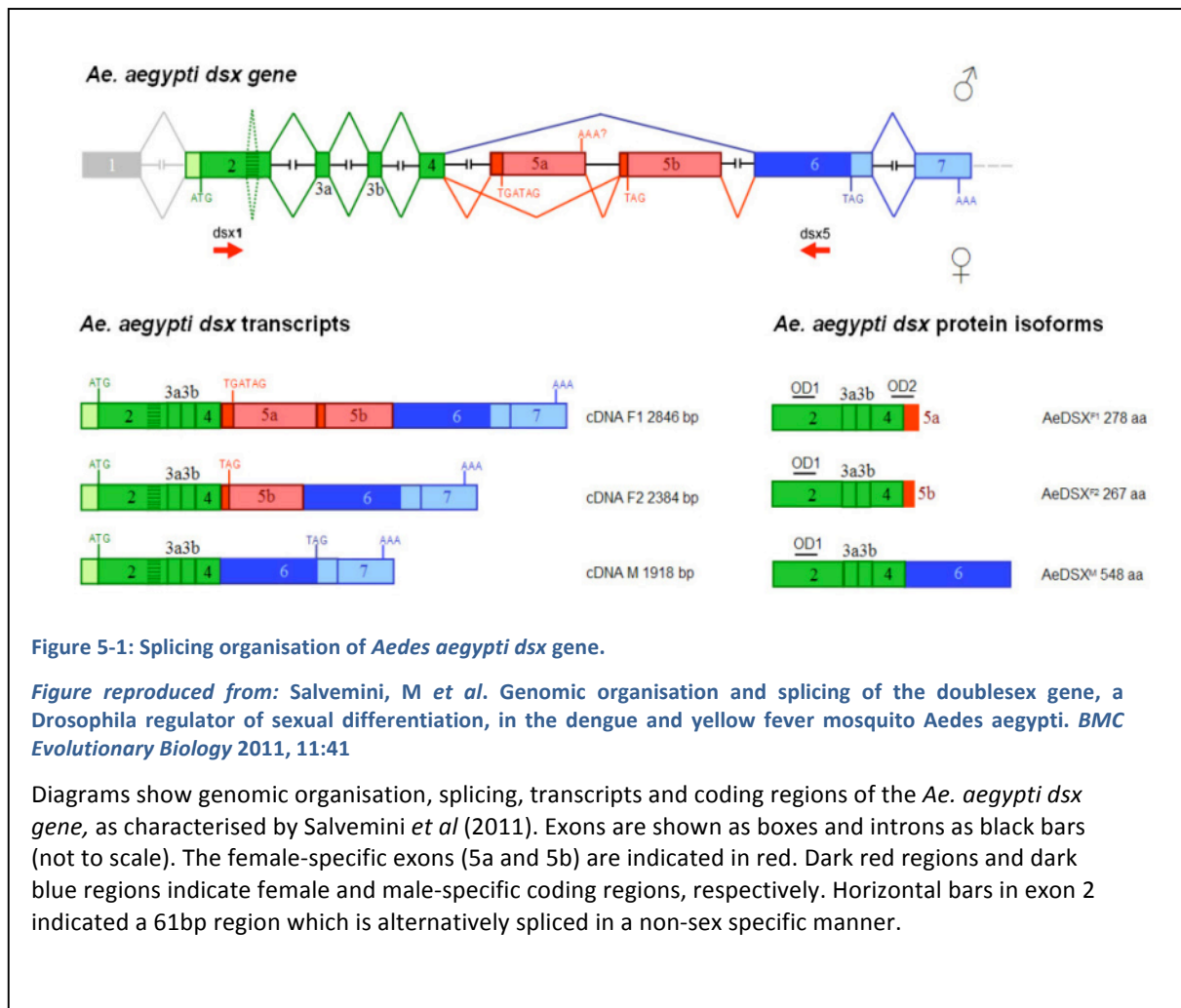


Figure 5-1: Splicing organisation of *Aedes aegypti dsx* gene.

Figure reproduced from: Salvemini, M *et al.* Genomic organisation and splicing of the doublesex gene, a *Drosophila* regulator of sexual differentiation, in the dengue and yellow fever mosquito *Aedes aegypti*. *BMC Evolutionary Biology* 2011, 11:41

Diagrams show genomic organisation, splicing, transcripts and coding regions of the *Ae. aegypti dsx* gene, as characterised by Salvemini *et al* (2011). Exons are shown as boxes and introns as black bars (not to scale). The female-specific exons (5a and 5b) are indicated in red. Dark red regions and dark blue regions indicate female and male-specific coding regions, respectively. Horizontal bars in exon 2 indicated a 61bp region which is alternatively spliced in a non-sex specific manner.

successful (Collado, 2013). However, the *Doublesex (dsx)* gene in *Aedes aegypti* has recently been characterised, and shown to generate male and female-specific transcripts through alternative splicing (Salvemini *et al.*, 2011). A schematic of the *Ae. aegypti dsx* gene as described by Salvemini *et al* is shown in Figure 5-1.

Sex-specific isoforms are obtained through the female-specific splicing of two exons (5a and 5b – shown in red in Figure 5-1). Two female-specific transcripts are produced: the longer, designated F1, contains both female-specific exons, while the shorter, designated F2, contains only the second, shorter female-specific exon. In males, both exons 5a and 5b are spliced out, to result in the shorter *dsx* M transcript. Three separate proteins are thereby produced:

AeDSX^{F1}, AeDSX^{F2} and AeDSX^M. All share a C-terminal domain encoded by the 5' shared exons (exons 2-4), with different N-terminal regions encoded by the 5' regions of exons 5a, 5b and 6, respectively, as shown in Figure 5-1.

Alternative splicing of the *dsx* gene has been exploited such as to confer female-specific expression of tTAV, and thus a repressible, female-specific lethal phenotype in *Ae. aegypti* (Collado, 2013). The approach used a *dsx* 'mini-gene', comprising the female-specific exons together with part of the surrounding intron sequences and shared exons. In transgenic insects, the mini-gene was found to be spliced in the same sex-specific manner as the native *dsx* gene. To generate female-specific tTAV expression, a start codon was engineered into the second female-specific exon, upstream of a tTAV transcript, such as to give an-frame tTAV transcript in females only. A similar *dsx*-based approach has also shown promise in the Diamondback moth (*Plutella xylostella*) (Jin et al., 2013).

The *dsx* gene is highly conserved among Diptera. Hence, the results obtained in *Ae. aegypti* suggested that alternative splicing of the *dsx* gene could prove a promising approach to achieving a female-specific system in *Ae. albopictus*.

This chapter describes three distinct approaches to developing a female-specific expression system in *Ae. albopictus*. Firstly, the results of an approach using the alternatively spliced regions of the *Actin4* gene, coupled with a putative multi-tissue promoter, are described. Secondly, results are presented of the transformation of *Ae. albopictus* insects with a construct containing the *Ae. aegypti dsx* mini-gene. Thirdly, this chapter reports on the isolation, characterisation, and use in a transgenic construct of the *Ae. albopictus dsx* gene, as an alternative approach to achieving female specific expression in the latter species.

5.2 METHODS

5.2.1 STRAIN REARING AND TRANSFORMATION

Strain rearing and transformation of insects was performed as described in General Methods (Chapter 2).

5.2.2 DNA CONSTRUCTS

Constructs OX4601, OX4489, OX3978 and OX4499 were made and kindly donated for use in this study by Scaife, S, Dafa'alla, T. and Phillips, C. (Oxitec Ltd). Construct OX5024 was synthesised by Scaife, S following sequencing and isolation of the *dsx* mini-gene fragments by the author, as described below. Construct OX5048 was synthesised by Dafa'alla, T. using the OX5024 construct as a template.

5.2.3 ON/OFF TETRACYCLINE ASSAYS

Phenotypic assays of insects reared in the presence or absence of tetracycline were performed as described in General Methods (Chapter 2), unless otherwise specified in the text.

5.2.4 RT-PCRS AND SPLICING ANALYSIS

2-step RT-PCRs were performed essentially as described in General Methods (Chapter 2). Unless otherwise specified in the text, samples used in cDNA preparation consisted of pools of 5 individuals (L3 larvae), 3 individuals (L4 larvae and male pupae) or 2 individuals (female pupae).

To analyse the splicing of transgenes in transgenic insects, RT-PCRs were performed using the primers outlined in Table 5-1. RT-PCRs on OX4601 samples were performed using primers 710)

Table 5-1: Primers for analysing splicing in constructs OX4601, OX4489, OX5024 and OX508

Primer name	Sequence	Target
710) AeA4ex1F	5'-GGAAACCGAGGATAACGACGAAGG-3'	Ae. aegypti Act4 (fwd)
1031) Diag2-tTAV2	5'-CTTCGTTCAACAGCTCCAACG-3'	tTAV (rev)
630) Agdsx-e3-f	5'-GAGATGATGCCCCTGATGTACG-3'	Ae. aegypti dsx 5' shared exon (fwd)
1102) Diag2-ubiq	5'-GGATGCCCTCCTGTCTGG-3'	Ubiquitin (rev)
Abdsx4-F1	5'-AAGCTACACTACCCCTGGGAGATGATGC-3'	Ae. albopictus dsx 5' shared exon (fwd)
895) AeMuAcR	5'-CCCAGGAAGGATGGCTGGAAGAG-3'	AAEL001928 exon 2 (rev)
1098) AeMuAcRTF	5'-GGCATTCTTAGTTCCGAAACCG-3'	AAEL001928 exon 1 (fwd)

AeA4ex1F, and 1031) Diag2-tTAV2. RT-PCRs on OX4489 samples were performed using primers 630) Agdsx-e3-f and 1102) diag2-ubiq RT-PCRs on OX5024 and OX5048 samples were performed using primers Abdsx4-F1 and 1102) Diag2-ubiq. As an endogenous control for cDNA quantity, mRNA from the muscle actin gene AAEL001928 was amplified using primers 895) AeMuAcR and 1098) AeMuAcRTF.

PCR products were analysed on 1% agarose gels and sequenced as described in General Methods (Chapter 2).

5.2.5 ANALYSING FLUORESCENCE PHENOTYPE OF TRANSGENIC LINES

To assay fluorescence phenotype in the presence or absence of tetracycline, heterozygous insects were reared in the presence or absence of 30 µg/ml chlortetracycline. Fluorescence was observed using a Leica M80 microscope (Leica Microsystems, U.K.) and images taken using a Canon PowerShot S5IS camera with an MM99 adaptor (Martin Microscopes, UK)).

5.2.6 CLONING AND SEQUENCING *AEDES ALBOPICTUS* *DSX* (*Aaldsx*) PARTIAL mRNA THROUGH DEGENERATE RT-PCR

First strand cDNA synthesis was performed on total RNA from male and female pupae samples (three pooled individuals per sample) as described in General Methods (Chapter 2). PCR was

Table 5-2: Table of primers for degenerate RT-PCR

Primer name	Sequence	Target	Target length
AbdsxPF1	<u>5'-ATGGTKTCDCARGATCGYTGG-3'</u>	<u>Aaldsx conserved-</u> <u>region</u>	
AbdsxPR1	<u>5'-CGSAGNGCCGYTGMAGSGCCAT-3'</u>	<u>Aaldsx conserved</u> <u>region</u>	250
AbdsxPR2	<u>5'-GCCGYTGMAGSGCCATSACSCG-3'</u>	<u>Aaldsx conserved</u> <u>region</u>	244
AbdsxPR3	<u>5'-GCCATSACSCGYTGYCGYTCGGC-3'</u>	<u>Aaldsx conserved</u> <u>region</u>	232
AbdsxPR4	<u>5'-CGYACGGCSGTMAGGCARCAYAA-3'</u>	<u>Aaldsx conserved</u> <u>region</u>	217

then performed on cDNA samples using the primers shown in Table 5-2:

PCR products were extracted and sequenced as described in General Methods (Chapter 2).

5.2.7 RAPID AMPLIFICATION OF cDNA ENDS (RACE)

Sequencing of the 5' and 3' ends of the *Aaldsx* mRNA was performed by 5' and 3' RACE using the Smarter RACE amplification kit (CloneTech laboratories, CA, USA). Reverse transcription was performed on RNA samples obtained as described in section 5.2.6, according to supplier's

instructions. Outer and Nested PCR reactions were then performed using the reaction conditions recommended by the supplier, with the following primers (Table 5-3):

Table 5-3: Table of primers for 5' and 3' RACE reactions

Primer name	Sequence	Target
Aabdsx5'RA-O	5'-GATCCATCACCCGTTGTCGTCGGC-3'	<i>Aaldsx</i> 5' shared exons (outer reverse primer)
Aabdsx5'RA-N	5'-ATCTTGAGACCGTGGTTGCGGCAGC-3'	<i>Aaldsx</i> 5' shared exons (nested reverse primer)
Aabdsx3'RA-O	5'-CCGCAACCACGGTCTCAAGATCGGC-3'	<i>Aaldsx</i> 5' shared exons (outer forward primer)
Aabdsx3'RA-N	5'-GCCGAACGACAACGGGTGATGGATC-3'	<i>Aaldsx</i> 5' shared exons (nested forward primer)

Amplification products were analysed on a 1% agarose gel and sequenced as described in General Methods (Chapter 2). Consensus sequences were obtained from three purified pJET plasmids per sample, by aligning sequences using the ContigExpress programme (Invitrogen, CA, USA).

5.2.8 RT-PCR AND SEQUENCING OF *AALDSX* mRNAs

Additional RT-PCRs were designed to amplify a near full-length mRNA from separate total RNA samples. First strand cDNA synthesis was performed on total RNA extracted from three male and three female pupae samples as described in General Methods (Chapter 2). PCR was then performed on cDNA samples using the primers Aabdsx-diagF and Aabdsx-diagR, and Aabdsx-diagR1 (Table 5-4). To obtain shorter mRNA amplification products, the primer pairs AabdsxdiagF-Abdsx2 and Abdsx4F1-Aabdsx-diagR were used to amplify the 5' and 3' ends of

the *dsx* mRNAs respectively. A third PCR using primers *Aabdsx*-diagF and *Abdsx*5bR was designed to amplify a product from female transcripts only (all primers shown in Table 5-4).

Table 5-4: List of primers used in sequencing *dsx* mRNA and introns

Primer name	Sequence	Target
Aabdsx-diagF	5'-ATGTTTCTCAAGATCGTTGGATGGTAAAG-3'	<i>Aabdsx</i> 5' shared region putative exon 2 (forward)
AabdsxF1-diagR	5'-CGTTTGCTAGGTAGATGTAGGCTGCTG C-3'	<i>Aabdsx</i> 3' shared region putative exon 7 (reverse)
AabdsxF2-diagR1	5'-CCAATAACTTTTTCCAGGAGTACGTTCCC-3'	<i>Aabdsx</i> 3' shared region putative exon 7 (reverse)
Abdsx1	5'-GATGACGAAGCTGGTGAGACAGTCG C-3'	<i>Aabdsx</i> 5' shared region putative exon 4 (forward)
Abdsx2	5'-GCTTGCCACCAACGATTCCGC-3'	<i>Aabdsx</i> 3' shared region putative exon 6 (reverse)
Abdsx4-F	5'-GGTGAGACAGTCGACGCA GCTCC-3'	<i>Aabdsx</i> 5' shared region putative exon 4 (forward)
Abdsx4-F1	5'-AAGCTACACTACCCCTGGGA GATGATG C-3'	<i>Aabdsx</i> 5' shared region putative exon 4 (forward)
Abdsx4-F2	5'-ATACTGAAAGGCGCCACGG GGA CG-3'	<i>Aabdsx</i> 5' shared region putative exon 4 (forward)
Abdsx5a-R2	5'-CGGATTGGCGTGTCTACTGCG-3'	<i>Aabdsx</i> 1 st female exon (reverse)
Abdsx5a-R1	5'-GATGCTGCTGCGTAGTTGATCGTA CTG-3'	<i>Aabdsx</i> 1 st female exon (reverse)
Abdsx5a-R	5'-CGTCAGTCAGATCTCTTCTGGG C-3'	<i>Aabdsx</i> 1 st female exon (reverse)
Abdsx5b-R2	5'-AGCCCCAGAATGGCTGCA GTTCGCTG-3'	<i>Aabdsx</i> 2 nd female exon (reverse)
Abdsx5b-R1	5'-ACGCTATCTCACAAGTAGAGCTACACGTGG-3'	<i>Aabdsx</i> 2 nd female exon (reverse)
Abdsx5b-R	5'-GTTGTTACAGTGACACTCTTCCCA C-3'	<i>Aabdsx</i> 2 nd female exon (reverse)
Abdsx5b-Fa	5'-GTCCAGTTTCTGACTTCGGTCG GATTCGG-3'	<i>Aabdsx</i> 2 nd female exon (forward)
Abdsx5b-Fb	5'-GTGGATTTCGGGTAGTCTGA GCGATCTGA C-3'	<i>Aabdsx</i> 2 nd female exon (forward)
Abdsx5b-F	5'-AGAATCGGTAGATGCCTGCTGCC-3'	<i>Aabdsx</i> 2 nd female exon (forward)
Abdsx6-R2	5'-CACCGGAAAGCTGCTGCAAGG CG-3'	<i>Aabdsx</i> 3' shared region putative exon 6 (reverse)
Abdsx6-R1	5'-TTCAGGGCAACACCGTTGTTGTCC-3'	<i>Aabdsx</i> 3' shared region putative exon 6 (reverse)
Abdsx6-R	5'-ACGTCATCGTCTCGGTG GTGCTGG-3'	<i>Aabdsx</i> 3' shared region putative exon 6 (reverse)
Abdsx6-F	5'-CCGACGCATAGCCTGTTTCACC -3'	<i>Aabdsx</i> 3' shared region putative exon 6 (forward)
Abdsx6-F1	5'-CAGCTTCCAGAAGGATATACCGA GCGA -3'	<i>Aabdsx</i> 3' shared region putative exon 6 (forward)
Abdsx6-F2	5'-CCCAGCCATTGCGACCAATCGTCOG-3'	<i>Aabdsx</i> 3' shared region putative exon 6 (forward)
MC14dsxF1-diagF	5'-GTCGTCTGCGCTGCGCCACG-3'	<i>Aabdsx</i> 1 st female exon (forward)
2366) Fla-PR1	5'-ACGCTCCNNNN N N N N NSCATG5-3'	Random primer mix
2367) Fla-PR2	5'-ACGCTCCNNNN N N N N NSGATC5-3'	Random primer mix
2368) Fla-PR3	5'-ACGCTCCNNNN N N N N NSACGT5-3'	Random primer mix
2369) Fla-PR4	5'-ACGCTCCNNNN N N N N NSAGCT5-3'	Random primer mix
2374) Fla-PR9	5'-ACGCTCCNNNN N N N N NSGCTC5-3'	Random primer mix
2375) Fla-PR10	5'-ACGCTCCNNNN N N N N NSGCAG5-3'	Random primer mix
2376) Fla-PR11	5'-ACGCTCCNNNN N N N N NSGTGTS-3'	Random primer mix
2377) Fla-PR12	5'-ACGCTCCNNNN N N N N NSCACT5-3'	Random primer mix

Cloning and sequencing of the resulting PCR products was performed as described in section 5.2.4. For sequencing the longer female-specific mRNAs, two additional primers, *Abdsx1* and *Abdsx2* (Table 5-4) were used, which flanked a ~1.5kb region in the mid-section of the mRNA.

Translations were performed *in silico* using the Vector NTI 2011 package (Invitrogen), and protein sequences compared using the ClustalW programme (University College, Dublin).

5.2.9 SEQUENCING *DSX* INTRONS

To partially sequence *dsx* introns, flanking reactions were performed using three nested primers for the 5' and 3' termini of the female-specific exons and the shared exons immediately surrounding them (primers *Abdsx4F* – *Abdsx6R*, Table 5-4). Further primers were designed to flank putative splicing sites in the shared regions identified by sequence comparisons with *Ae. aegypti dsx*. However, these flanking reactions did not succeed in amplifying specific products and are therefore not shown.

Genomic DNA (gDNA) was extracted from *Ae. albopictus* wild-type pupae samples (2 individuals per sample) using the NucleoSpin Tissue kit (Macherey-Nagel, Duren, Germany). gDNA was then digested using either *Xho* I, *Nhe* I, *Bgl* II or *Nde* I (New England Biolabs, MA, USA). gDNA flanking the exons was then amplified using the gene-specific primers described and one of two sets of random reverse primers (2366-2369 or 2374-2377,). Amplification products obtained following three nested PCRs were cloned and sequenced using the procedure described in 5.2.4.

5.2.10 SYNTHESISING A *DSX* MINIGENE AND CONSTRUCT OX5024

For the following steps, primer design and amplification of a *dsx* mini-gene was carried out by the author. The OX5024 construct was synthesised by Scaife, S (Oxitec Ltd).

To create a *dsx* mini-gene, three overlapping fragments of the *dsx* gene were amplified from *Ae. albopictus* gDNA (prepared as described in 5.2.9), as follows, using primers described in Table 5-5:

- Primers MC5 and MC6: 131bp at the 3' end of the upstream shared exons;
- Primers MC7 and MC8: the two female-specific exons together with 290bp at the 5' end of the preceding intron, and all of the succeeding intron;
- Primers MC9 and MC10: a 145bp fragment from the 5' end of the shared exons downstream of the female-specific regions.

These fragments together comprised the *Aaldsx* mini-gene. Primers MC8 and MC9 added a start codon at the 3' end of the female-specific region. Primers MC1)PacltetOF and MC11)Not1HspR were used to amplify a cassette comprising tetO and a *hsp70* minimal promoter from construct OX4489. Primers MC3)NotlUbiF and MC4)NheISv40R were used to amplify a *Ubiquitin-tTAV-Sv403'UTR* cassette from the same construct (Table 5-5). The *tetO-hsp70* cassette and *Ubiquitin-tTAV-Sv403'UTR* would flank the 3' and 5' ends, respectively, of the *dsx* minigene. The OX4489 vector was then digested with Nhe I and Not I and fragments assembled through single-step enzymatic assembly, as described (Gibson et al., 2009).

5.2.11 STATISTICAL ANALYSIS

All statistical analysis was performed using the “R” software, as described in General Methods (Chapter 2).

Table 5-5: Primers for synthesising dsx mini-gene construct OX5024

Primer name	Sequence	Target
MC1)PacI tetOF	5'-TGTATCTTAACGCGAGTTAATTAAAGGTTTCGACTTTCAC-3'	tetO-hsp70 fragment (forward)
MC2)AscI HspR	5'-CCGCTGGTCTGGGGCGCGCCGAGATTGTTTAGCTTGTTTACAG-3'	tetO-hsp70 fragment (reverse)
MC3)NotI UbiF	5'-GCGGCGCGCCCCAGACCAGCGGCCGCCAGATCTTCGTCAGACCCCTG-3'	Ubiquitin-tTAV-SV40 fragment (forward)
MC4)NheI Sv40R	5'-TTAATCATATGAGATCTATCTGCTAGCGTTAAGATACATTGATGAG-3'	Ubiquitin-tTAV-SV40 fragment (reverse)
MC5)AscI dsxExon4F	5'-AACAAGCTAAACAATCTGCGGCGCGCCGAAGTGGTGAAGACAGTCGC-3'	1st dsx fragment (forward)
MC6)dsxIntron4R	5'-CTTCAAAACAACCTCTGGCCTGTCTTCTCGCGCGAG-3'	1st dsx fragment (reverse)
MC7)dsxIntron4F	5'-CTCGCGCGAGAAGACAGGCCAGGAGTTGTTTTGAAGGTG-3'	2nd dsx fragment (forward)
MC8)dsxEx5ATGR	5'-GCCGTTTTTGGCCATGGTGGTTTAGCAGGCATCTACCGATTCTAC-3'	2nd dsx fragment (reverse)
MC9)dsxExon5ATGF	5'-TCGGTAGATGCCTGCTAAACCACCATGGCCAAAACGGCTGCGACAG-3'	3rd dsx fragment (forward)
MC10)dsxExon6R	5'-GGTCTTGACGAAGATCTGGCGGCCGCTCGACCGCCGTTGTTGTCTG-3'	3rd dsx fragment (reverse)
MC11)NotI HspR	5'-ATCATATGAGATCTATCTGCTAGCGTGCGGCCGCTGGTCTGGGGCGCGCC-3'	tetO-hsp70 fragment (reverse)

5.3 RESULTS AND DISCUSSION

5.3.1 ALTERNATIVELY-SPLICED REGIONS OF THE *ACTIN4* GENE ARE NOT SPLICED IN A SEX-SPECIFIC MANNER WHEN ISOLATED FROM THE *ACTIN4* PROMOTER ELEMENTS

To test the ability of *Actin4* alternatively spliced elements to confer sex-specific expression in isolation (i.e., independently of the *Actin 4* promoter), DNA construct OX4601 (tetO-VP16, Hex1 γ -AaeA4-tTAV) was synthesised (Figure 5-2).

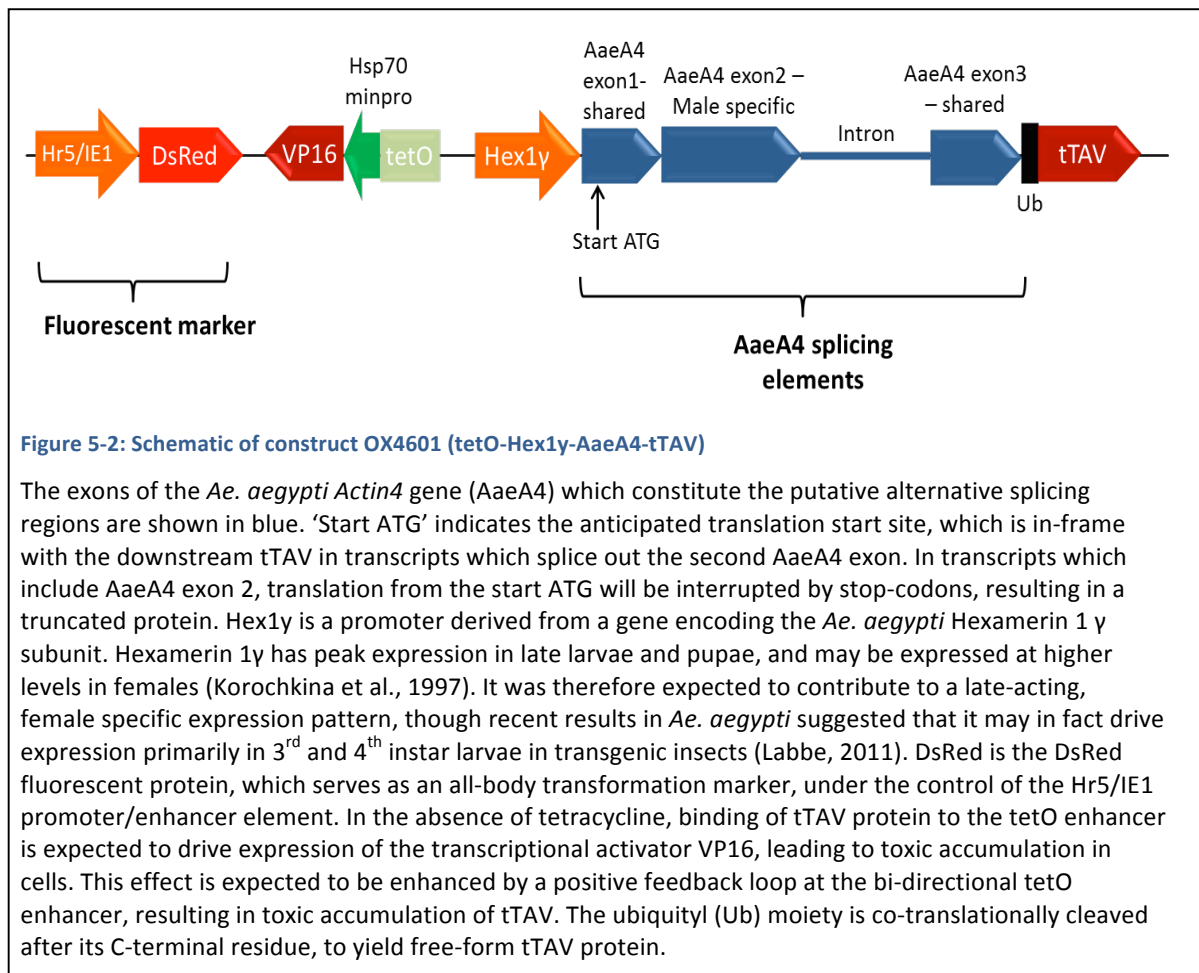


Figure 5-2: Schematic of construct OX4601 (tetO-Hex1 γ -AaeA4-tTAV)

The exons of the *Ae. aegypti* *Actin4* gene (AaeA4) which constitute the putative alternative splicing regions are shown in blue. 'Start ATG' indicates the anticipated translation start site, which is in-frame with the downstream tTAV in transcripts which splice out the second AaeA4 exon. In transcripts which include AaeA4 exon 2, translation from the start ATG will be interrupted by stop-codons, resulting in a truncated protein. Hex1 γ is a promoter derived from a gene encoding the *Ae. aegypti* Hexamerin 1 γ subunit. Hexamerin 1 γ has peak expression in late larvae and pupae, and may be expressed at higher levels in females (Korochkina et al., 1997). It was therefore expected to contribute to a late-acting, female specific expression pattern, though recent results in *Ae. aegypti* suggested that it may in fact drive expression primarily in 3rd and 4th instar larvae in transgenic insects (Labbe, 2011). DsRed is the DsRed fluorescent protein, which serves as an all-body transformation marker, under the control of the Hr5/IE1 promoter/enhancer element. In the absence of tetracycline, binding of tTAV protein to the tetO enhancer is expected to drive expression of the transcriptional activator VP16, leading to toxic accumulation in cells. This effect is expected to be enhanced by a positive feedback loop at the bi-directional tetO enhancer, resulting in toxic accumulation of tTAV. The ubiquitin (Ub) moiety is co-translationally cleaved after its C-terminal residue, to yield free-form tTAV protein.

The construct was injected into an *Ae. aegypti* WT background. 1220 embryos were injected.

There were 108 adult survivors from which 15 separate lines were obtained. Given the large number of separate insertion events, preliminary investigations were carried out on G2

individuals from each line, reared in the presence or absence of tetracycline (Table 5-6). These investigations found no evidence of the expected female-specific lethal phenotype in insects

Table 5-6: Preliminary survival analysis of OX4601 lines

G2 individuals from 7 OX4601 lines were reared in the presence (“On Tet”) or absence (“Off Tet”) of 30 µg chlortetracycline. Population sizes were not pre-determined, hence the feeding regime was non-standardised. Pupae were picked, screened, and the numbers of male and female transgenic and wild-type individuals were recorded. Pupation rates are shown as a percentage of the total pupae recorded in each experiment (transgenic pupae of a given sex were expected to comprise 25% of the starting population in each case). The transgene in lines H1, J and S appeared to be strongly male linked; hence, transgenic males constitute close to 50% of a heterozygous population.

Line	Sex	Pupation On Tet (transgenic pupae as % total pupae – n – recorded)	Pupation Off Tet (transgenic pupae as % total pupae – n – recorded)
F	♂	21.8% (n=239)	0 (n=192)
	♀	16.7% (n=239)	0 (n=192)
G	♂	25.2% (n=305)	0.8% (n=200)
	♀	21.6% (n=305)	1.6% (n=200)
H1	♂	47.4% (n=234)	50.4% (n=263)
	♀	0.4% (n=234)	0.4% (n=263)
H2	♂	30.2% (n=305)	22.4% (n=174)
	♀	23.6% (n=305)	24.7% (n=174)
I	♂	24% (n=321)	45.5% (n=275)
	♀	18.4% (n=321)	14.9% (n=275)
J	♂	50.4% (n=521)	41.7% (n=434)
	♀	1.4% (n=521)	0.7% (n=434)
T	♂	29.6% (n=189)	21.1% (n=213)
	♀	31.2% (n=189)	23.9% (n=213)
S	♂	48% (n=200)	34.9% (n=169)
	♀	4% (n=200)	5.9% (n=169)

reared in the absence of tetracycline. Instead, the lines exhibited a bi-sex lethal phenotype, which was fully penetrant in some, but not all, cases.

Lines F and G exhibited a fully or near-fully penetrant lethal phenotype in the absence of tetracycline, which manifested itself during larval development –though primarily in older (3rd and 4th instar) larvae, with some pupal lethality also recorded. Line S also exhibited some lethality in the absence of tetracycline, though this was less penetrant. Lines H1, H2, I, J and T did not appear to show any lethality prior to pupation, though eclosion rates in lines J and T did appear moderately reduced in relation to wild-type (data not shown). Lines H1, J and S all appeared to be strongly male-linked, with almost all transgenic insects being male.

To investigate further whether the expected sex-specific splicing of the Actin 4 elements was occurring, RT-PCRs were performed on male and female insects from two of the OX4601 (tetO-*VP16*, Hex1y-*AaeA4-tTAV*) lines which had shown fully penetrant lethality (lines F and G). It was found that neither of the two lines exhibited the expected sex-specific splicing, with both of the expected male and female splice forms being expressed in male and female insects (Figure 5-3). This result, together with the lack of a clear sexually divergent phenotype in any of the OX4601 lines observed, indicated that the Actin 4 splicing elements – as incorporated in the architecture of the OX4601 construct – were not sufficient in themselves to result in female-specific expression.

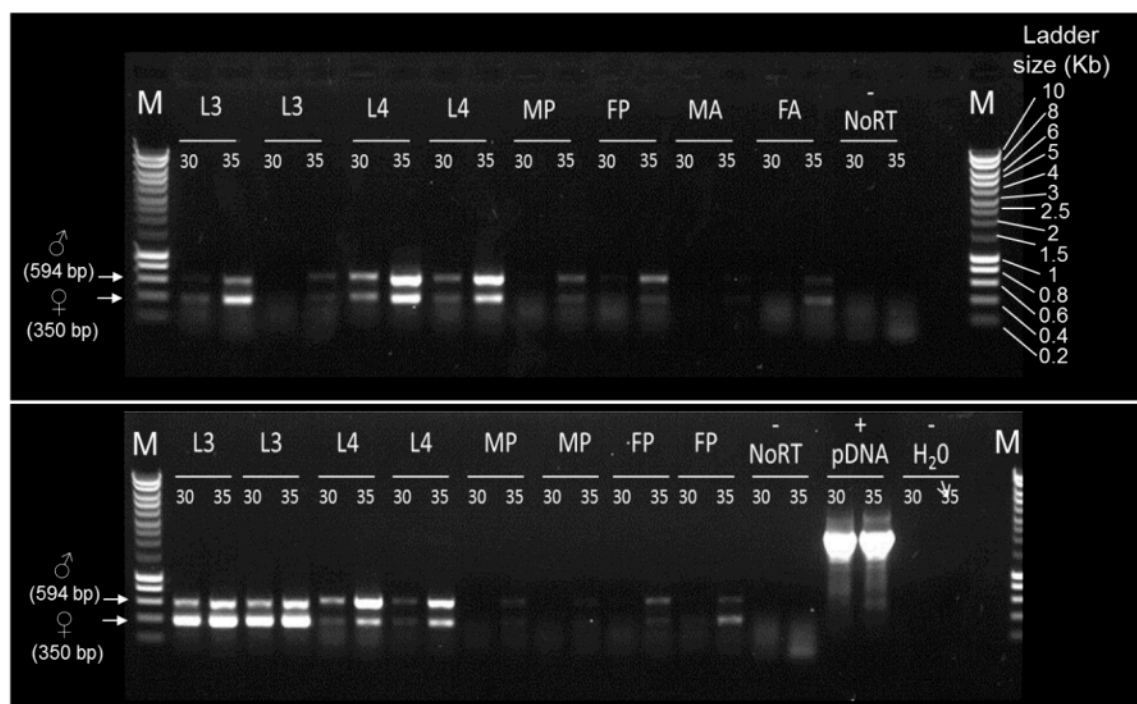


Figure 5-3: Actin4 splicing in OX4601 (Hex1y-Act4-tTAV-tetO-VP16) lines

2-step RT-PCRs were performed on samples of heterozygous transgenic individuals from two OX4601 lines (F and G), at different developmental stages. L3 & L4=3rd and 4th instar larvae; MP and FP = male and female pupae; MA and FA = male and female adult. M = SmartLadder molecular weight marker (Eurogene). PCR was performed using primers 710 and 1031 (see Methods) which amplify the 4601 Actin 4 exons plus part of the ubiquitin and tTAV exons. Expected amplicon sizes for male and female-specific transcripts are indicated. A = OX4601 line F; B = OX4601 line G.

Controls: - NoRT = Male pupae sample without reverse transcriptase; + pDNA = DNA from purified OX4601 plasmid;

H₂O = water

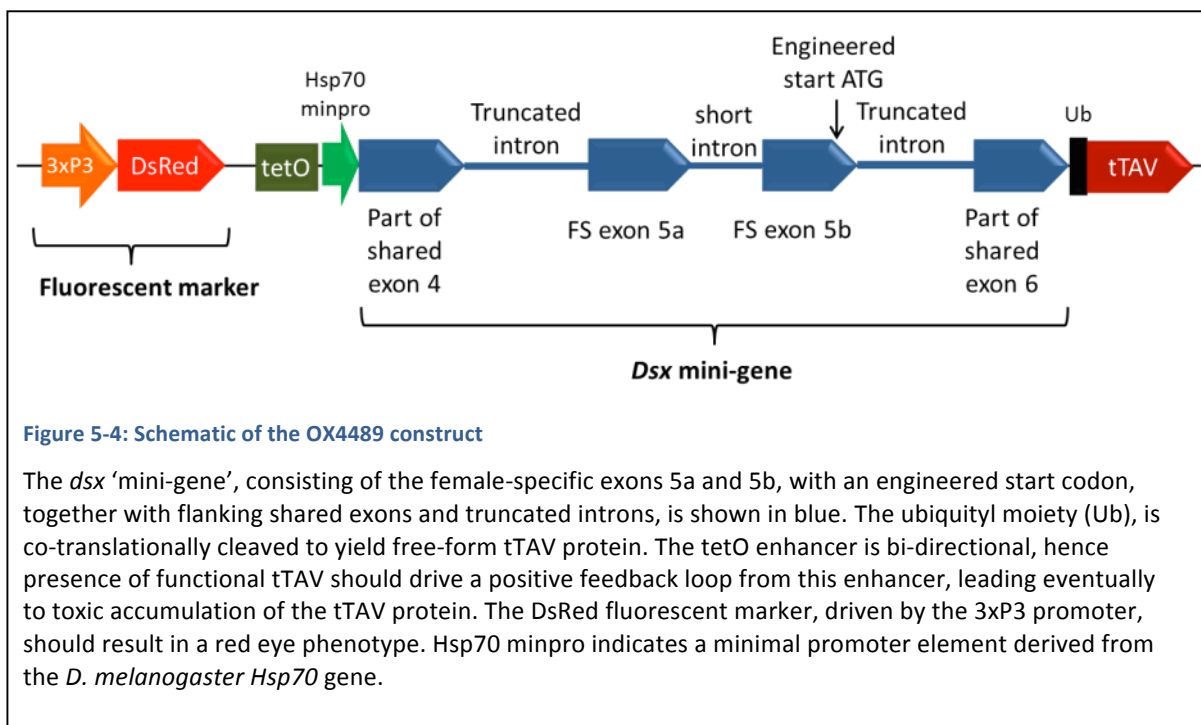
Lines F and G do show some promise in conferring bi-sex, repressible lethality – itself an important goal for future product development (see Chapters 3 and 4). However, the OX4601 construct clearly does not consistently result in a highly penetrant lethal phenotype, and as such was not considered worthy of further investigation in this regard.

5.3.2 AN *AE. AEGYPTI* *DSX* MINI GENE IS NOT SPLICED IN A SEX-SPECIFIC MANNER IN TRANSGENIC *AE. ALBOPICTUS*

The construct OX4489 is based on a *dsx* mini-gene, incorporating the sex-specific splicing regions of the *Ae. aegypti dsx* gene (*Aaedsx*), fused with a Ubiquitin-tTAV cassette. The latter is in-frame with a start codon engineered into the female-specific *dsx* exon, such that a

functional tTAV transcript is expected only where the female-specific exon is spliced into the transcript (Figure 5-4).

The construct also contains a bi-directional tetO enhancer element, such that expression of tTAV is expected to drive a positive feedback loop resulting in toxic over-expression of the



tTAV protein (as found in the OX513A conditional lethal line (Phuc et al., 2007b)). This was expected to result in a female-specific, conditional (i.e., tetracycline-repressible) lethal phenotype.

The OX4489 construct was injected into *Ae. albopictus* wild-type embryos. 800 embryos were injected, resulting in 24 adult survivors. Transgenic individuals were found in three separate G0 pools. One of these, pool C, was judged to contain lines from two separate insertions on the basis of fluorescence phenotype, yielding four lines in total.

Although the OX4489 construct was a putative female-specific lethal, earlier results with this construct in *Ae. aegypti* indicated that no lethal phenotype was present in either sex. Preliminary testing of OX4489 *Ae. albopictus* lines in the absence of tetracycline likewise did

not result in any lethality (results not shown). However, OX4489 lines in *Ae. aegypti* were found to exhibit a sexually divergent fluorescence phenotype when reared in the absence of tetracycline (Collado, 2013). The tetO enhancer is bi-directional, and therefore capable of acting as a bi-directional expression module when flanked by minimal promoters (Krestel et al., 2001). However, VP16 is also known to be capable of activating expression at distal promoters (Seipel et al., 1992). Results reported by Collado (2013) thus indicate that VP16 acts to up-regulate *DsRed* expression from the distal up-stream 3xP3 promoter in females expressing functional tTAV protein in the OX4489 construct. Sex-specific expression in *Ae. albopictus* from the same construct would therefore also be expected to generate a similar sex-specific pattern of *DsRed* expression. However, OX4489 transgenics in *Ae. albopictus* show no apparent difference in fluorescence phenotype between sexes (Figure 5-5).

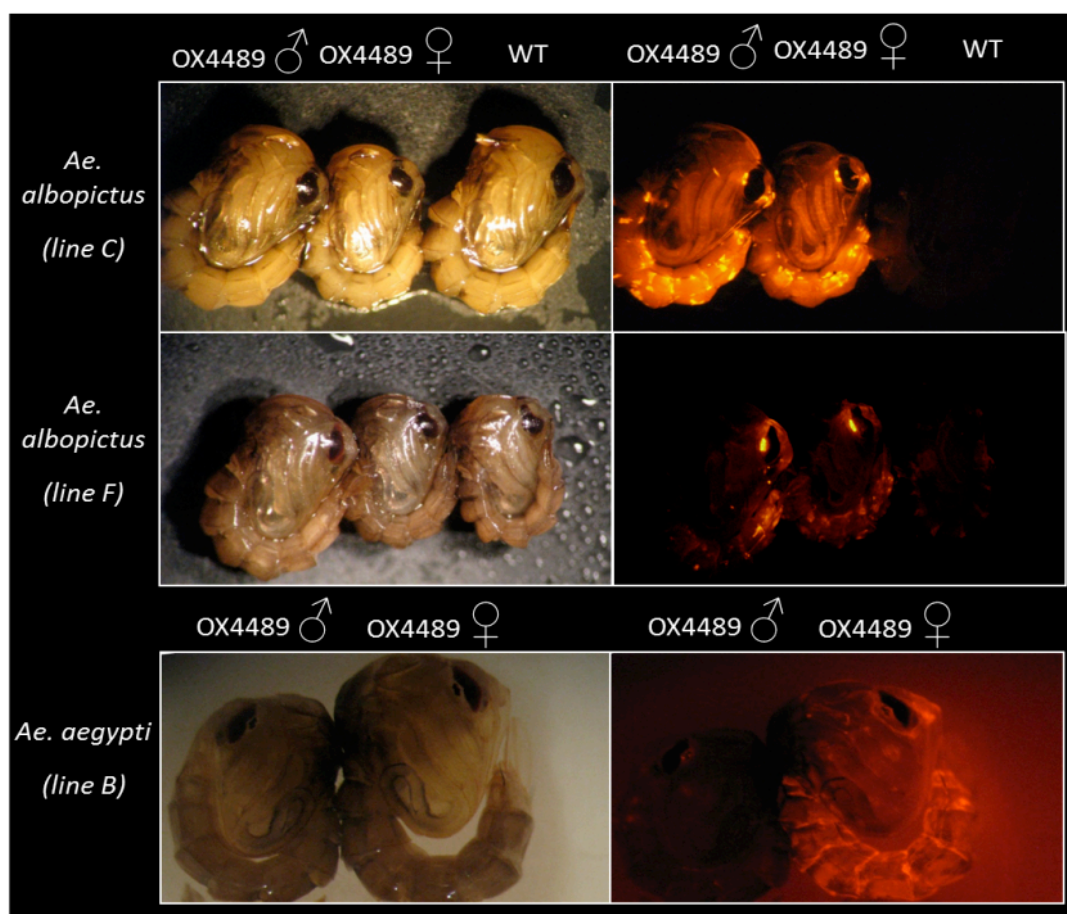


Figure 5-5: Fluorescence phenotype of OX4489 *Ae. albopictus* and OX4489 *Ae. aegypti* when reared off Tet

Transgenic individuals *Ae. albopictus* expressing the OX4489 (*Aaedsx-tTAV-tetO-DsRed*) were reared in the absence of tetracycline and pupae photographed alongside WT pupae using a fluorescence microscope. Shown are individuals from OX4489 lines C and F, as indicated to the left of panels. Left-hand panels show bright-field images, right-hand images are taken using a red filter, showing DsRed expression. Lower panels show male and female pupae from an *Ae. aegypti* line (OX4489B) for comparison (images of OX4489B courtesy of A. Collado).

To assess whether the *Aaedsx-tTAV* mRNA was being spliced in the expected sex-specific manner in *Ae. albopictus*, each of the four OX4489 (*Aaedsx-tTAV*) lines was crossed to an effector line, OX3978D, which expressed a tetO-*AmCyan* cassette. Insects heterozygous for both constructs were expected to exhibit tetracycline-repressible expression of the *AmCyan* fluorescent marker, in a manner corresponding to the pattern of tTAV expression in the OX4489 lines. In addition, RT-PCRs were performed on male and female pupae samples isolated from each of the OX4489 *Ae. albopictus* lines.

Visual inspection of insects heterozygous for both OX4489 (*Aaedsx-tTAV*) and OX3978D (*tetO-AmCyan*) using a fluorescence microscope failed to detect any differences in AmCyan expression between male and female insects – either in the presence or absence of tetracycline (Figure 5-6). This indicated a lack of sex-specific expression of tTAV from the OX4489 construct. RT-PCRs of individuals bearing the *dsx-tTAV* construct confirmed a lack of sex-specific splicing: in all cases, the expected female-specific splice was detected in both male and female samples, and no band corresponding to the expected male-specific splicing was detected (Figure 5-7).

In insects carrying both OX4489 (*Aaedsx-tTAV*) and OX3978D (*tetO-AmCyan*) constructs, the intensity of AmCyan expression appeared to increase during pupation, with strongest fluorescence seen in pupae >24 hrs old. This may result from accumulation of tTAV transcript as a result of the positive feedback loop present in the OX4489 construct. This phenomenon may also explain the variation in levels of tTAV expression which was observed in RT-PCRs of insects bearing the OX4489 construct, which may be observed in Figure 5-7.

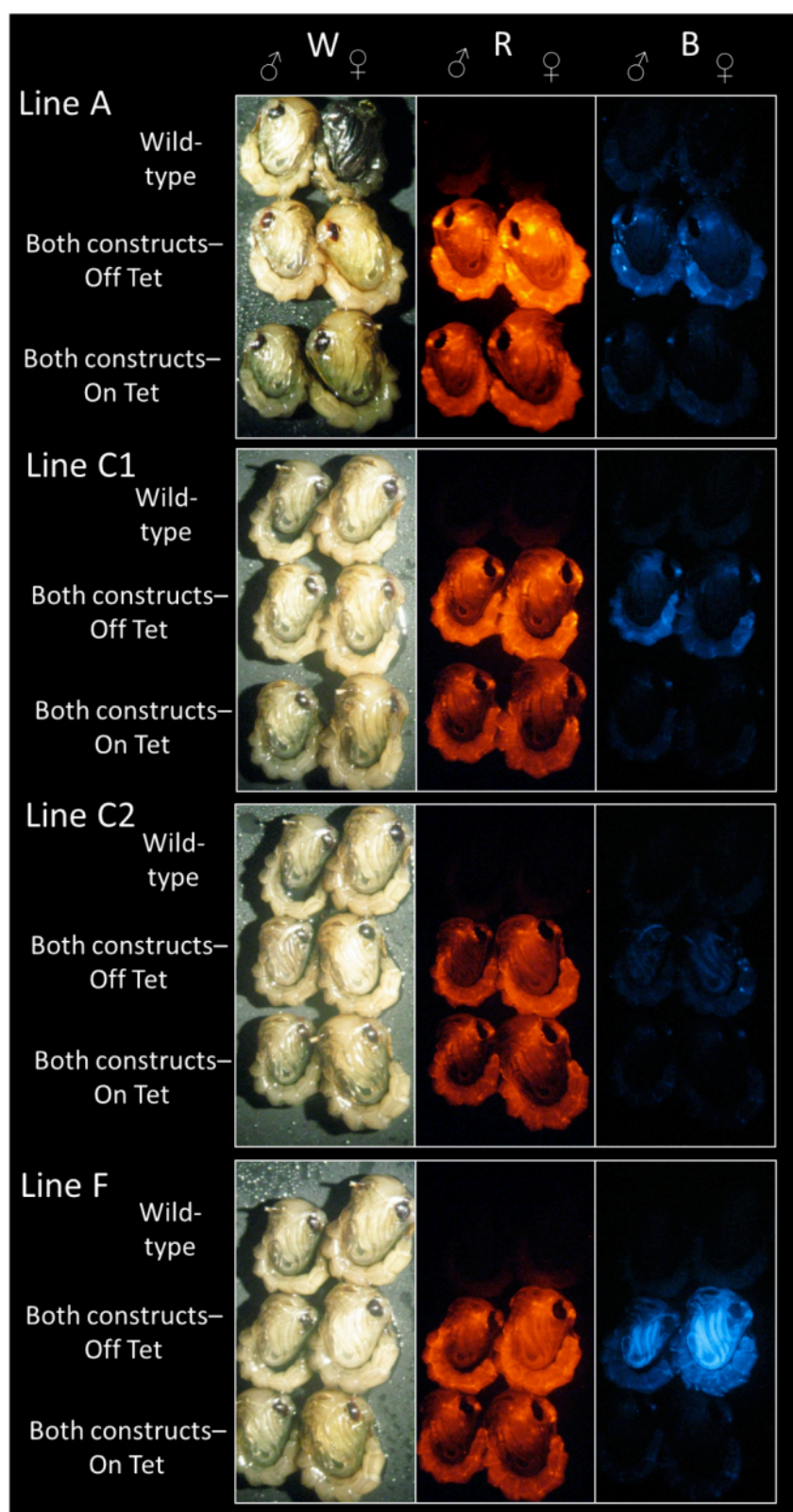


Figure 5-6: Fluorescence in OX4489xOX3978 F1 progeny

Heterozygous individuals from four OX4489 lines – A, C1, C2 and F – were crossed with heterozygotes from the OX3978 line and progeny reared in the presence ('On Tet') or absence ('Off Tet') of tetracycline. For each OX4489 line (indicated at left of panels) images show male and female F1 progeny carrying both constructs, alongside wild-type individuals. W=white light; R=red filter; B=blue filter.

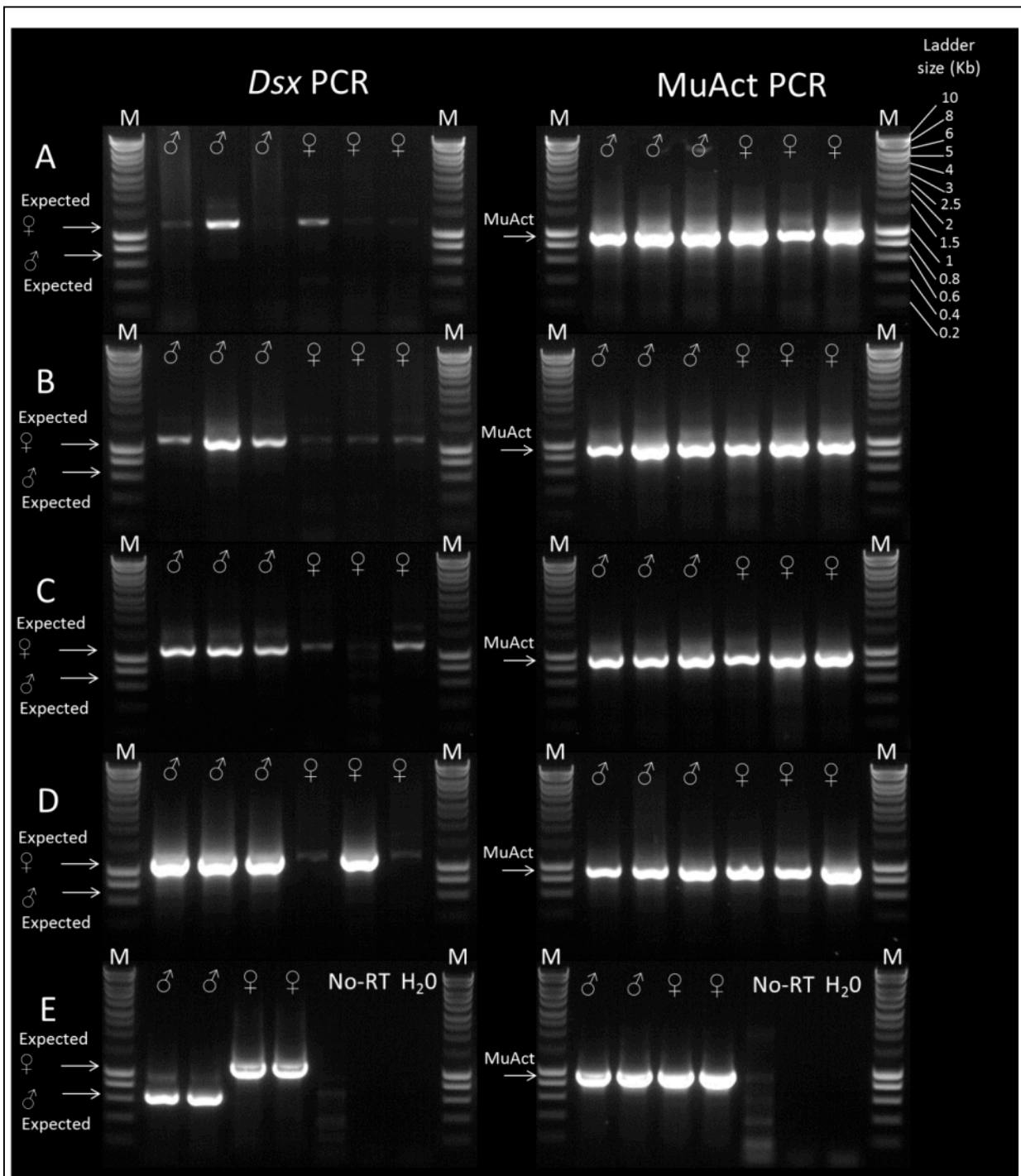


Figure 5-7: RT-PCR of *dsx* splicing in 4489-albo lines

RT-PCR was performed on male and female pupae samples (3x male pupae or 2x female pupae) from OX4489 lines, using the *dsx* primers 630 and 1102, and Muscle Actin (*MuAct*) control primers 895 and 1098, as indicated at the top of the figure. M = SmartLadder molecular weight marker (Eurogene); sizes of bands are indicated. Expected amplicon sizes for male and female *dsx* transcripts, and the expected muscle actin transcript are indicated by arrows. Panels A-D: PCRs for 4489 lines A, C1, C2 and F respectively. E: PCRs for male and female pupae from the *Ae. aegypti* line 4489B, showing the expected splice bands for males and females. No-RT and H₂O controls are also shown. Primer sequences are given in the Methods section (Table 5-1).

The lack of differentiated expression between males and females shown by both RT-PCR and outcrossing to an effector line indicates that the *Ae. aegypti* *dsx* mini-gene is not spliced in the expected sex-specific manner in *Ae. albopictus*. This was unexpected, in view of the sex-specific splicing achieved with the same construct in *Ae. aegypti*. The two species are closely related, and this in conjunction with the highly conserved nature of the *dsx* gene (Salvemini et al., 2011) had led to the expectation that the *Ae. aegypti* mini-gene would function in an equivalent manner in an *Ae. albopictus* background. Instead, the results indicate an unanticipated species specificity, which may relate to differences between the *dsx* orthologues present in each species. Isolation of the native *dsx* gene therefore remained a potential approach for achieving female-specific expression in *Ae. albopictus*.

5.3.3 A PUTATIVE *AE. ALBOPICTUS* *DSX* mRNA WAS IDENTIFIED THROUGH DEGENERATE RT-PCR

There is currently no published genome information for *Ae. albopictus*, which excluded the possibility of an *in silico* analysis to identify putative *dsx* orthologues in this species. In order to identify the *Ae. albopictus* *dsx* gene, an alignment of cDNA sequences from known *dsx* orthologues in various dipterans was conducted in order to identify conserved areas of sequence. Three areas of highly conserved sequence were identified in the shared exons at the 5' end of the *dsx* transcript. These areas were used as the basis for designing degenerate primers to amplify the equivalent region in the *Ae. albopictus* *dsx* transcript (Figure 5-8). Five separate PCRs were then performed on *Ae. albopictus* cDNA using different primer pairs, as described in Methods. The fourth of these PCRs resulted in amplification of a 220 bp fragment which showed a high degree of homology with the 5' end of the *Ae. aegypti* *dsx* cDNA.

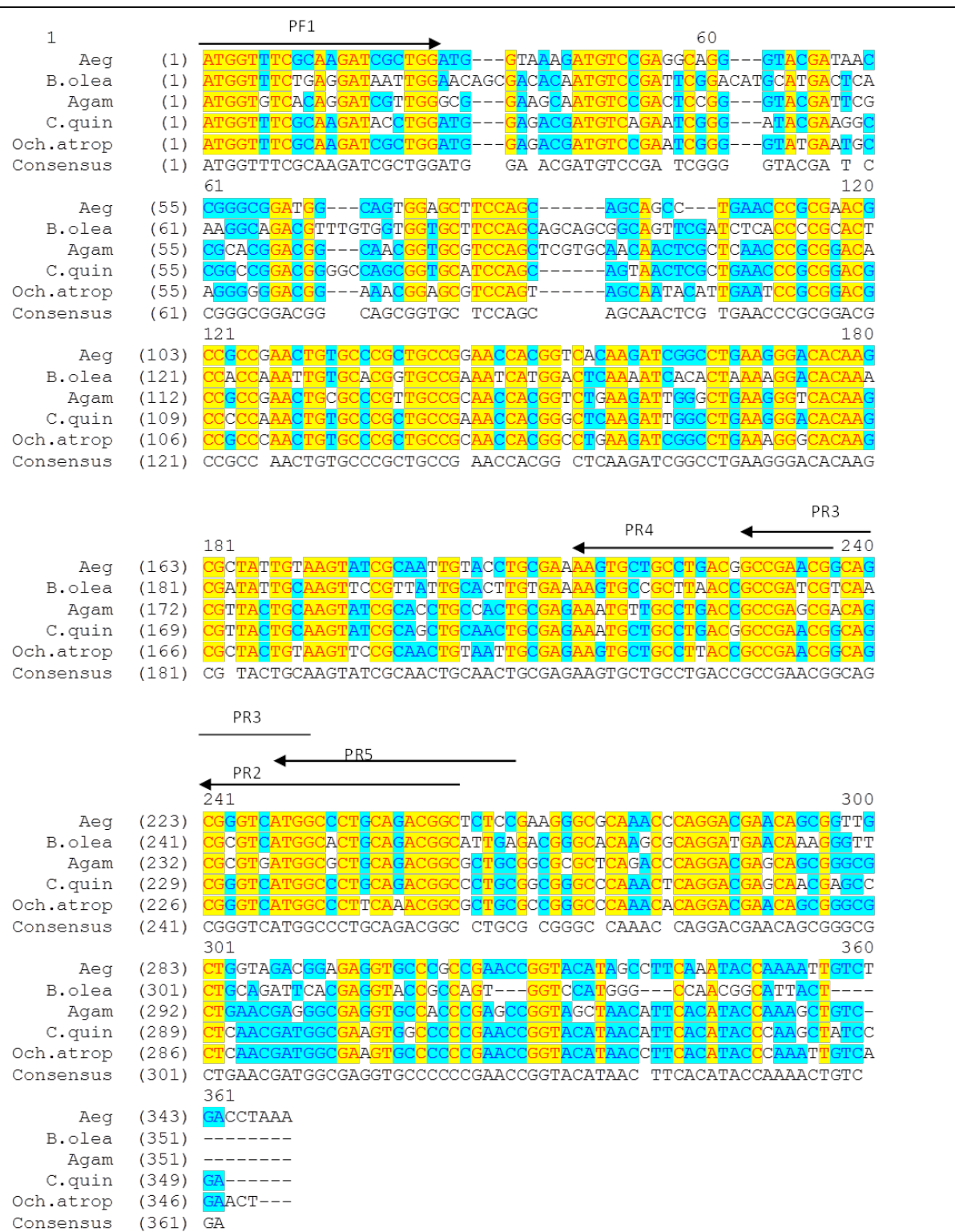


Figure 5-8: Inter-species alignment of *dsx* orthologues and design of primers for degenerate PCR

Male-specific isoforms of *dsx* cDNA sequences for *Ae. aegypti* (Aeg), *Bactrocera oleae* (B. olea), *Anopheles gambiae* (A.gam), *Culex quinquefasciatus* (C. quin) and *Aedes (Ochlerotatus) atropalpus* (Och. atrop) were obtained from a search of the NCBI nucleotide database (<http://www.ncbi.nlm.nih.gov/>). Sequences were aligned using the Align X[®] software package (Invitrogen); yellow highlighting indicates complete sequence identity, blue highlighting indicates three or more matched bases. Arrows designated PF1, PR2, PR3, PR4, PR5 indicate the target sequences of the primers *Abdsx*PF1, *Abdsx*PR1-PR4, respectively, shown in the Methods section (Table 5-2).

5.3.4 mRNA SEQUENCING IDENTIFIED MALE AND FEMALE SPECIFIC *AE. ALBOPICTUS DSX* TRANSCRIPTS

Having identified and sequenced a partial mRNA from the putative *Ae. albopictus dsx* gene, 3' and 5' RACE was then used to obtain a full-length cDNA transcript. 3'RACE reactions successfully amplified male and female cDNA products. Sequencing showed that these products exhibited strong identity with the *Ae. aegypti* M1 and F1 *dsx* isoforms. 5'RACE reactions failed to amplify any *dsx* product. However, the region amplified through the 3' RACE reaction was predicted to contain the entire coding region, along with the female-specific regions essential for designing a *dsx* mini-gene.

To confirm the sequences identified through RACE, additional RT-PCRs on 3 male and 3 female pupae samples were performed using primers for the 5' and 3' ends of the RACE sequence. A single PCR product was obtained from male samples and two PCR products obtained from female samples, which approximately corresponded in size to the *Ae. aegypti* male (M1) and female (F1 and F2) mRNAs as previously described (Salvemini et al., 2011). cDNAs from each male and female sample using the primer pair *dsxF-dsxR1* were then sequenced as described in Methods.

Using the sequences thus obtained, additional PCRs were designed to give shorter amplification products encompassing the 5' and 3' ends of the *dsx* transcript (Figure 5-9).



Figure 5-9: 2nd RT-PCR of 5' and 3' *dsx* mRNAs, showing male and female transcripts

Two further PCR reactions were performed on cDNA obtained from additional wild-type single male and female samples (♂1-♂4 and ♀1-♀4 respectively). Primers *Abdsx*-diagF (P1) and *Abdsx*2 (P4) amplified the 5' region of the *dsx* mRNAs, while primers *Abdsx*4-F (P2) and *Abdsx*R1 (P5) amplified the 3' region, as indicated. Primers *Abdsx*-diagF and *Abdsx*5bR (P3) were designed to amplify only the female transcript (FS PCR). The approximate target sites of primers P1-P5 on the *Aaldsx* transcript are indicated in Figure 5-10. Sizes, obtained from sequencing, for the male band (M), female F2 band (F2) and female F1 band (F1) are indicated. As discussed below, sequencing of transcripts identified a second splice donor site in the F1 exon, which gave rise to a slightly shorter F1 transcript, marked F1(S). Sequencing also showed that some F1 transcripts retained the short 206 bp intron which lies between the F1 and F2 female exons. This may explain the detection of a second, larger F1 band in the FS PCR, as indicated.

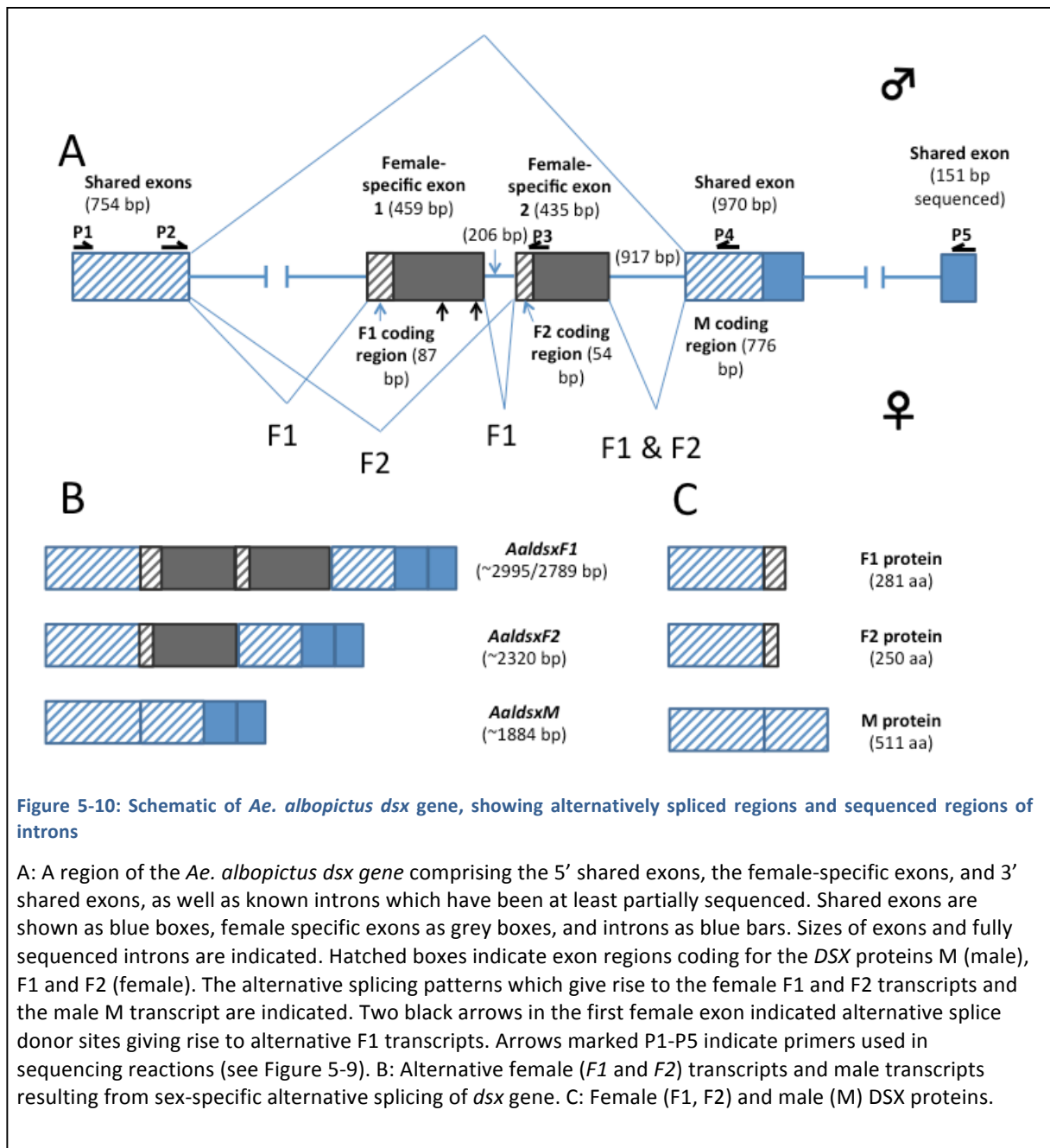
These PCRs were performed on 4 single male and female pupae samples. They confirmed the presence of two larger female products and a shorter male-specific product.

To confirm that the female transcripts were not present in males, a further RT-PCR was performed using a reverse primer in the female-specific exons (Figure 5-9 – FS PCR). The expected transcripts were detected in females, but no transcript detected in males. In summary, sex-specific splicing of the *dsx* transcript in *Ae. albopictus* appears to result in the following principal transcripts (though as discussed below, additional female transcripts may also be present):

- A single male transcript of 1884 bp², which was designated *Aaldsx* M1
- A large female transcript of 2779 bp, which was designated *Aaldsx* F1
- A smaller female transcript of 2320 bp, which was designated *Aaldsx* F2

A schematic of the *Ae. albopictus dsx* gene, showing splicing patterns, is shown in (Figure 5-10).

² The areas sequenced are truncated with respect to the full-length mRNA, in that the 5'UTR is not present. Nonetheless, as discussed below, the sequences obtained comprise the entire coding region of the *Ae. albopictus dsx* gene, and include the female-specific splicing region which is required for synthesising a functional *dsx* mini-gene.



As in the *Ae. aegypti dsx* gene (see Figure 5-1), the female specific region of the *Ae. albopictus dsx* gene appears to comprise two female-specific exons: both exons are spliced into the larger, F1 transcript, whereas only the second, smaller exon is spliced into the smaller F2 transcript. In the F1 transcript most frequently detected in *Ae. albopictus*, the two female-specific exons are separated by a small 206 bp intron – similar in size to the 208 bp intron which separates the female-specific 5a and 5b exons in *Ae. aegypti*. However, alternative splice forms were also detected, in which the F1 exon was truncated at its 3' end by 19 bp or

167 bp, respectively. These appeared to result from the use of alternative splice donor sites 5' to that used in the more abundant transcript.

As a first step towards further characterisation of the *Ae. albopictus dsx* transcripts, sequences were aligned with the published sequences for the corresponding M1 (AeadsxM1 [GenBank: DQ440534]), F1 (AeadsxF1 [GenBank:DQ440532]) and F2 (AeadsxF2 [GenBank: DQ440533]) transcripts from *Ae. aegypti*, as characterised by Salvemini et al. (2011). The alignment revealed a high level of sequence identity between the *Ae. albopictus* and *Ae. aegypti dsx* orthologues across the entire transcript sequence. Sequences were particularly conserved, as expected, within the protein coding region located at the 5' end (not shown).

To characterise the *Ae. albopictus dsx* protein isoforms, translations were performed *in silico* to obtain protein sequences for the M1, F1 and F2 proteins (*N.B:* as the ORF terminates early in the F1 exon, only two female-specific proteins are produced, since the protein coding regions of the alternative F1 transcripts are identical (Figure 5-10)). Alignment of these protein sequences with published sequences for *Ae. aegypti* again revealed a high level of sequence identity (Figure 5-11). Indeed, protein sequences corresponding to the 5' shared (non-sex-specific) exons were virtually identical in each species, with the exception of a 6 aa region which included the addition of 3 proline residues in both male and female *Ae. albopictus* protein isoforms. Both male and female transcripts contained an alternatively-spliced region of 63 bp (21 aa) which was detected in sequences obtained in some samples but not in others. This region corresponds to an identical region in the *Ae. aegypti dsx* transcripts, which is also spliced in a non-sex specific manner, as reported by Salvemini *et al.*(2011) (See Figure 5-1).

The male specific region of the *Ae. albopictus* M1 *dsx* protein isoform shows a much lower degree of sequence identity with the *Ae. aegypti* M1 protein than that observed in either of the female-specific variants. This finding is consistent with the observation reported by

Salvemini *et al* that the male-specific region of *Ae. aegypti* M1 showed a low overall level of conservation with respect to male *dsx* protein isoforms from *A. gambiae* and *D. melanogaster*.

A

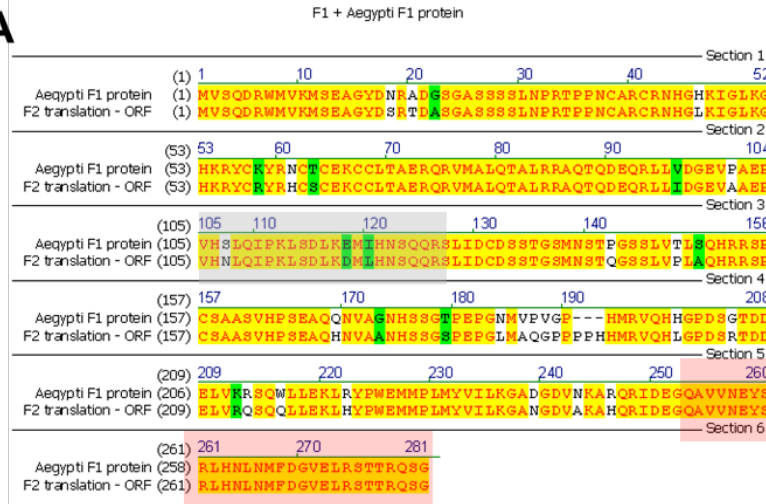
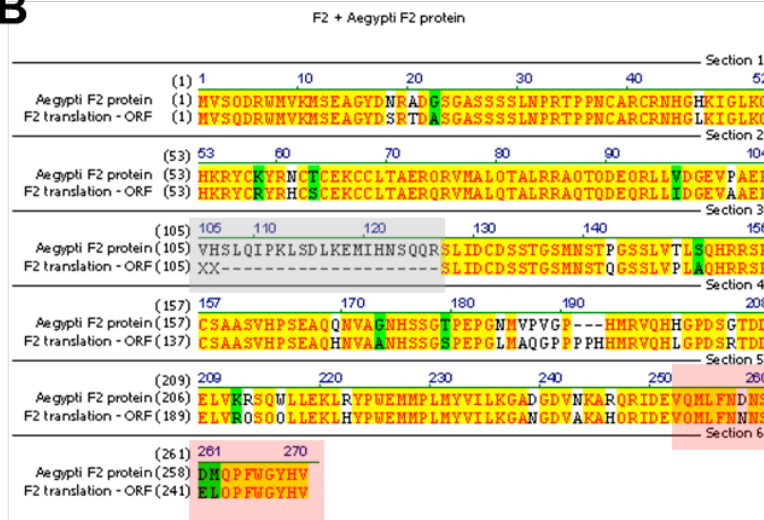


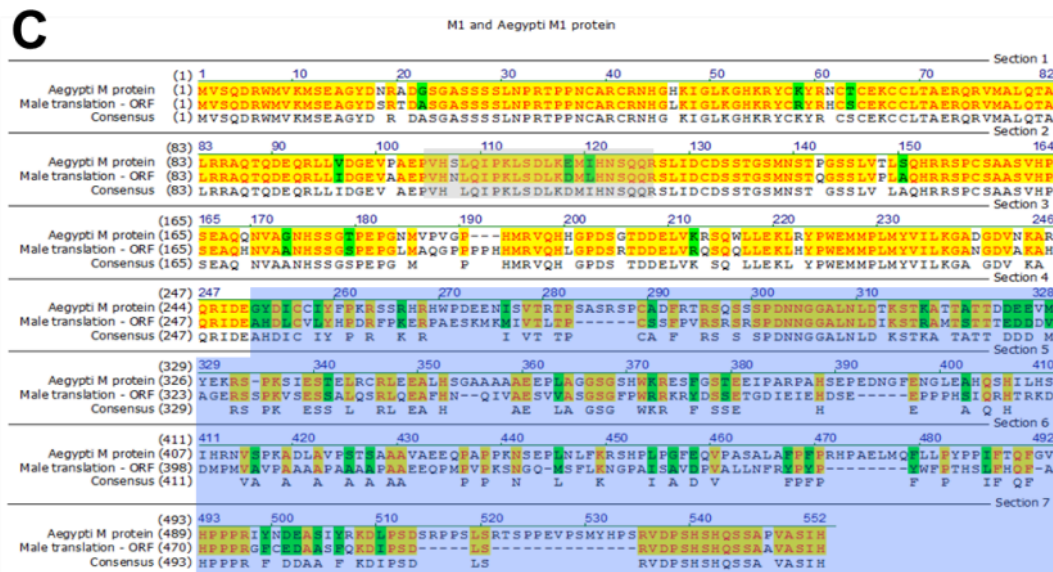
Figure 5-11: Comparison of *Ae. albopictus dsx* and *Ae. aegypti dsx* protein isoforms

Protein sequences obtained from an *in silico* translation of open reading frames from the *AaldsxF1* (A), *AaldsxF2* (B) and *AaldsXM1* (C) mRNAs were aligned with published sequences from the *Ae. aegypti* protein isoforms *AeadsxF1*, *AeadsxF2* and *AeadsXM1*. Yellow highlighting indicates sequence identity (top row – *AaldsxF1*; bottom row – *AeadsxF1*). Pink and blue shaded areas indicate female and male specific regions, respectively, in each protein. Grey shaded areas demarcate a 21 bp non-sex specific alternatively spliced region

B



C



5.3.5 CHARACTERISING EXON-INTRON BOUNDARIES IN THE *AE. ALBOPICTUS* *DSX* GENE REVEALED SOME DIVERGENCE FROM THE *AE. AEGYPTI* ORTHOLOGUE

In order to synthesise a *dsx* mini-gene which would retain sex-specific splicing *in vivo*, sequences or partial sequences were required for those introns which flanked the alternatively spliced regions. A comparison of the male (M1) and two female specific mRNAs (F1 and F2) was sufficient to identify the boundaries of shared and female-specific exons. Flanking PCRs using random reverse primers were then used to obtain partial sequences for the introns bordering these regions, as described in Methods.

The resulting sequences revealed a number of differences in the exon-intron structure of the alternatively spliced region in the *Ae. albopictus* vs the *Ae. aegypti dsx* genes. Though small, these differences could be of significance in view of the failure of an *Ae. aegypti dsx* minigene to be spliced correctly in *Ae. albopictus*.

A key distinction is the relatively short (<1 kb) intron separating the female F2 exon and the 3' shared exons in the *Aaldsx* gene, whereas in *Ae. aegypti* the equivalent exon exceeds 10 kb. Since this was the only intron the size of which was amenable to complete sequencing, the sizes of remaining introns in the *Aaldsx* gene are unknown. In addition, the 206 bp intron separating the two female-specific exons (equivalent to the small intron of 208 bp separating exons 5a and 5b in *Ae aegypti*) was occasionally present in sequenced transcripts, suggesting that in *Ae. albopictus* this region is occasionally retained in female transcripts (Figure 5-10). It also appears that up to three 5' splice donor sites can alternatively be used in splicing this intron, with alternative sites at 19 bp and 167 bp, respectively, upstream of the primary site.

Initial flanking sequencing on two separate pooled samples indicated that the large intron separating the shared and female-specific exons had conserved regions of approximately 300 bp and 600 bp at its 5' and 3' termini (shown in Figure 5-10). Outside of this region, sequences from the two pooled samples showed a greater level of variation, which suggested that intron

sequence of importance for correct splicing of the *dsx* gene might be found within these regions at the immediate 5' and 3' ends of the intron. Subsequently, additional sequencing suggested that these conserved regions may extend further into the intron than initial results suggested, which may be of use in re-engineering *dsx* constructs in future (see Discussion for further treatment of this).

In their investigation of the *Ae. aegypti dsx* gene, Salvemini *et al* (2011) identify a number of sequence elements in and around the female specific exons which they suggest could be of importance in splicing. However, the identification of these elements is based largely on probing for canonical TRA/TRA2 binding sites. Although the TRA/TRA2 proteins and their associated binding sites on the *dsx* gene are widely conserved among dipterans, no orthologue of *tra* has yet been identified in *Ae. aegypti* or *Ae. albopictus*. It was therefore doubtful whether an equivalent bioinformatics analysis on the *Aal**dsx* gene would yield results useful to the synthesis of a functional mini-gene.

5.3.6 AN *AE. ALBOPICTUS DSX* MINI-GENE WAS SPLICED IN A SEX SPECIFIC MANNER *IN VIVO*, THOUGH 'LEAKY' MALE SPLICING WAS OBSERVED

The sequenced *Ae. albopictus dsx* gene was used to construct a putative female-specific lethal construct for use in *Ae. albopictus*.

A *dsx* mini-gene was amplified from *Ae. albopictus* gDNA and incorporated into construct OX5024, as described in Methods. The mini-gene comprised the female-specific regions of the *dsx* gene, with an engineered start codon added to the second female specific exon, partial sequence from the shared exons upstream and downstream of the female-specific regions, together with the whole of the intron upstream and part of the intron downstream of the female-specific regions.

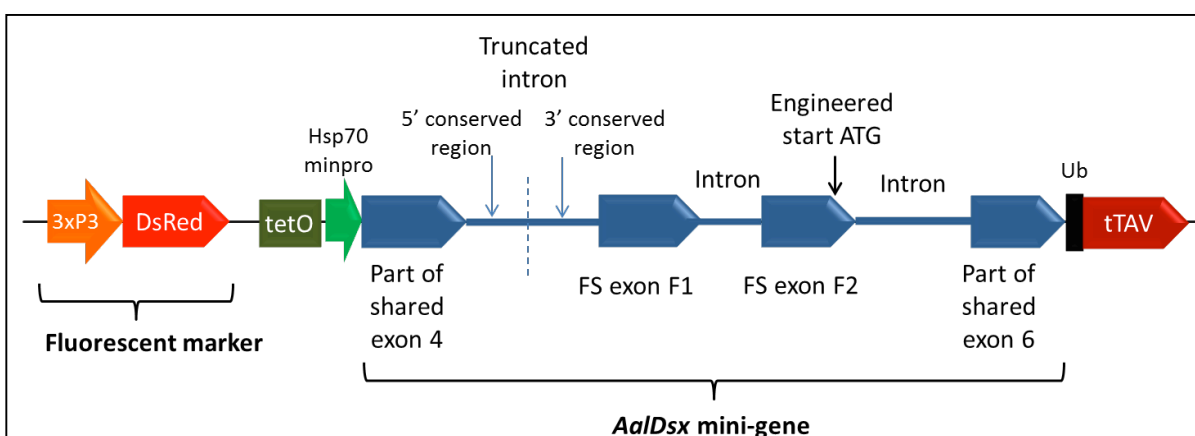


Figure 5-12: Schematic of construct OX5024, showing the *Ae. albopictus* (*Aal*) *dsx* mini-gene

As in construct OX4489, a start ATG codon has been added at the 3' end of the female-specific F2 exon (FS exon F2), which should be included in all female transcripts, such as to give an in-frame *dsx-ubi-tTAV* protein only in female insects. The ubiquitin (Ub) moiety is cleaved co-translationally to yield free-form tTAV protein. Also as in construct 4489, The tetO enhancer is bi-directional, hence presence of functional tTAV should drive a positive feedback loop from this enhancer, leading eventually to toxic accumulation of the tTAV protein. The truncated intron 5' of the female-specific (FS) exons consists of a 302 bp and 687 bp fragments from the 5' and 3', respectively, of the native intron, which initial sequencing showed to be highly conserved between wild-type strains.

Construct OX5024 is based on construct OX4489, and comprises the *Ae. albopictus dsx* mini-gene described above, fused to a ubiquitin-tTAV cassette (Figure 5-12).

As with construct OX4489, the presence of an engineered start codon in the female-specific exons is expected to result in a complete tTAV protein in females only. The construct is therefore putatively a female-specific lethal, since tTAV acting on the bi-directional tetO enhancer could lead to accumulation of toxic levels of the tTAV protein in females.

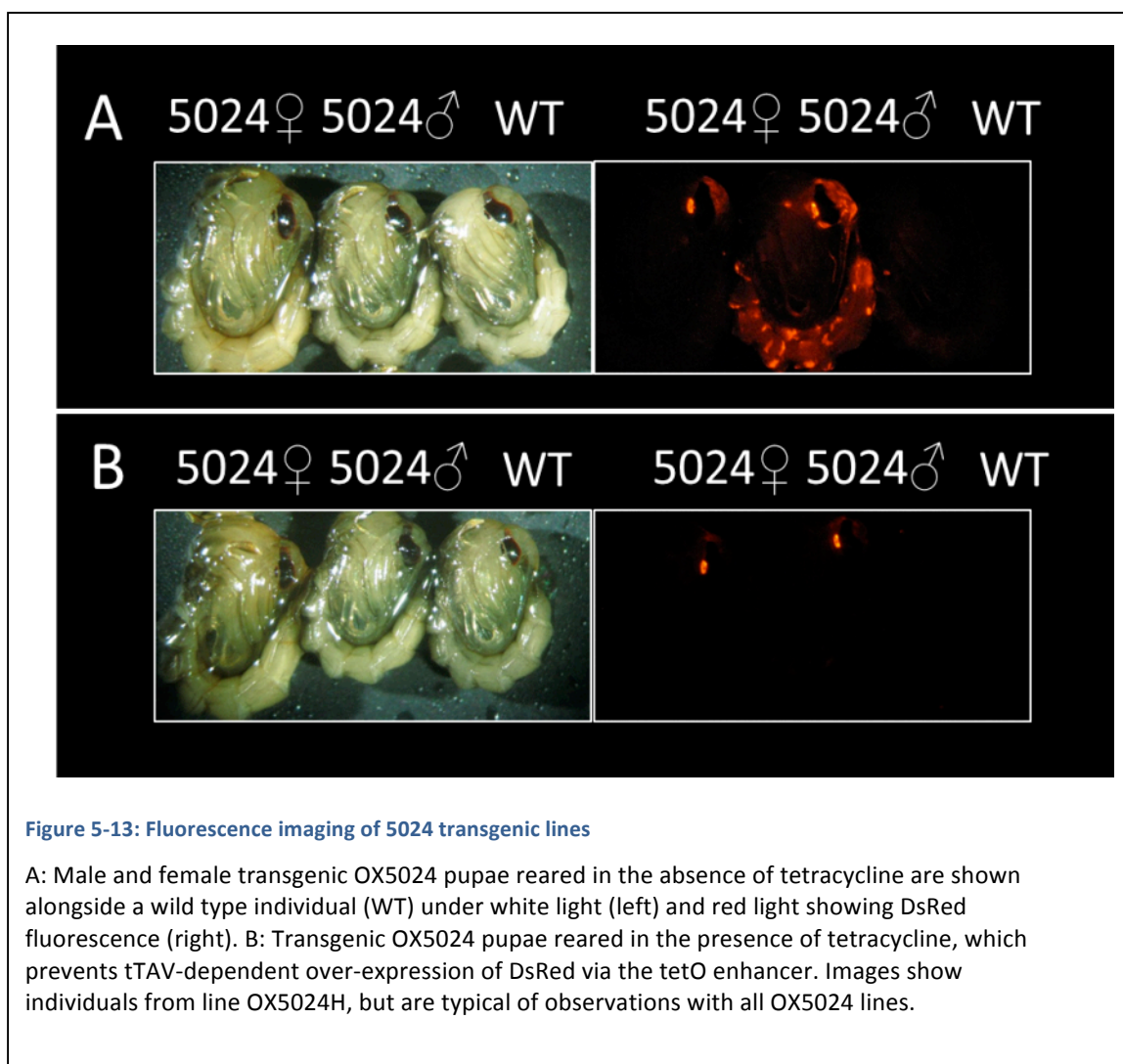
Injection data for OX5024 and OX5048 (described below) is shown in table Table 5-7.

Table 5-7: Injection data for *Aaldsx* constructs**

Construct	Eggs injected	G0 survival (to adult)	Transgenic G1 pools
OX5024 (<i>albopictus</i>)	2584	76	7
OX5024 (<i>aegypti</i>)	1492	46	6
OX5048	118	8	3

Injections with OX5024 yielded six transgenic lines for downstream analysis. After initial tests indicated that the lines were non-lethal in either males or females, G2 individuals from each line were reared in the absence of tetracycline to assay for any sex-specific expression of *DsRed* – as had been noted in *Ae. aegypti* lines bearing the 4489 construct. Unexpectedly, all six lines showed bright, all-body fluorescence in males, but not in females (Figure 5-13). This indicated the presence of functional tTAV in males but not in females, which was the converse of the expected female-specific expression.

RT-PCRs on G2 individuals from each line confirmed the presence of the expected male-specific transcript in males, and the expected female F2 transcript in females (Figure 5-14).



However, the female F2 transcript was also present in male samples, indicating that sex-specific splicing was not occurring as expected. Sequencing of male and female bands from lines A and H confirmed that the putative male and female F2 products did indeed match the expected sequences for these products. However, on re-examination of the original construct sequence, it was discovered that the positioning of the engineered start codon was forward-shifted by a single base pair, such that translation initiation at the engineered start site would no longer result in an in-frame tTAV protein. Moreover, it was noted that two putative start codons (albeit with non-canonical upstream Kozak sequences) in the shared exons would give rise to an in-frame tTAV protein in the male, but not female, splice form. This likely explains the unanticipated male-specific DsRed fluorescence in male insects. Finally, no product for the larger female (F1) transcript was detected. This was confirmed with additional RT-PCRs which specifically targeted the female F1 transcript.

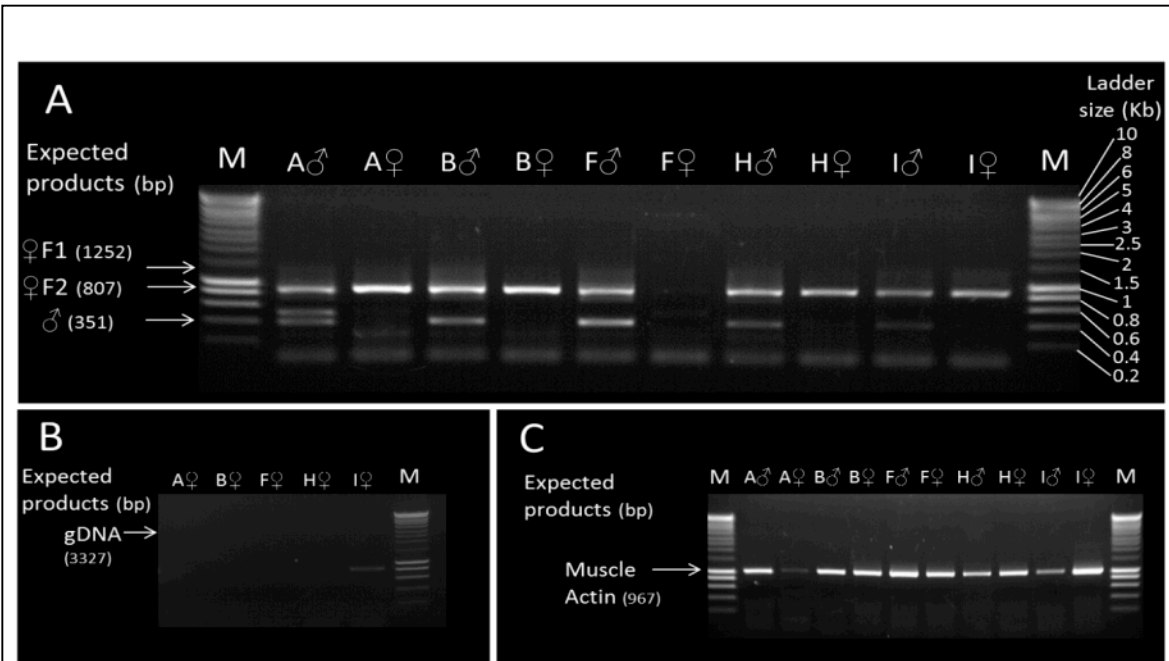


Figure 5-14: RT-PCRs of OX5024 lines

A: RT-PCR amplifying part of the putative sex-specific splicing regions of the OX5024 (*Aaldsx-tTAV*) construct. Male and female samples from each of five OX5024 lines (A, B, F, H & I) are shown. Expected product sizes for the female-specific (♀F1 and ♀F2) and male-specific (♂) transcripts are indicated. M= SmartLadder molecular weight marker (Eurogene): sizes of bands are indicated at right of gel. B: The same PCR using control samples prepared in the absence of reverse transcriptase. The expected size of the amplicon from any contaminating gDNA is indicated. C: RT-PCR for the endogenous muscle actin gene indicating overall levels of cDNA present in each sample.

5.3.7 A MODIFIED *AE. ALBOPICTUS* *DSX* CONSTRUCT SHOWS IMPROVED SPECIFICITY OF SPLICING

One possible explanation for the ‘leaky’ splicing, or lack-of female-specific splicing, in the OX5024 construct was that the added ATG start codon might be interfering with correct splicing of the female-specific regions (although it is notable that a similar design had not appeared to interfere with splicing in *Ae. aegypti*). To investigate this possibility, a new construct, OX5048, was designed, in which the added start codon was 5’ of the entire *dsx* mini-

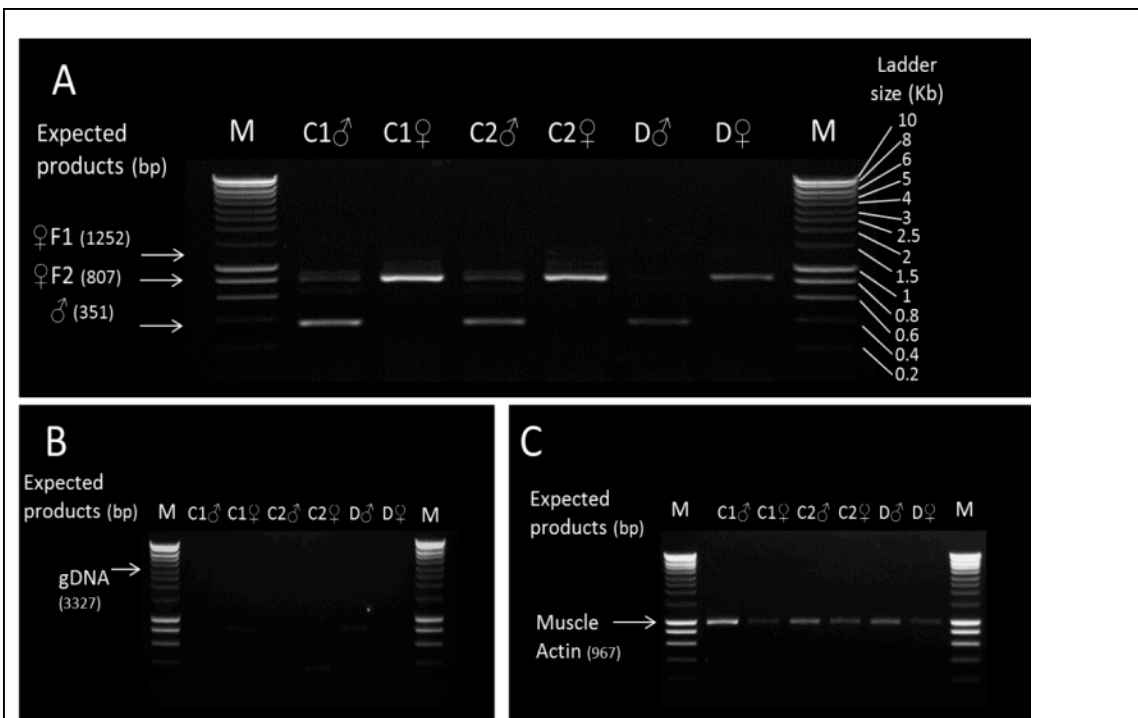


Figure 5-15: Sex-specific splicing of construct OX5048 (*Aaldsx-tTAV*)

A: RT-PCR amplifying part of the putative sex-specific splicing regions of the OX5048 (*Aaldsx-tTAV*) construct. Male and female samples from each of the three OX5048 lines are shown. Expected product sizes for the female-specific (♀F1 and ♀F2) and male-specific (♂) transcripts are indicated. B: The same PCR using control samples prepared in the absence of reverse transcriptase. C: RT-PCR for the endogenous muscle actin gene indicating overall levels of cDNA present in each sample.

gene. A number of mutations were also made in the downstream sequence to remove potential start codons which would be in-frame with the downstream tTAV transcript. Injections with OX5048 yielded three separate lines. Two of these lines, labelled C1 and C2,

were derived from the same G0 cross, but were classed as different insertions on the basis of their different fluorescence phenotypes. G3 samples from each line were used in RT-PCRs to test for sex-specific expression of the *dsx* transcript (Figure 5-15). In all three lines, differential splicing between the sexes was clearly evident. The expected male transcript was found to be strongly expressed in all male samples, and entirely absent from female samples. Female samples showed strong expression of the expected female-specific F2 transcript (though not the larger F1 transcript). Sequencing confirmed that bands of the expected size did indeed derive from the expected male and female transcripts. However, as with the first *Aaldsx* construct, OX5024, splicing of the OX5048 construct appeared 'leaky', with some female F2 transcript also being evident in male samples. It is notable that the brightness of bands in the PCR shown in Figure 5-15 indicates that, unlike the original *Aaldsx* construct, OX5024, the male splice form is present in markedly higher amounts than the female transcript in male OX5048 samples. This result suggests a higher degree of specificity in splicing the modified *Aaldsx* mini-gene present in the OX5048 construct. Although these PCRs cannot provide a quantitative assessment of transcription levels, observations of the fluorescence in male and female the OX5048 samples further suggest that this apparent differential expression is reflected in the relative levels of tTAV being produced in male and female insects. When reared in the absence of tetracycline, males from each of the three lines do show some increased DsRed fluorescence relative to samples reared in the presence of tetracycline, indicating that functional tTAV protein is indeed produced in males (Figure 5-16). However, fluorescence appears to be less bright in males relative to female insects, particularly in line D.

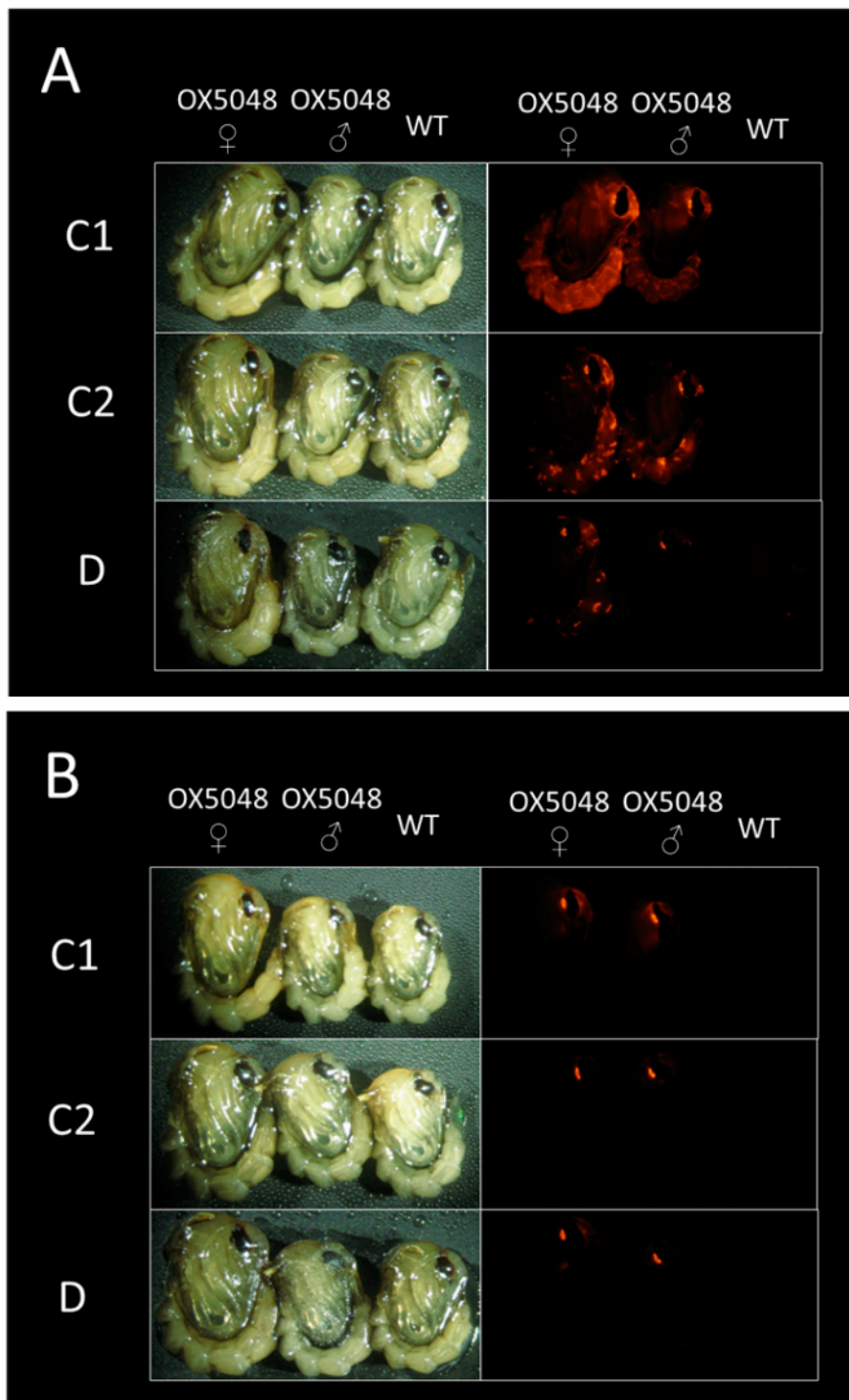


Figure 5-16: Fluorescent images of OX5048 (*Aaldsx-tTAV-tetO-DsRed*) lines

A: Male and female pupae from OX5048 lines (*Aaldsx-tTAV-tetO-DsRed*) reared in the absence of tetracycline. Transgenic insects are shown alongside a wild-type (WT) individual, under white light (left-hand panels) or red filter (right-hand panels) which shows DsRed fluorescence. Panels C1, C2 and D show pupae from OX5048 lines C1, C2 and D, respectively. B: Male and female pupae from OX5048 lines reared in the presence of tetracycline, which blocks tTAV-mediated over-expression of DsRed via the tetO enhancer.

Although splicing of the *dsx* mini gene in OX5048 lines was clearly 'leaky', it was considered that the observed female-male differential in expression levels of the female-specific transcript might be sufficient to confer a degree of female-specific lethality, when crossed to a tetO-lethal effector line. To investigate this hypothesis, heterozygous individuals from OX5048 line D were crossed with heterozygous individuals expressing the OX4499 construct, which contains an effector cassette in which the tetO enhancer was linked to the pro-apoptotic gene *Michelob_X* (*mx*). The presence of functional tTAV in F1 progeny carrying both constructs was therefore expected to drive *mx* expression, inducing lethality. OX5048D was selected as the splicing and phenotypic analysis described above had identified it as the most 'promising' OX5048 line, in terms of its exhibiting a clear differential in expression strength of tTAV between males and females.

F1 progeny from this cross were reared in the presence or absence of 30 µg/ml chlortetracycline (tet) as described (Methods). Rates of pupation and eclosion for male and female individuals carrying both constructs and for wild-type individuals were recorded. Pupation rates of insects carrying only one construct were also recorded, before insects were discarded. In the absence of tetracycline, overall pupation rates were found to be significantly reduced for insects carrying the OX4499 (tetO-*mx*) construct relative to both wild-type and insects carrying the OX5048 (*dsx-tTAV*) construct only

Both males and females carrying both constructs exhibited a reduced rate of pupation in the absence of tetracycline (Figure 5-17 – A). However, overall pupation rates were also found to be significantly reduced for insects carrying the OX4499 (tetO-*mx*) construct only, relative to both wild-type and insects carrying the OX5048 (*dsx-tTAV*) construct only. There was no significant difference between overall pupation rates of insects carrying the OX4499 construct and those carrying both constructs at $p < 0.05$. (GLM, pupation rate by genotype, p (z) = 0.095, 6.5×10^{-5} and 6.79×10^{-6} for OX4499, OX5048 and WT insects respectively, relative to those

carrying both constructs). In the presence of tetracycline, there were no significant differences in the pupation rates of different genotypes.

A complicating factor was that the OX5048 transgene in line D proved to be male linked, producing a male sex bias in progeny containing both constructs. However, a GLM model indicated that there was no significant interaction between tetracycline treatment and sex, indicating that the relative survival of females reared in the absence of tetracycline was no different to that of males. (GLM with Poisson error for pupation rates of insects with both constructs, followed by stepwise removal of factors to obtain best-fit model. Differences in variance between the saturated model and a model lacking the “sex”:“tetracycline treatment” interaction term was analysed by Anova: $\chi^2 = 0.23$.).

Eclosion rates for insects carrying both constructs and for wild type insects were measured as a proportion of total pupae eclosing (data not shown). For insects carrying both constructs, eclosion was not significantly different between males and females either in the presence or absence of tetracycline (GLM with binomial error for eclosion rates followed by stepwise removal of factors; difference in variance between saturated and null models was analysed by Anova: $\chi^2 = 0.43$)

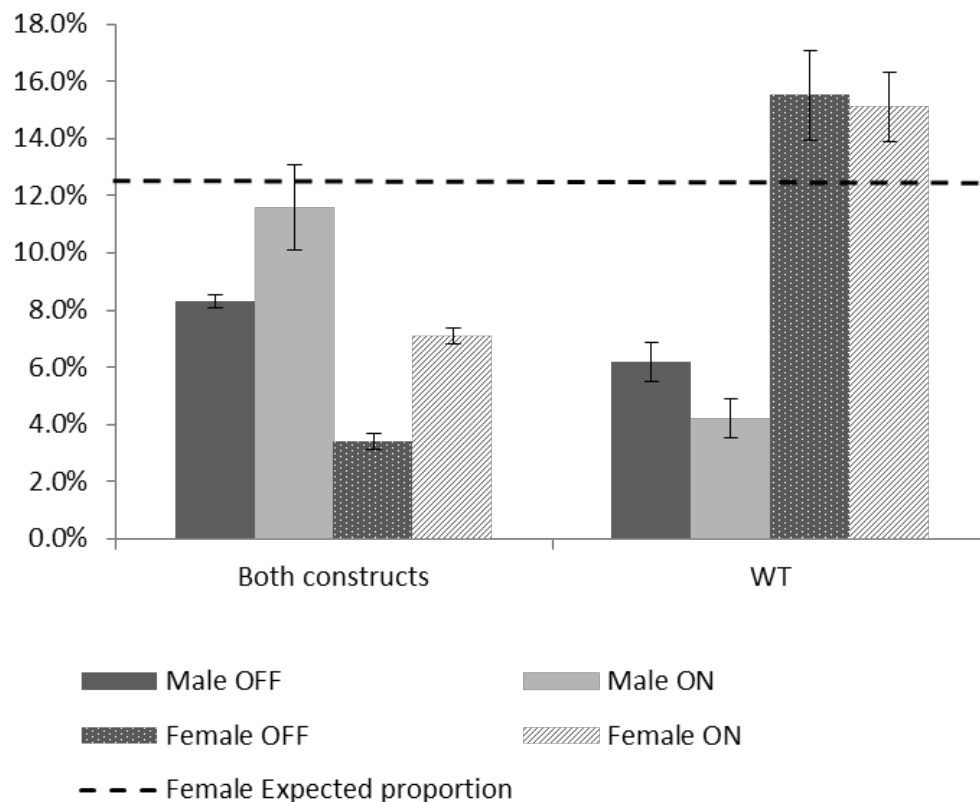


Figure 5-17: Pupation rates of OX5048 x OX4499 F1 progeny

F1 progeny resulting from crosses between heterozygous OX5048 transgenic individuals and heterozygous OX4499 transgenic individuals were reared in the presence ('ON') or absence ('OFF') of tetracycline. Pupae were screened on the basis of fluorescence phenotype for the presence of one or both constructs. Wild-type individuals and individuals carrying both constructs were then sexed and counted, before being placed separately into cages and allowed to eclose. Pupae expressing only one construct were counted and then discarded (data not shown). Bars show mean numbers of male and female pupae (as indicated), as a proportion of the starting population. The expected proportion of males and females of a given phenotype predicted by Mendelian inheritance was 12.5%, as indicated (dashed line).

Statistical analysis was performed by interrogating GLM models (detail reported in main text). No significant difference in off-tet survival of transgenic insects was recorded between sexes (Anova of GLM models following stepwise removal of factors; $p=0.23$). No significant difference was found in eclosion rates between sexes, regardless of tetracycline treatment ($p=0.43$).

It therefore appears that splicing of the OX5048 construct results in reduced levels of the full-length 'female' *dsx-tTAV* transcript in male transgenic insects in comparison to females. However, the differential is not sufficient to confer markedly different survival rates between males and females in the context of a tTAV-tetO repressible lethal system.

5.3.8 SPLICING OF *AE. ALBOPICTUS* *DSX* IN *AE. AEGYPTI* DEMONSTRATES RECIPROCAL LOSS OF FUNCTION OF *DSX* GENES BETWEEN THE SPECIES

The OX5024 construct was also used to transform an *Ae. aegypti* wild-type background, to assess whether the observed loss of function of the *Ae. aegypti dsx* mini-gene in an *Ae. albopictus* background was a bi-directional phenomenon. Six separate lines were established, of which four were derived from separate G0 pools, and two were derived from shared G0 pools on the basis of a distinct fluorescence phenotype.

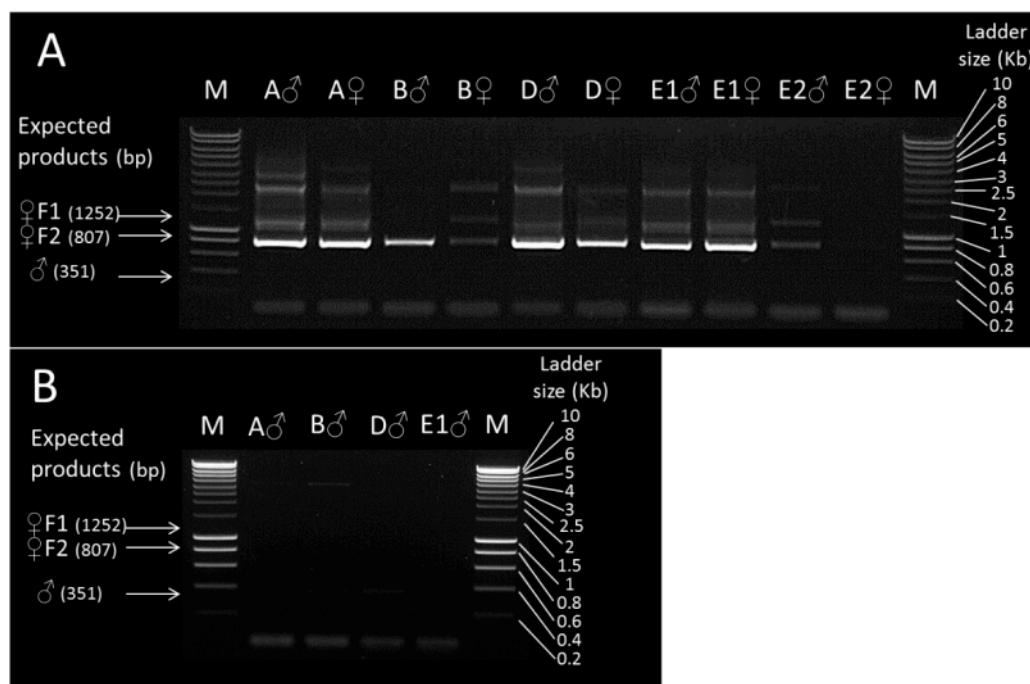


Figure 5-18: Splicing of *Aabdsx*-tTAV expression in OX5024 (*Ae. aegypti*) lines

A: RT-PCR amplifying part of the putative sex-specific splicing regions of the OX5024 (*Aabdsx*-tTAV) construct. Male and female samples from each of the five OX5024 (*Ae. aegypti*) lines are shown (A – E2, as indicated above lanes). M= SmartLadder molecular weight marker (Eurogentech). Expected product sizes for the female-specific (♀F1 and ♀F2) and male-specific (♂) transcripts are indicated. B: The same PCR using control samples prepared in the absence of reverse transcriptase. The identity of larger, faint bands in many samples is unclear, since these were not sequenced.

Samples from each line were used in RT PCRs to test for sex-specific splicing of the *dsx* transcript. Results indicate that there is a complete loss of sex-specific splicing of the *Aabdsx* mini-gene in an *Ae. aegypti* background (Figure 5-18). Both male and female samples appear to process the expected female transcript only, with no transcript corresponding to the expected male-specific splice form observed in either sex. As noted previously, a sequence error in the OX5024 construct meant that only the male-specific transcript resulted in tTAV expression – probably from an alternative start codon located 5' to the female-specific region of the *dsx* mini-gene – whereas the start codon located in the female transcript was out-of-frame with the down-stream tTAV protein. It was therefore predicted, on the basis of the RT-

PCR results described, that no tTAV protein would be produced in either sex. The unidentified larger bands seen in many samples in Figure 5-18 is unexplained, and may simply result from non-specific primer binding. However, one possibility is that they result from specific amplification of un-spliced *dsx* mRNA.

Finally, an examination of the fluorescence phenotype of male and female insects in the presence and absence of 30 µg/ml tetracycline did not, for either sex, reveal any increase in DsRed fluorescence intensity, as would be expected in the presence of functional tTAV (Figure 5-19).

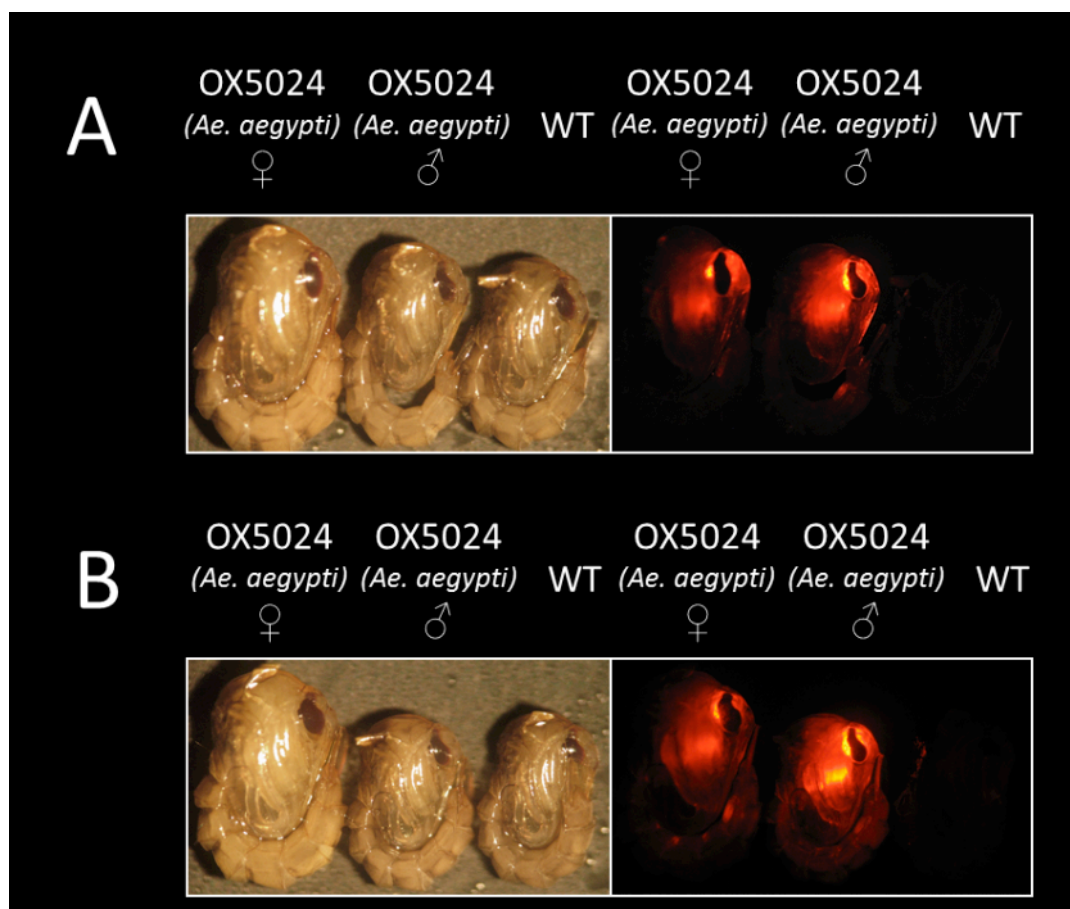


Figure 5-19: Lack of sex differentiated fluorescence in OX5024 (*Ae. aegypti*) lines

Male and female pupae from OX5024 (*Aaldsx*-tTAV-tetO-DsRed) *Ae. aegypti* lines reared in the presence or absence of tetracycline. Transgenic insects are shown alongside a wild-type (WT) individual, under white light (left-hand panels) or red filter (right-hand panels) which shows DsRed fluorescence. A: No tetracycline; B: 30 µg/ml tetracycline. Transgenic individuals shown are from OX5034 (*Ae. aegypti*) line A, but are typical of other lines examined.

5.4 CONCLUSIONS: PROSPECTS FOR ENGINEERING FEMALE-SPECIFIC LETHALITY IN AEDES INSECTS

A genetic sexing mechanism would be a highly desirable component of an improved dominant-lethal based approach to insect population control. In its optimal form, to maximise benefits to rearing efficiency, the genetic sexing mechanism would act at an early stage in development, enabling easy removal of females from the release generation and increasing rearing capacity. Engineering expression systems based on sex-specific splicing of the genes *Actin4* and the sex-determining factor *Doublesex (dsx)* are proven approaches to achieving female specific expression in the disease vector species *Ae. aegypti* (Fu et al., 2010). In the related vector species, *Ae. albopictus*, an engineered gene cassette based on the promoter and alternatively spliced elements of the *Actin4* gene has also been used to generate a repressible, female-specific flightless phenotype in transgenic mosquitoes (Labbé et al., 2012).

This chapter describes the results of three parallel approaches to developing novel female-specific expression systems in the vector species *Ae. aegypti* and *Ae. albopictus*. The first of these approaches was an attempt to engineer a female-specific expression system based on the alternatively-spliced elements of the *Ae. aegypti Actin4* gene when isolated from the native *Actin4* promoter. The second used sex-specific splicing elements of the *Ae. aegypti dsx* gene, and the third used equivalent elements from the *Ae. albopictus dsx* orthologue in an attempt to generate a female-specific expression system in the latter species.

5.4.1 THE ALTERNATIVELY SPLICED ELEMENTS OF THE *ACTIN4* GENE DO NOT APPEAR SUFFICIENT TO DIRECT SEX-SPECIFIC SPLICING IN THE ABSENCE OF THE NATIVE *ACTIN4* PROMOTER.

The *Ae. aegypti Actin4* gene is strongly expressed in the flight muscles of developing females (Munoz et al., 2004). Gene expression systems in which elements of the *Actin4* gene have been

used to generate female-specific expression have been based on both the *Actin4* promoter and sex-specifically spliced regions of the gene. This has essentially reproduced the expression profile of the native gene, resulting in a female flightless phenotype in transgenic systems (Fu et al., 2010, Labbé et al., 2012).

Construct OX4601 was designed to de-couple the sex-specific splicing of the *Actin4* gene from the tissue-specific expression driven by the native promoter, such as to result in a more ubiquitous expression profile. However, RT-PCRs clearly indicate the absence of sex-specific splicing in OX4601 transgenic insects, and no transgenic line showed any evidence of a sex-specific phenotype. These results indicate that the *Actin4* alternative splicing elements are in themselves insufficient to direct sex specific splicing. Since sex-specific splicing has been observed in constructs incorporating the *Actin4* promoter, it is likely that sequences within the promoter are necessary to direct the native male and female splicing. An alternative explanation may be that sex-specific splicing is itself restricted to the flight muscles – for example through tissue-specific expression of splicing factors. This would in theory confer a female-flightless phenotype on OX4601 lines; however, such an effect would be difficult to distinguish from the bi-sex mortality also present.

In summary, these results do not preclude the possibility of being able to de-couple the sex-specific splicing of the *Actin4* gene from its limited native expression profile. Nonetheless, they suggest that significant additional research, including deletion analysis of the *Actin4* promoter in order to isolate the elements required for sex-specific splicing, would be required in order to do so.

5.4.2 AN *AE. AEGYPTI* *DSX* MINI GENE IS NOT SPLICED IN A SEX-SPECIFIC MANNER IN *AE. ALBOPICTUS*

A *dsx* mini-gene incorporating the sex-specifically spliced elements of the native *Ae. aegypti* *dsx* gene has previously been shown both to be spliced in a sex-specific manner and to direct

sex-specific expression in *Ae. aegypti*. The *dsx* gene is highly conserved amongst Diptera; it was therefore expected that the *Ae. aegypti dsx* mini-gene would preserve its function in the closely related vector species *Ae. albopictus*. Results presented in this chapter clearly indicate that this is not the case. Transgenic *Ae. albopictus* individuals did not splice the *Ae. aegypti dsx* gene in a sex-specific manner, nor did they exhibit the expected sex-specific expression of tTAV, as indicated by crosses to a tetO-marker line. This result was surprising in view of previous results demonstrating cross-species conservation of function with similar constructs: sex-specific splicing elements of the *Ae. aegypti Actin4* gene have previously been shown to retain their function when transplanted into an *Ae. albopictus* background (Labbé et al., 2012), while a sexing construct based on the *dsx* gene of the moth *Bombyx mori* was spliced in the expected sex-specific manner and was found to direct sex-specific expression in the Diamond-back moth *Plutella xylostella* – a species of a different genus.

The native *Ae. albopictus dsx* gene (*Aaldsx*) has been identified and partially sequenced through the work reported in this chapter. The sequence of the *Aaldsx* cDNA is highly similar to the cDNA sequence of the *Ae. aegypti dsx* gene (*Aaedsx*) reported previously, and codes for a near-identical protein sequence, with the exception of the C-terminal region of the male-specific protein. It therefore appears that subtle differences in sequence are sufficient to disrupt splicing behaviour in the non-native species. The exact location of the key elements necessary for correct splicing is unknown. Following bioinformatics analysis, Salvemini *et al* suggest that a number of sequence elements within and around the female specific exons of the *Aeldsx* gene may be involved (Salvemini et al., 2011). However, these are based largely on canonical binding sites for the TRA/TRA2 proteins, which, while widely conserved amongst many dipteran species, have yet to be identified in *Ae. aegypti* or *Ae. albopictus*. An equivalent bioinformatics analysis has yet to be performed on the *Aaldsx* gene. However, since it is unlikely that *dsx* splicing between two such closely related species relies on a substantively different mechanism, it seems equally unlikely that the observed differences in splicing

between the two orthologues will have their origin in the presence or absence of canonical binding sites for a conserved upstream effector.

5.4.3 USE OF AN *AALDSX* MINI-GENE SHOWS SOME PROMISE AS A MECHANISM FOR ACHIEVING SEX-SPECIFIC EXPRESSION IN *AE. ALBOPICTUS*

Two attempts to achieve sex-specific expression in *Ae. albopictus* through transgenesis with an *Aaldsx* mini-gene are described in this chapter. Results with construct OX5024 (*Aaldsx*-tAV-tetO [1st iteration]) showed little promise of achieving female-specific expression: although a male-specific splice-form was produced, female transcript was also produced in both sexes. A second construct, OX5048 (*Aaldsx*-tAV-tetO [2nd iteration]) was designed with a start codon 5' of the *dsx* mini-gene, against the possibility that adding the start codon to the female specific region, as in construct OX5024, might be responsible for disrupting splicing. Although results with this construct could not provide a clear answer to this question, transgenic individuals do show sex-specific splicing of the *dsx* mini-gene in a manner similar to that expected. Some female transcript is still produced in male insects, but RT-PCRs appear to indicate that it is present in lower amounts in males compared with females. Furthermore, transgenic insects show some evidence of a sexually-divergent phenotype in the off-tetracycline expression of the DsRed fluorescence marker. This suggests the presence of higher levels of functional tAV protein in females compared with males. Nonetheless, results from crosses between OX5048 line D and a tetO-*MichX* line show that this differential, if indeed present, is not sufficient to confer female-specific lethality in the context of a RIDL system.

Together, these results indicate that, while female-specific expression based on *dsx* splicing has clearly yet to be realised in *Ae. albopictus*, an *Aaldsx* mini-gene may yet prove a viable avenue by which to reach this goal. It is not clear why the *Aaldsx* mini-gene as used in constructs OX5024 and OX5048 was not sufficient to confer the native *dsx* splicing as seen in equivalent constructs in *Ae. aegypti*. Based on experience with the Actin4 gene, as reported in

this chapter, one possibility could be that sequences in the promoter region are required for the native splice forms. However, this was clearly not the case in *Ae. aegypti*, and seems unlikely in view of the fact that the promoter is a considerable distance upstream of the sex-specific splicing regions in the native gene (in contrast, the Actin4 promoter is immediately adjacent to the alternatively spliced elements). A further possibility is the presence of a number of small but potentially critical sequence mis-matches between the *dsx* mini-gene and the most recent consensus wild-type sequence, which were identified following additional sequencing of the sex-specific splicing regions of the native *dsx* gene. These were mainly single base pair differences, but notably, they included a 10 bp region at the 5' end of the short intron which separates the female-specific regions. Because of its location, this region may be important in directing female-specific splicing. These differences may have arisen from errors in the original sequencing, or from the presence of rare alternative splice forms in the transcripts originally sequenced. Additional sequencing of the intron preceding the female-specific regions was also performed on eight single individual samples from two different wild-type strains, to elucidate the extent of the earlier-identified conserved regions at the 5' and 3' ends of this intron. The sequencing indicated that these conserved regions might extend further into the intron than previously thought, particularly at its 5' end. Based on this additional sequencing of the *dsx* gene, a new construct has been designed which incorporates the new sequence, including the potentially important region located in the short intron, noted above. It also incorporates a larger area of flanking intron upstream of the female-specific exons, extending to 600 bp at the 5' end and 500 bp at the 3' end of this intron.

Should this construct prove able to confer female-specific expression in *Ae. albopictus*, it will represent an important step along the path to a 'second-generation' dominant-lethal product in this species – as outlined in Chapter 2. Use of constructs based on the *Ae. aegypti dsx* gene has shown great promise as a means of developing a female-specific lethal phenotype in *Ae. aegypti*. Unlike lines based on the Actin4 promoter, which result in female flightlessness, lines

developed using the *dsx* construct exhibit a true female-specific lethal phenotype. Moreover, the *dsx* splicing regions can be combined with a variety of different promoter elements, offering the possibility of female-specific expression which acts early in development. In future product lines, this would facilitate the early elimination of females from the release generation – a highly valuable attribute in a next-generation dominant-lethal strategy in either species.

6 DEVELOPING AN AUXIN-BASED INDUCIBLE LETHAL SYSTEM

Note on the following text: *The work presented in this chapter was undertaken as a collaborative project with the molecular biology team at Oxitec Ltd, funded by the Technology Strategy Board. Construct design and synthesis was undertaken by the molecular biology team, as noted in the Methods section. All other work presented herein was the sole work of the author.*

6.1 INTRODUCTION

Previous chapters have discussed prospects for refining two key advantages of a genetic approach to traditional SIT-based pest control; namely, late-acting lethality and a genetic sexing mechanism.

These chapters have discussed the development of such systems in isolation. However, as discussed in Chapter 2, ongoing development of dominant-lethal based products will focus on incorporating one or more such systems to arrive at a product which offers the full suite of potential enhancements which such systems can bring to the traditional SIT approach.

The dominant lethal approaches discussed previously in this thesis incorporate a tetracycline-repressible system to ensure that expression of the lethal gene occurs only under restrictive (absent tetracycline) conditions. However, combining more than one system in a single product line would be greatly facilitated by development of an orthogonal expression control mechanism to that of the tetracycline-repressible system (Tet-Off system). For example, a desirable feature of a genetic sexing system would be early elimination of females in the release generation, which would increase rearing efficiency and production capacity. In contrast, late-acting lethality in the field should enhance the efficiency of a dominant-lethal based approach by avoiding density-dependent effects, as discussed in Chapters 3 and 4. The

development of a system orthogonal to a tetracycline-based one would allow both these features to be combined in a single product strain by placing early-acting, female-specific lethality under a different control mechanism to late-acting, bi-sex – or female-specific – lethality.

Development of an alternative genetic control mechanism for dominant-lethal based systems is therefore an important goal of future product development. Moreover, whereas the Tet-Off system relies on repression, during rearing, of a constitutively lethal system, an orthogonal system could be based on induction of a constitutively repressed system. This has some differences, which could offer additional advantages for some applications, summarised in Table 6-1.

Table 6-1: Advantages of developing an orthogonal approach to the Tet-Off system for RIDL

Advantages to developing new genetic control systems for RIDL	
General benefits of an orthogonal system	Benefits specific to an inducible system
Flexibility to develop multiple components of a dominant lethal system, such as genetic sexing and bi-sex lethality, which would be under the control of different systems and therefore activated independently.	Environmental contamination (provided on a small scale) ceases to be a concern, since presence of the inducer would simply re-enforce the lethal phenotype resulting from lack of tetracycline.
Tetracycline is a commonly-used antibiotic: theoretical – though small – possibility of environmental presence from animal feed etc.	The inducing factor would be used only at the point where it is needed, rather than being incorporated into an on-going rearing programme – offering potential cost savings.
Large-scale use of tetracycline over long periods may be considered undesirable due to potential for antibiotic resistance, and implications for human health.	Rapid induction offers the potential for more finely-tuned control over timing of activation.

In the context of use in a RIDL-based pest control approach, there are certain criteria which any novel inducible system would be required to meet. The inducer should be relatively cheap and readily available; neither the inducer itself nor the expressed components of the system should show toxicity to humans or to potential predators; it should preferably have a proven track record of safety for humans and animals. Finally, while less of a concern in the context of an inducible system, minimal levels of environmental presence would be an additional advantage. This last would avoid larval death among transgenic insects in the field, in the case of an early-acting inducer.

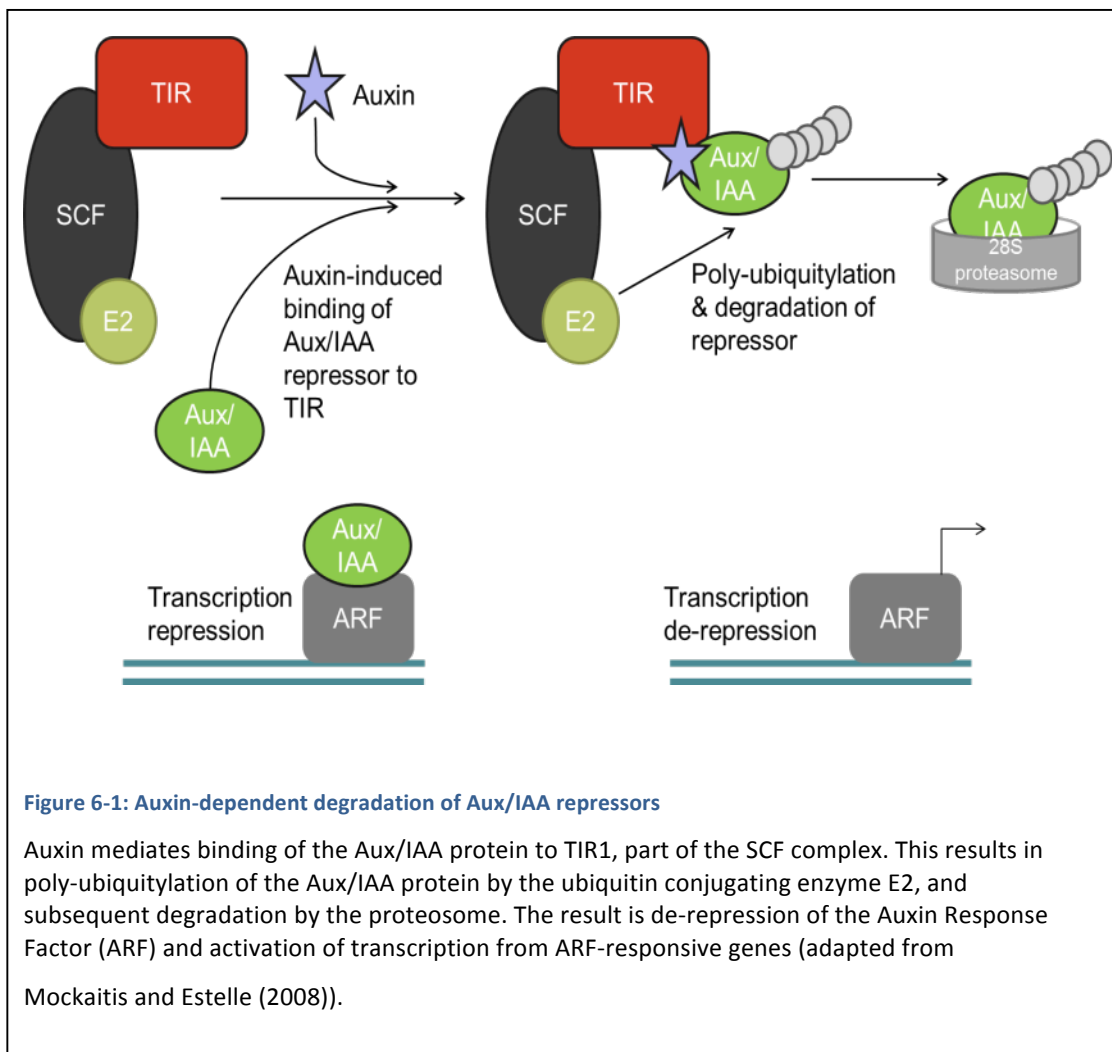
There exist a number of possible approaches which might meet some or all of these criteria. These include small-molecule responsive transcription factors, analogous to the Tet-Off system, such as the vanillic acid system (Gitzinger et al., 2011); binary transcription-factor based systems such as the 'Q' system (Potter et al., 2010); light-activated transcription factors (Motta-Mena et al., 2014); or RNA-based expression control mechanisms, such as 'Aptazymes' (Ketzer et al., 2014).

Hormone-based signalling pathways also offer highly sensitive response mechanisms which have been incorporated into novel transcription activation systems (Nordstrom, 2003). However, the potential for un-desired effects associated with the use of mammalian or insect hormones is likely to render such systems unsuitable.

In contrast, plant signalling pathways, such as auxin-based signalling, may offer the sensitivity and specificity of mammalian and insect hormone-based systems, while being more readily adaptable to a large-scale rearing programme. Auxins are used in a wide variety of herbicides, and as a consequence are cheap, readily available, and have low human toxicity (Park et al., 2011). This, combined with the absence of any auxin-based system (and hence presumed lack of biological activity) in animals, renders them promising candidates for use in an inducible

expression system. Auxin-inducible activation of a transgenic system could therefore be a promising approach to developing an inducible lethal phenotype in insects.

Auxins are plant hormones involved in a wide variety of plant growth and development pathways. They are able to induce rapid changes in gene expression in selected plant tissues through degradation of proteins belonging to the Auxin/Indole-3-acetic acid (Aux/IAA) family (Figure 6-1) (reviewed in Guilfoyle (1999)). The auxin-binding protein Transport Inhibitor Resistant 1 (TIR1) (Dharmasiri et al., 2005, Kepinski and Leyser, 2005) is a member of the F-box protein family. It binds the SCF complex to form the ubiquitin ligase complex E3. TIR1 has been shown to bind Aux/IAA proteins in an Auxin-dependent manner, which results in poly-ubiquitylation and degradation of the Aux/IAA protein by the E3 ubiquitin ligase complex (Tan et al., 2007).



While auxin responsiveness mediated by TIR1 is specific to plants, substrate degradation mediated by F-box protein-SCF complexes is conserved throughout eukaryotic species (Craig and Tyers, 1999). Recently, an auxin-based protein degradation system has been developed which allows for auxin-inducible protein degradation in non-plant cells, based on ectopic expression of the TIR1 protein, which was then shown to specifically degrade an IAA-GFP fusion protein in an auxin dependent manner (Nishimura et al., 2009). The authors demonstrated functionality of this system in a wide variety of non-plant eukaryotic cell lines, suggesting that the system could be transposed to the majority of eukaryotes.

Adaptation of this mechanism, such that auxin-dependent binding of TIR and an IAA family fusion protein could induce gene expression in transgenic insects, has the potential to offer a sensitive, rapidly-inducible system of gene expression. This chapter reports on the

development and testing of three novel, auxin-inducible gene expression systems in transgenic *Aedes aegypti* mosquitoes.

6.2 MATERIALS AND METHODS

6.2.1 STRAIN REARING AND TRANSFORMATION

Ae. aegypti wild-type strains, rearing and transformation were as described in General Methods (Chapter 2).

6.2.2 DNA CONSTRUCTS

Constructs OX4786, OX4787 and OX4797 were made for use in this investigation by Tarig Dafa'alla and Caroline Phillips, Oxitec Ltd.

6.2.3 AUXIN BLOOD FEEDING AND SURVIVAL ASSAY

To administer auxin in a blood meal, 10 µl of a 100X solution of natural auxin (Indole-3-acetic acid – 'IAA') or the synthetic analogue (1-naphthaleneacetic acid – 'NAA') in 100% EtOH was added per 1 ml blood, for a final IAA/NAA concentration of 500 µM. Control specimens received blood meals containing diluent alone (10 µl of 100% EtOH per 1 ml blood).

Cages containing 100 wild-type females and 100 wild-type males were fed on treatment or control blood 3 days post-eclosion. 40 blood-fed females (visually inspected) were placed in separate cages for 48 hours to allow egg development, before being placed in individual entomological tubes containing wetted cotton for egg laying. After 48 hours the number of eggs in each tube was recorded. The first 25 tubes containing eggs from each treatment were selected and eggs hatched overnight using a vacuum chamber (Nalgene, Rochester, N.Y.). Hatched larvae were counted the following day.

To assay survival of the hatched larvae, larvae from females in each treatment were placed in two pools of ~750 individuals at a density of 0.75 larvae/ml (the second pool from the IAA treatment contained only ~500 larvae, which were reared at the same density). Larvae were

fed on crushed dry fish food (TetraMin flake food from Tetra GmbH) administered according to the standard feeding regime described previously (Chapter 2, Methods).

Pupae were picked manually and sexed under a microscope. Males and females from each pool were placed in separate cages and allowed to eclose.

6.2.4 AUXIN LARVAL REARING

To assay the effect of auxin in larval rearing water, wild-type *Ae. aegypti* eggs were hatched and 1000 larvae counted into rearing trays 1 day post-hatch. Auxin (IAA or NAA in 100% EtOH) was then added to rearing water to a final concentration of 50 μ M or 250 μ M. The equivalent volume of EtOH only was added to separate trays as a control treatment. Five repeats were performed for each treatment and the control treatment. Insects were fed on crushed dry fish food (TetraMin flake food from Tetra GmbH) administered according to the standard regime described previously (General Methods). Pupae from each tray were picked, sexed and counted, before being placed in separate cages and allowed to eclose. Following sex separation, a selection of pupae from each treatment was photographed under a light microscope using a Canon PowerShot S5IS camera with an MM99 adaptor (Martin Microscopes, U.K)). Cephalothorax widths were recorded using the Image J analysis software (NIH, USA).

6.2.5 RT-PCR OF AUXIN LINES

Total RNA extraction and cDNA synthesis was performed as described in General Methods (Chapter 2). PCR was then performed on cDNA samples using the primers shown in Table 6-2:

Table 6-2: Summary of primers used in Chapter 6

Primer name	Sequence	Target	Target Length
98)su-amcyanf	5'-CCAGACCTCCACCTTCAAGGTGACC-3'	<i>AmCyan</i> (OX4786)	517 bp
119)Sucyanr2	5'-CACGCTGTTGCCGCCCTTGTCC-3'		
98)su-amcyanf	5'-CCAGACCTCCACCTTCAAGGTGACC-3'	<i>AmCyan</i> (OX4787)	591 bp
1492)AmCy-r2	5'-TTCTTGGGTCGAGATCTGAGTCC-3'		
3108) DiagOsTIR	5'-GCGCTCGATCTCGTACCAGAC-3'	<i>TIR-Gal80</i> fragment (OX4786)	574 bp
3126)	5'-CACGAAGAAGATCCCAGAGCTG-3'		
Seq2Gal80			
3134)	5'-CAGCAGCTGCAAGGACCTG-3'	<i>TIR-Gal4DBD-</i> <i>Ubiquitin</i> fragment (OX4787/OX4797)	853 bp
Seq4OsTIR	5'-GGATGCCCTCCTTGTCCTGG-3'		
1102) Diag2Ubiq			
1431) K10-short-1	5'-TTGCGATTACCAAGTGATTG-3'	<i>Rpr</i> (OX4787)	833 bp
974) Diag-rpkr	5'-CACCGTGGTGCTGGAGACC-3'		
895) AeMuAcR	5'-CCCAGGAAGGATGGCTGGAAGAG-3'	AAEL001928 (Muscle actin)	3141bp (gDNA)
1098)	5'-GGCATTCAATCTAGTTCCGAAACCG-3'		1066 bp (cDNA)
AeMuAcRTF			

6.2.6 ON/OFF AUXIN TESTING OF TRANSGENIC LINES

For survival assays, heterozygous populations were hatched and 100 larvae counted into pots 1-day post hatch. For auxin-treated populations, a solution of NAA in 100% EtOH was then added to rearing water to a final concentration of 100 μ M, unless otherwise specified in the text. Larvae were fed according to the standard feeding regime described previously (General Methods). Pupae were picked and screened before being placed in cages and allowed to eclose. For fluorescence assays, heterozygous populations were reared in the presence or absence of NAA, which was added to rearing water to a final concentration of 100 μ M. Larvae were fed on ground TetraMin food *ad libitum*. Fluorescence was observed using a Leica M80 microscope (Leica Microsystems, U.K.) and images taken using a Canon PowerShot S5IS camera with an MM99 adaptor (Martin Microscopes, U.K)).

6.2.7 SURVIVAL ASSAY OF OX4786 X OX4787 TEST CROSSES

Heterozygous populations of OX4787 lines A and H and OX4786 line I were hatched and transgenic individuals selected based on fluorescence using a Leica M80 microscope. Males from OX4787 lines A and H were crossed with females from OX4786 line I. F1 progeny from these crosses were hatched and reared in hatch pots at 1 larvae/ml on standard diet (General Methods), using 200 larvae per replicate. Insects were reared until pupation, and separated according to fluorescence using a Leica M80 microscope. Pupae were then placed into separate cages and allowed to eclose.

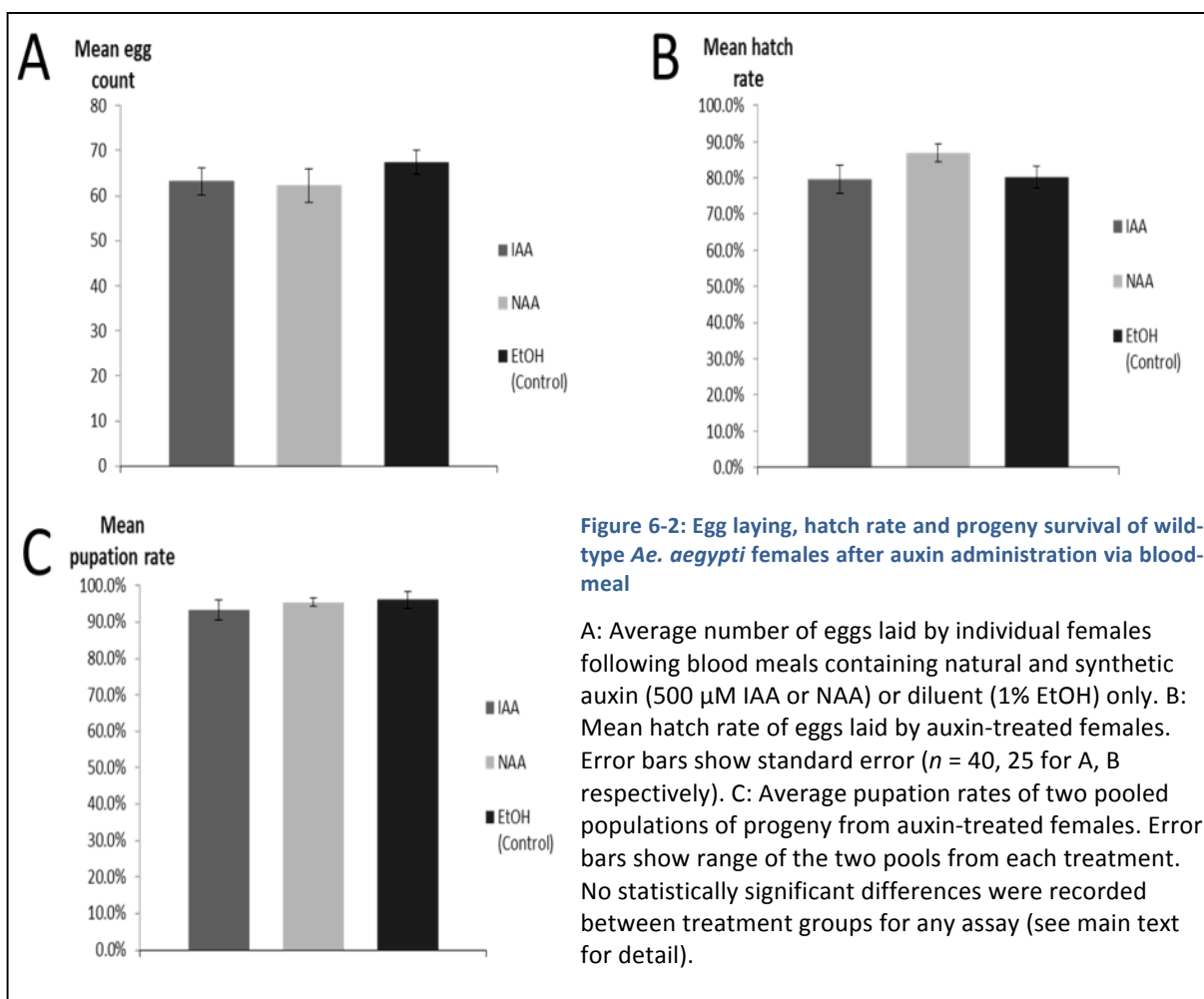
6.2.8 STATISTICAL ANALYSIS

All statistical analysis was performed using the “R” software for statistical analysis (R Development Core Team, 2010), as described in General Methods (Chapter 2).

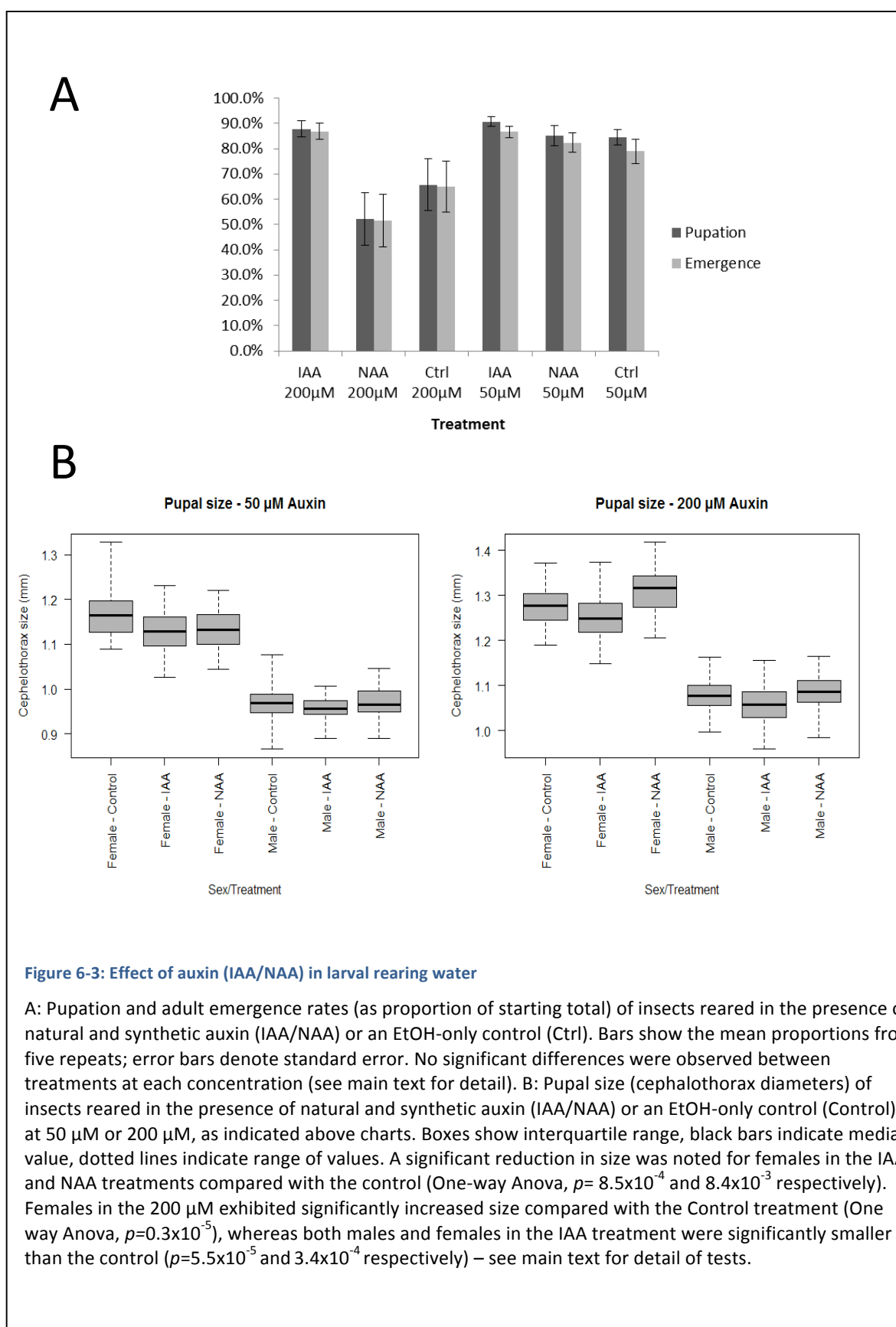
6.3 RESULTS AND DISCUSSION

6.3.1 EVALUATION OF THE EFFECT OF NATURAL AND SYNTHETIC AUXIN ON WILD-TYPE *AEDES AEGYPTI*

A pre-requisite for the use of auxin in a novel gene expression system is a lack of significant toxic effect on mosquitoes during rearing. The effects of two possible delivery mechanisms were assayed: maternal transmission via the blood-meal, and administration to larval rearing water.



Maternal transmission of an inducing factor could allow induction of a genetic control mechanism at the earliest possible stage in development: the developing embryo. To assay for any potential toxicity of this approach using auxin, 500 μ M of the natural auxin (IAA) or its synthetic analogue (NAA) was administered to females via a blood-meal, as described in Methods. No significant effect was observed between treatments on the fecundity of females, as measured by mean egg counts (Kruskal-Wallis rank sum test, $\chi^2 = 2.64$, $p = 0.267$). Similarly, no significant effect was observed on their fertility, as measured by hatch rate of eggs laid (GLM with quasi-binomial error, $t = -0.14$ and 1.57 , $p = 0.89$ and 0.13 for IAA and NAA treatments respectively, vs control). Progeny from treated females were pooled into two rearing trays per treatment to assay pupation; hence, no statistical calculations were performed. However, there did not appear to be any obvious impact of the differently treated blood on pupation rates of resulting progeny (Figure 6-2).



To test an alternative delivery approach for the auxin-inducing factor, auxin (IAA or NAA) was added to larval rearing water 1 day post-hatch, as described in Methods (Figure 6-3). No significant difference was found in pupation/emergence rates between treatment groups and control (GLM for emergence rates by treatment, with quasi-binomial error: at 50 μ M, $t = 1.11$ and 1.35 , $p = 0.28$ and 0.20 for IAA and NAA respectively, vs control; at 200 μ M, $t = 0.83$ and -1.11 , $p = 0.42$ and 0.28 for IAA and NAA respectively, vs control). It should be noted, however, that at 200 μ M, effects may have been masked by large amounts of variation in the EtOH control and NAA treatments, with some repeats showing very high levels of mortality. In the 200 μ M treatment, EtOH was added to rearing water to a volume of 1%. This appeared to impact on the survival of larvae, with pupation rates in the EtOH control treatment averaging 65%. Toxicity of the EtOH solvent therefore appears to be a limiting factor in obtaining auxin concentrations in rearing water beyond 200 μ M (though it is notable that the presence of IAA, rather than NAA, appeared to have a protective effect). Nonetheless, the auxin-induced degradation system has been shown to be capable of a 95% reduction in levels of the target protein at just 20 μ M auxin (Nishimura et al., 2009), hence this level of toxicity is not expected to be a limitation on the ability to induce the system in transgenic insects.

An assay for pupal size revealed a significant reduction in the size of female pupae reared on 50 μ M IAA/NAA with respect to the control treatment (one-way ANOVA on $-1/x$ transformed data, followed by Tukey-HSD post-hoc tests; $p = 0.00085$ and 0.0086 for IAA vs Ctrl and NAA vs Ctrl, respectively), whereas no significant difference was recorded between male populations (one-way ANOVA followed by Tukey-HSD post-hoc test; $p = 0.24$ and 0.85 for IAA vs Ctrl and NAA vs Ctrl, respectively). However, although statistically significant, these differences did not appear to be large; moreover, the findings stood in contradistinction to results at 200 μ M treatment: at 200 μ M, a small, but significant increase in pupal size was observed for females reared on NAA (One-way ANOVA followed by Tukey-HSD post-hoc test; $p = 0.3 \times 10^{-5}$ for NAA vs

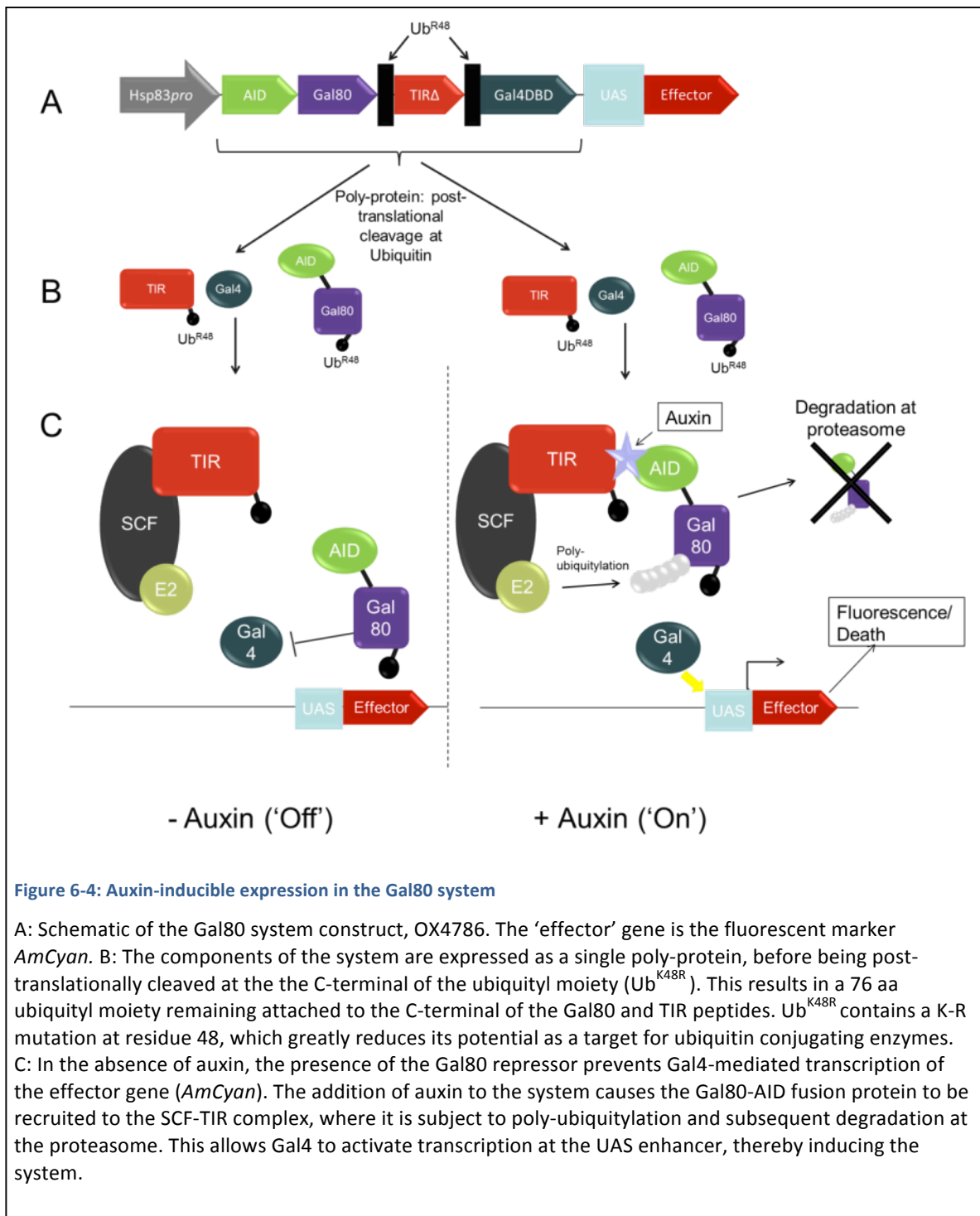
Ctrl), whereas both males and females reared on IAA were significantly smaller than those of the control treatment (One-way ANOVA followed by Tukey-HSD post-hoc test; $p = 5.5 \times 10^{-5}$, $p = 3.4 \times 10^{-4}$ for IAA treated males and females, respectively, vs control).

It was necessary at this stage to determine whether or not gross fitness effects resulted from auxin treatment. In this context, it is important to bear in mind the distinction between observations of statistical significance, and those of *biological* significance; i.e. – for the purposes of this study – the gross effects which would be of concern in designing an auxin-regulated expression system. Indeed, given the size of the effects observed, despite some statistically significant differences in measured pupal sizes, it was considered unlikely that these results represented a major impact on fitness.

6.3.2 CONSTRUCTS FOR AUXIN-INDUCIBLE EXPRESSION

As noted at the beginning of this chapter, the design and synthesis of all constructs used in this chapter was the work of the Oxitec molecular team (led by Tarig Dafa'alla, Oxitec Ltd).

To develop an auxin-inducible expression system, two contrasting approaches were pursued, both of which were based on auxin-dependant induction of a Gal4-UAS system. The first approach (referred to henceforth as 'the Gal80 system') used the canonical auxin-induced degradation pathway to mediate degradation of the transcriptional repressor Gal80 (Lue et al., 1987), thereby inducing the Gal4/UAS expression system (Figure 6-4).



A fusion protein was synthesised consisting of Gal80 fused to an IAA-family peptide (henceforth referred to, after Nisimura *et al*, as the Auxin-inducible degron – 'AID') which was expected to bind TIR in the presence of auxin and subsequently be degraded, thereby removing the Gal80 repressor.

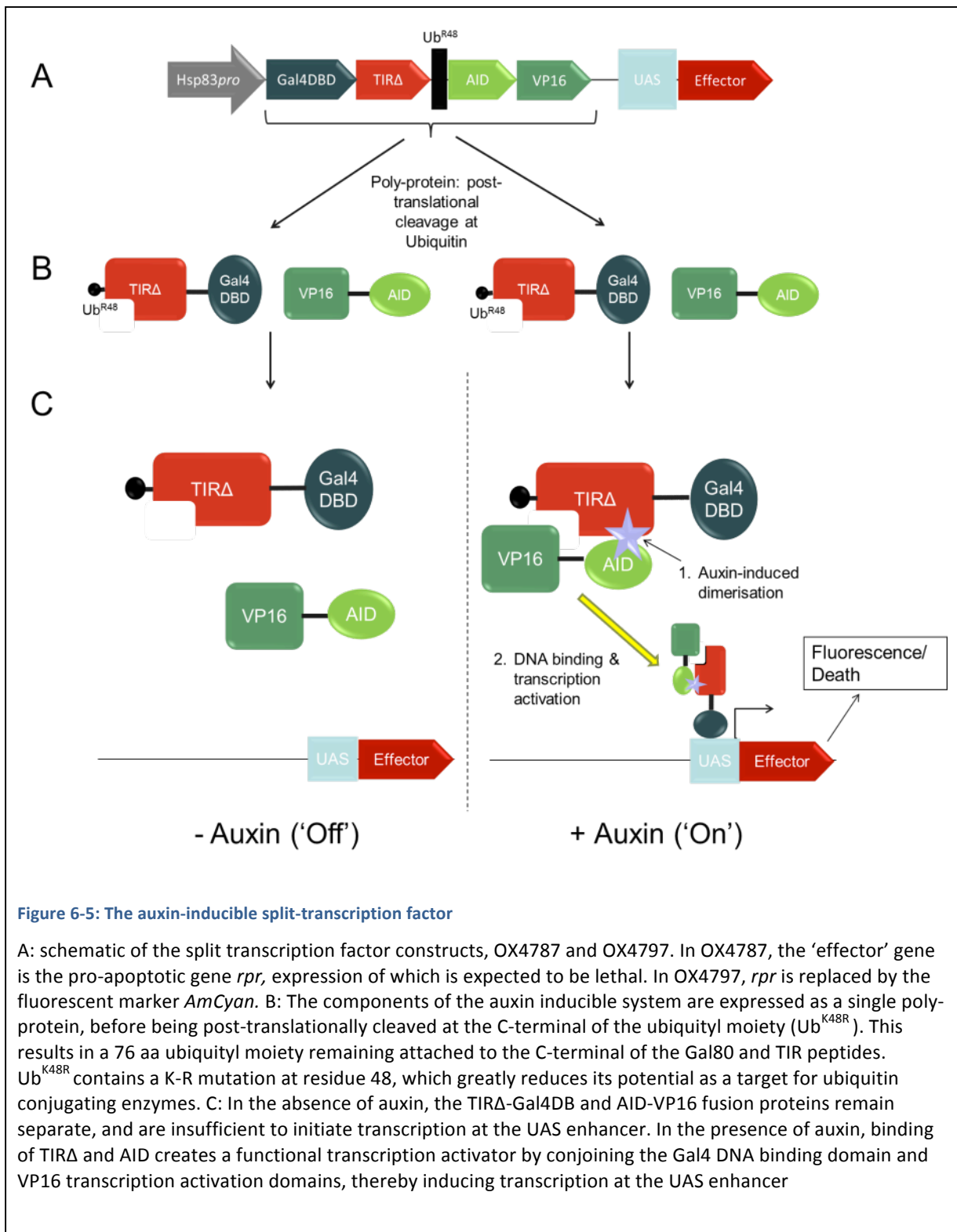
The second approach (referred to henceforth as the split-transcription factor system) employs a modified TIR protein in which the SCF binding F-box domain had been removed (Figure 6-5). Structural and biochemical studies on the human Skp2 F-box protein and the yeast F-box protein Cdc4p have indicated that the F-box region is critical for SCF binding, with point mutations in helix 1 of the F-box domain being sufficient to ablate SCF binding (Bai et al., 1996, Schulman et al., 2000). Furthermore, structural analysis of the TIR protein shows the F-box domain as comprising a small – ~40 aa – region at the N-terminus of the protein, with little apparent interaction with the auxin-binding domain (Tan et al., 2007). It was therefore expected that F-box deletion would un-tether TIR from the SCF-ubiquitin ligase complex, thereby enabling auxin-induced binding of TIR to an AID-fusion protein, without subsequent ubiquitylation and degradation of that protein.

Two chimeric proteins were then synthesised by fusing the modified TIR protein (TIR Δ) to the DNA-binding domain of Gal4 (Gal4DBD), and by fusing the transcription activation domain of the transcriptional activator VP16 to an AID peptide. A modular transcriptional activator was thereby produced, designed to be dependent on auxin-induced binding of the Gal4DBD-TIR Δ and AID-VP16 fusion proteins to activate transcription from a UAS enhancer (Figure 6-5).

To test the Gal80 system in transgenic insects, construct OX4786 was made (Figure 6-4 – A). The construct incorporates the AID-Gal80 fusion protein, the auxin-binding TIR protein, the transcriptional activator Gal4 and a minimal promoter-UAS enhancer driving the fluorescent protein AmCyan. It was expected that transgenic insects carrying this construct would not express AmCyan under non-induced conditions, since Gal80 would block Gal4-mediated transcription activation from the UAS element. In the presence of auxin, it was expected that the Gal80-AID fusion protein would bind TIR and be degraded, thus enabling transcription of AmCyan.

To test the split transcription factor system, constructs OX4787 and OX4797 were made (Figure 6-5 – A). Each construct incorporates the Gal4 DNA-binding domain (Gal4DBD) -TIRΔ fusion protein, and the AID-VP16 fusion protein, together with a UAS enhancer driving expression of an effector – either the fluorescent marker *AmCyan* (OX4797), or the pro-apoptotic gene *reaper* (*rpr*) (OX4787). In each case, it was expected that transcription of the effector, *AmCyan* or *rpr*, would be dependent on the presence of auxin, which would induce binding of the two fusion proteins and hence VP16-mediated transcription activation from the UAS enhancer.

In order to ensure high, as well as stoichiometric, levels of transgene expression, the various components of the systems described were expressed as a single poly-protein, under the control of the hsp83 promoter. Separate proteins of interest were obtained using the ubiquitin fusion approach. Here, a 76 aa ubiquityl moiety is used to conjugate two proteins of interest. The ubiquityl moiety is subjected co-translational cleavage after its C-terminal residue by endogenous de-ubiquitylating enzymes (DUBs) (Bachmair et al., 1986). This liberates free-form N-terminal protein, but results in a ubiquityl moiety remaining attached to the C-terminal protein (TIR and Gal80 in the Gal80 system, TIRΔ in the split-transcription factor system). While such moieties can serve as targets for ubiquitin-conjugating enzymes (and hence have reduced stability), the ubiquityl moiety used here contains a lysine to arginine mutation at lysine 48 (Ub^{K48R}), a key locus for the formation of isopeptide bonds in the formation of poly-ubiquitin chains. This increases the stability of the resulting protein-Ub fusion (Suzuki and Varshavsky, 1999). However, see Conclusions for a fuller consideration of the possible impact of this design on the functionality of the resulting proteins.



6.3.3 TRANSFORMATION AND CHARACTERISATION OF AUXIN-INDUCIBLE LINES

To assess the activity of a putative auxin-inducible system *in vivo*, wild-type *Ae. aegypti* embryos were injected with constructs OX4786, OX4787 and OX4797. Survival and transformation rates are summarised in Table 6-3. Three transgenic lines were established carrying construct OX4786 (Gal80 system), labelled OX4786A, I and J respectively. Four lines were established carrying construct OX4797 (split transcription factor - *AmCyan*), labelled OX4797F, G, H and T. Ten lines were established carrying construct OX4787 (split transcription factor - *rpr*), of which four (OX4787A, F, H, N) were used in downstream analysis.

Table 6-3: Injection data for auxin construct

Construct	Eggs injected	G0 survival (to adult)	Positive G1 pools
OX4786	1500	44	3
OX4787	1250	154	10
OX4797	1574	107	4

6.3.4 PHENOTYPE OF OX4786 LINES

Construct OX4786 (Gal80 system) was designed as a putative auxin-inducible system in which the presence of auxin would trigger expression of the AmCyan fluorescent protein. It was therefore expected that transgenic lines carrying the OX4786 construct would display AmCyan fluorescence in the presence of auxin, but not in its absence. The auxin analogue NAA has been shown to be more stable than IAA (Dunlap et al., 1986), and might therefore prove a more effective inducer. Accordingly, heterozygous populations from lines OX4786A, I and J were reared in the presence or absence of 100 μ M NAA. Transgenic individuals from lines 4786A and I were found to exhibit strong all-body AmCyan fluorescence, but this occurred both in the presence and absence of auxin. In contrast, transgenic individuals from line 4786J showed no detectable AmCyan fluorescence, whether reared in the presence or absence of auxin (Figure 6-6).

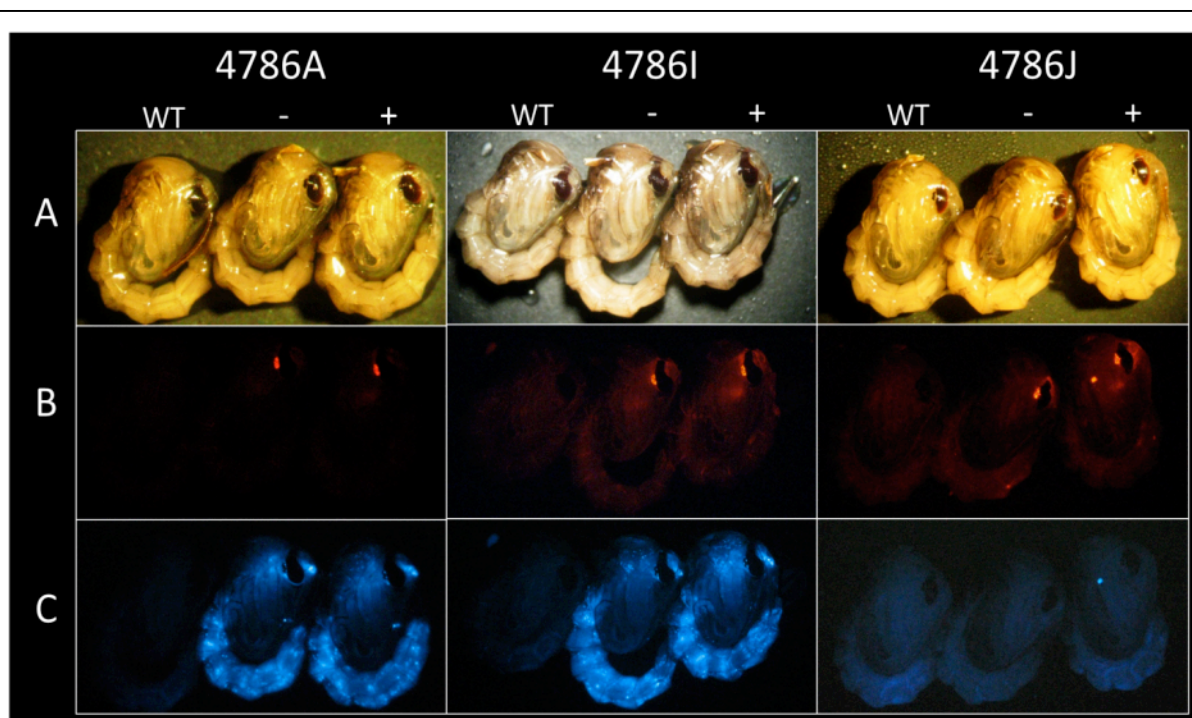


Figure 6-6: Fluorescence imaging of 4786 transgenic pupae

Images are of individuals from OX4786 lines A, I and J, as indicated. Each image shows a representative transgenic individual reared in the absence (-) or presence (+) of 100 uM NAA, alongside a wild-type individual (WT). A=Bright field; B=Red filter, showing 3xP3-DsRed transformation marker; C=Blue filter

To further assay for any auxin-inducible expression of the AmCyan marker, RT-PCRs were performed on samples of transgenic pupae reared in the presence or absence of NAA. As expected, the TIR-Gal80 fusion protein transcript, constitutively expressed under the control of the *hsp83* promoter, was detected in all pupae samples. This indicated that the non-induced components of the auxin-inducible system were being expressed both in the presence and absence of NAA. However, none of the lines showed any detectable difference in the level of *AmCyan* transcript in samples reared in the presence or absence of NAA. Although the approach used was not strictly quantitative, this result was in accordance with the observed fluorescence of transgenic individuals: together, the results indicate that *AmCyan* expression was not induced in the presence of NAA.

Of particular note was the clear difference in fluorescence phenotype between lines A and I, which showed strong AmCyan fluorescence, and Line J, which did not. Although only a semi-

quantitative assay, the RT-PCRs shown in Figure 6-7 do not appear to indicate any clear differences in the strength of constitutive gene expression (the *Gal80-TIR* amplicon) driven by the *hsp83* promoter. However, the level of *AmCyan* expression in line J appears lower than that in lines A and I, as might be expected from the fluorescence phenotypes (though the presence of a smaller, non-specific amplicon in the RT-PCR for line J makes these results difficult to interpret with confidence). Since the *UAS-AmCyan* cassette lies close to the 3' arm of the DNA insert, it is possible that it might be more susceptible to the influence of enhancer elements in the surrounding chromosomal DNA. Nonetheless, there is no clear evidence to suggest that the differences in fluorescence observed between OX4787 lines relate merely to variation in the overall strength of transgene expression, due to positional effects.

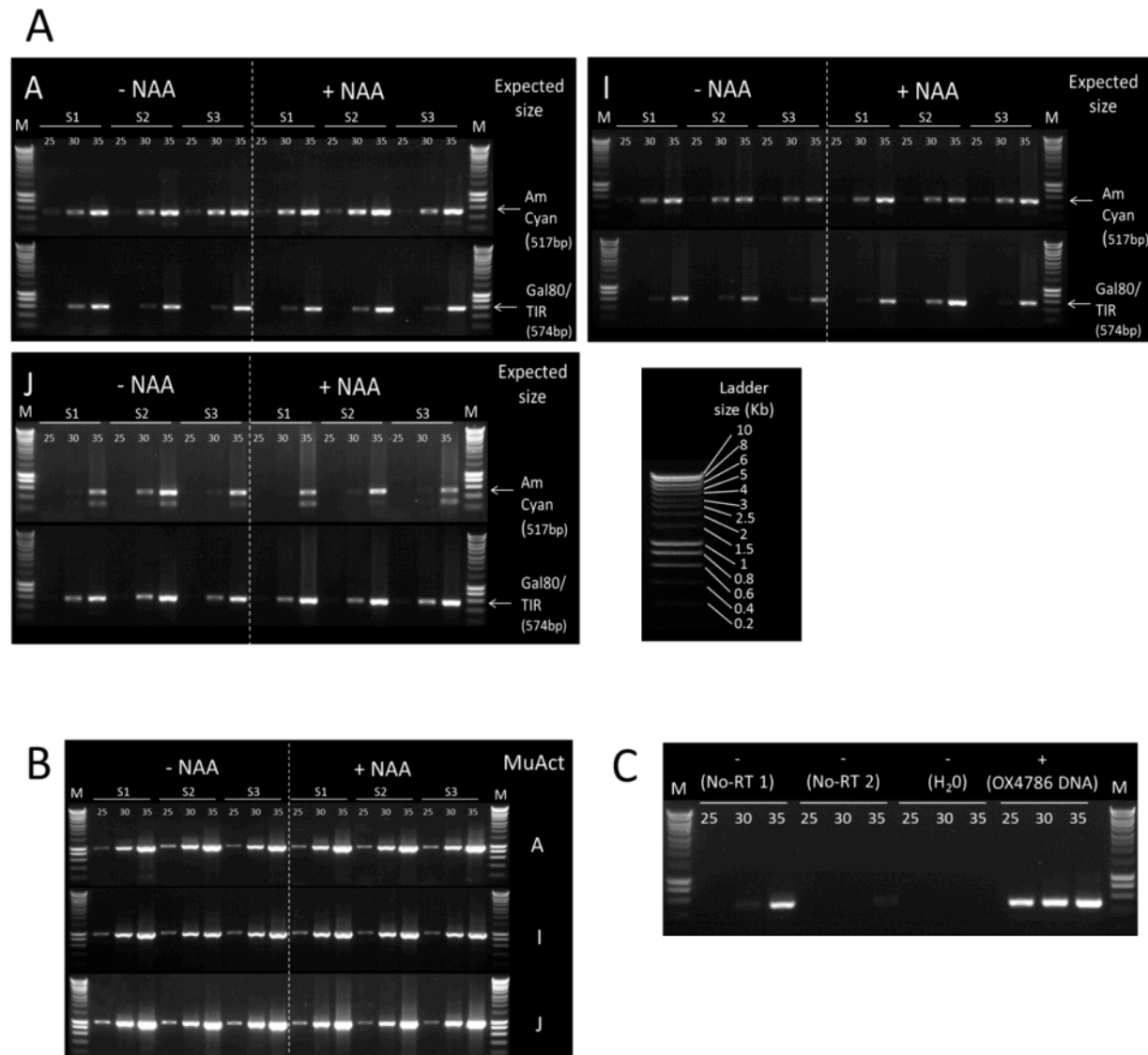


Figure 6-7: RT-PCR of OX4786 (Gal80 system) transgenic lines

A: RT-PCR was performed on pupal samples from OX4787 lines A, I and J, as indicated in each panel. Each sample consisted of 3 pooled transgenic male pupae, reared in the presence or absence of NAA (+NAA/-NAA, as indicated above panels). PCR was performed on 3 samples for each line/treatment (S1, S2, S3), using primers directed to AmCyan or the TIR-Gal80 fusion transcript (see Table 6-2 for description of primers used). The sizes of expected amplification products are indicated to the right of each gel. To provide a semi-quantitative assay, samples were removed from PCR reactions after 25, 30 and 35 cycles, as indicated above each lane. M = SmartLadder molecular weight marker (Eurogene). Sizes of bands in the marker ladder are shown at right of panels. B: PCR amplifying a region of AAEL001928 (muscle actin) – an endogenous control gene. C: positive and negative control PCRs (No-RT = no reverse transcriptase added). The presence of a positive AmCyan band in the first no-RT control (and very faintly in the second), indicates that some of the cDNA samples may have had gDNA contamination. However, gDNA should have resulted in a larger (3 kb) band in the endogenous control PCRs. Since no such band was detected, it was assumed that the level of contamination was low and therefore unlikely to affect results.

6.3.5 PHENOTYPE OF OX4787 AND OX4797 LINES

Constructs OX4787 and OX4797 both comprised a split-transcription factor auxin-inducible expression system, in which the presence of auxin would trigger the expression of a downstream effector (see Figure 6-5): in OX4787 this effector was the pro-apoptotic protein Reaper, which was expected to cause lethality when expressed; in OX4797 the effector gene was the fluorescent protein AmCyan. It was therefore expected that transgenic insects carrying the OX4787 construct would die in the presence of auxin, whereas transgenic individuals carrying the OX4797 construct would exhibit AmCyan fluorescence.

Accordingly, heterozygous populations from lines OX4797F, G, H and T were reared in the presence or absence of 100 μ M NAA. Transgenic pupae from each pool were then examined under a fluorescence microscope. Varying amounts of low-level AmCyan fluorescence could be observed in samples from both treatments for line H. However, no consistent difference was observed in the level of AmCyan fluorescence exhibited by pupae reared in the presence or absence of NAA, in any of the four lines (Figure 6-8).

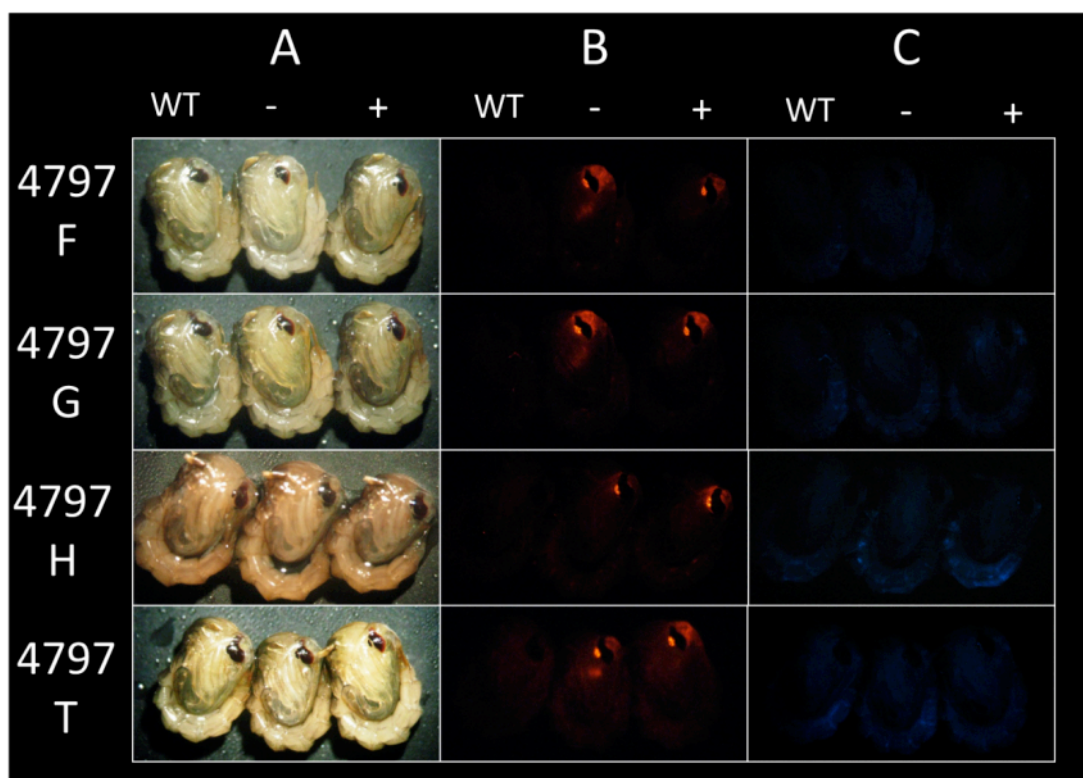
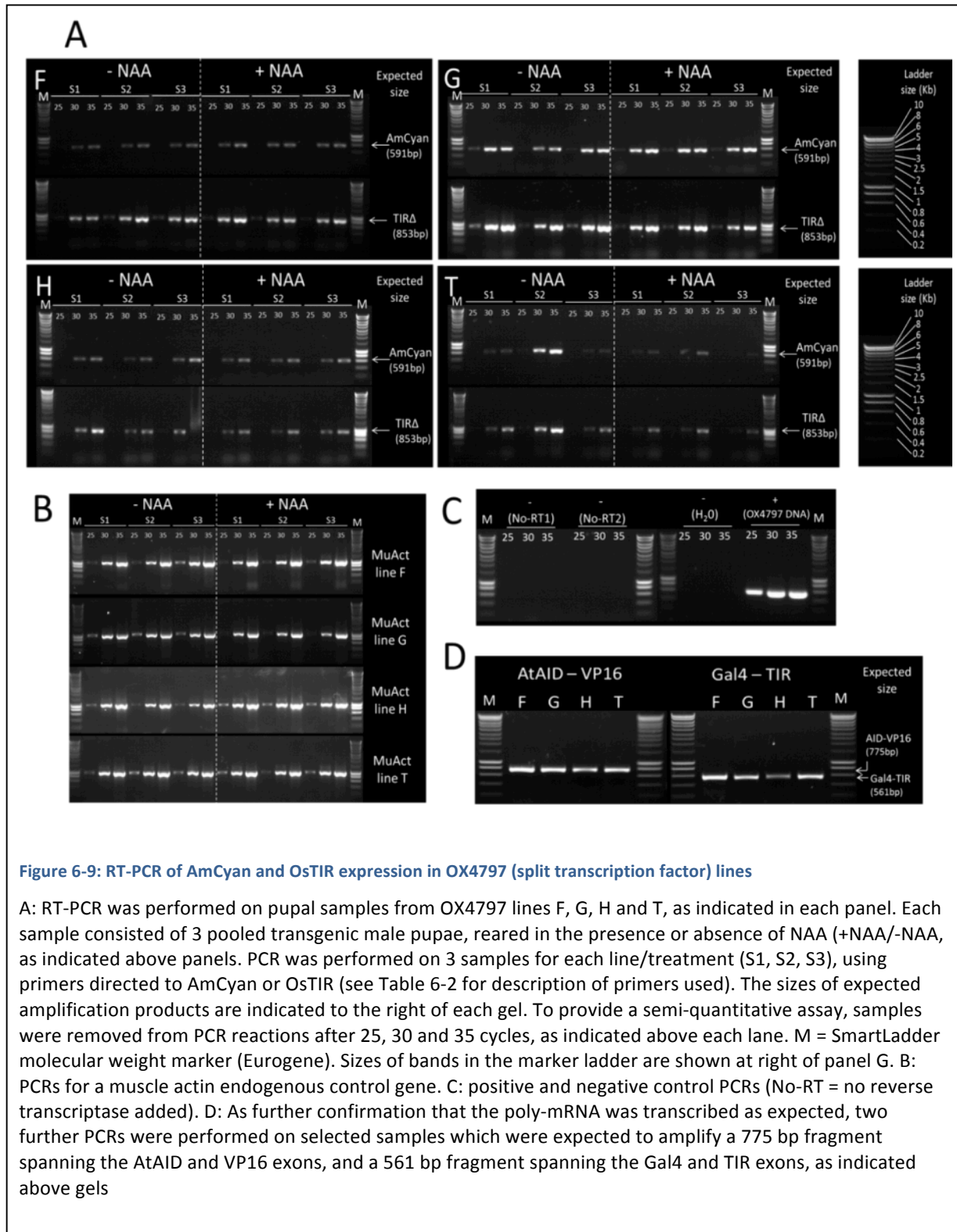


Figure 6-8: Fluorescence imaging of OX4797 transgenic pupae

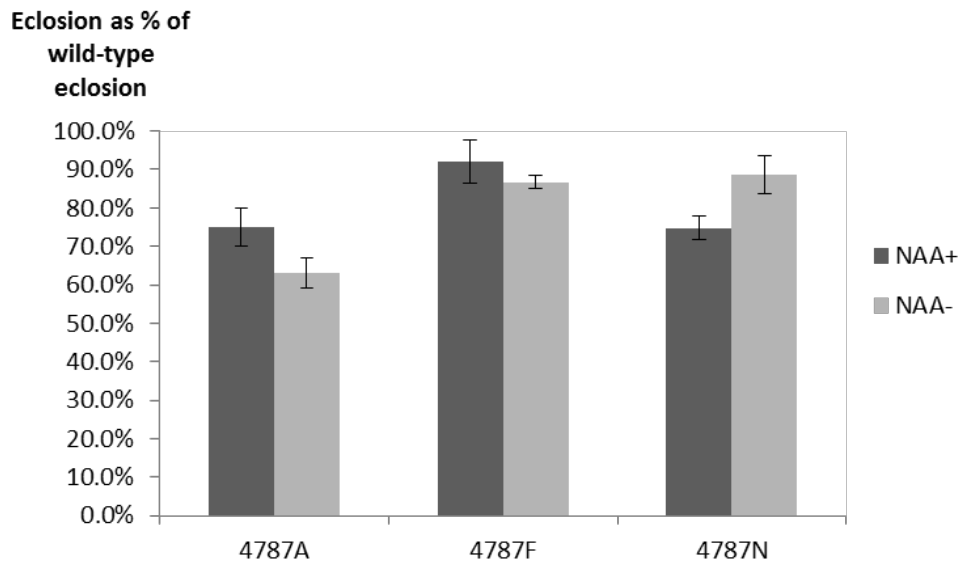
Images are of individuals from OX4797 lines F, G, H and T, as indicated. Each image shows a representative transgenic individual reared in the absence (-) or presence (+) of 100 μ M NAA, alongside a wild-type individual (WT). Panel A=Bright field ; panel B=Red filter, showing 3xP3-DsRed transformation marker; panel C=Blue filter.

As a further assay for *AmCyan* expression in OX4797 lines, RT-PCRs were performed on samples reared in the presence or absence of 100 μ M NAA. Expression of the *TIRΔ* transcript, which was constitutively expressed under the control of the Hsp83 promoter, was detected in all samples, regardless of treatment. Expression of the *AmCyan* marker, which should have been induced in samples reared in the presence of NAA, was also detected in all samples. However, expression levels did not appear to vary between treatments (Figure 6-9).



Lines containing construct OX4787 were expected to show auxin-inducible expression of the pro-apoptotic gene *reaper* (*rpr*), and hence exhibit a lethal phenotype in the presence of auxin. Heterozygous populations from lines OX4787A, F, H and N were therefore reared in the presence or absence of the auxin analogue NAA. Lines A, F and N were tested in the presence of 100 μ M NAA, which was added to rearing water 1 day post-hatching, as in previous assays (see Methods). In an effort to expose larvae to a higher dose of auxin, a heterozygous population from line OX4787H was hatched and reared in the presence of 250 μ M NAA. However, a high level of mortality was observed in both transgenic and wild-type individuals subjected to this treatment. Of the four lines tested, none showed the expected auxin-inducible lethal phenotype (Figure 6-10).

A



B

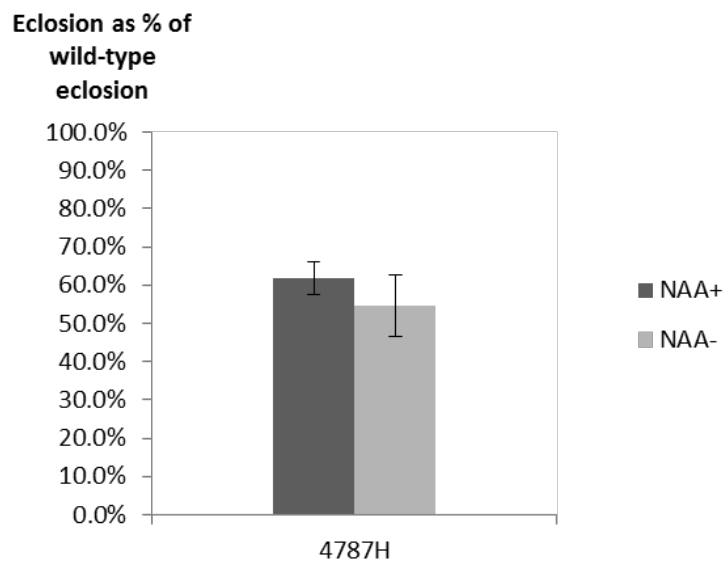
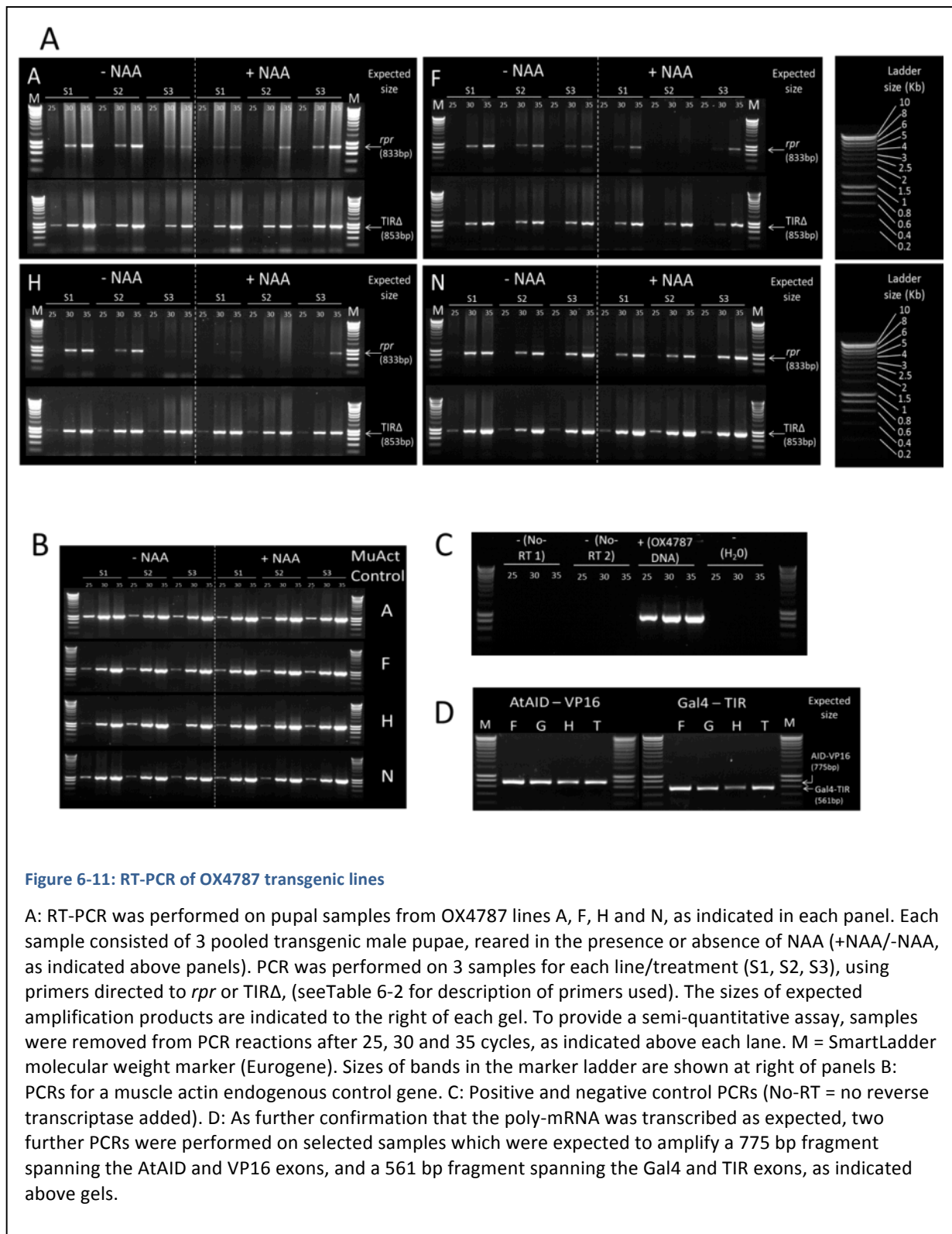


Figure 6-10: Pupation and survival of OX4787 lines reared in the presence or absence of NAA

A: Heterozygous populations from lines OX4787 A, F and N were reared in the presence (NAA+) or absence (NAA-) of 100 μ M NAA. Bars show the mean eclosion (of five repeats) of transgenic insects, normalised by wild-type eclosion. Error bars show standard error. B: A heterozygous population from line OX4787H was reared in the (NAA+) or absence (NAA-) of 250 μ M NAA. Bars show the mean eclosion (of three repeats) of transgenic insects, normalised by wild-type eclosion. Error bars show standard error. No significant differences were found between eclosion rates in the presence or absence of NAA for lines F, N and H (Anova on GLM models following stepwise removal of factors; $p=0.19, 0.06, 0.81$ respectively). Line A showed a marginally significant increase in eclosion in the presence of NAA ($p=0.03$). See main text for detail.



Transgenic insects from all lines, in particular lines A and H, exhibited a higher level of mortality than that seen for wild-type insects. However, this occurred both in the presence and absence of NAA: no significant differences were found in the survival to eclosion of NAA-treated and non-treated insects for lines F, N or H, whereas line A in fact showed a small but significant increase in survival in the presence of auxin (One-way ANOVA on GLM with quasi-binomial error, following Henderson-Tilton mortality adjustment: $p(F) = 0.03, 0.19, 0.06, 0.81$ for lines A, F, N and H respectively).

To further assay for auxin-induced expression of the *rpr*, RT-PCRs were performed on pupae samples from NAA-treated and non-treated populations for each OX4787 line. As was the case for both OX4786 and OX4797 lines, a semi-quantitative RT-PCR revealed no apparent differences in the level of *rpr* expression exhibited by NAA-treated samples in comparison to non-treated samples (Figure 6-11). Some *rpr* expression is evident in all lines both in the presence and absence of auxin, which is consistent with the lethality observed in Figure 6-10.

In summary, neither of the two split transcription factor constructs appeared capable of conferring auxin-inducible expression in transgenic insects.

6.3.6 CROSSES BETWEEN OX4787 AND OX4786 INDICATE FUNCTIONAL GAL4/UAS ELEMENTS IN BOTH CONSTRUCTS

The auxin-inducible systems described have a complicated architecture. As a result, it is difficult to ascertain which elements were not functioning as expected, to result in the observed lack of function of each system.

One element in each system more amenable to direct testing was the Gal4/UAS expression system, whereby a de-repressed Gal4 (Gal80 system) or a chimeric transcription factor containing the Gal4 DNA binding domain (split transcription factor system), was expected to induce expression of an effector through binding the *UAS* enhancer element. In each system,

as discussed above, activation of the *UAS* enhancer and expression of the downstream effector gene was expected only in the presence of auxin. One possible explanation for the strong AmCyan fluorescence in OX4786 lines A and H (Gal80 system, *AmCyan* effector) is therefore that Gal4 is active in these lines, and was driving *AmCyan* expression via the *UAS* element.

To test this hypothesis, heterozygous individuals from OX4786 line H were crossed to heterozygous individuals from OX4787 lines A and H (split transcription factor system, *rpr* effector). The OX4787 construct also contained a *UAS* enhancer element, which was expected to lead to expression of the pro-apoptotic gene *rpr* in the presence of a functional Gal 4 transcriptional activator. Hence, if functional Gal4 was indeed being expressed in OX4786 transgenic insects, the presence of both OX4786 (*UAS-rpr*) and OX4787 (*Hsp83-Gal4*) alleles in F1 progeny was expected to be a lethal condition. This would also assay for the presence of a functional *UAS-rpr* cassette in the OX4787 lines tested. A schematic for this assay, illustrating the expected outcomes, is shown in Figure 6-12. As noted in Figure 6-12 (B), a complicating factor is the shared transformation marker resulting in red-eye fluorescence in insects carrying either the OX4786 construct or OX4787. Lethality in F1 progeny inheriting both constructs could therefore only be assessed as a displacement from the expected ratio of F1 progeny exhibiting the blue body/red eye fluorescence: this phenotype would characterise both individuals carrying both constructs and those carrying the OX4786 construct alone.

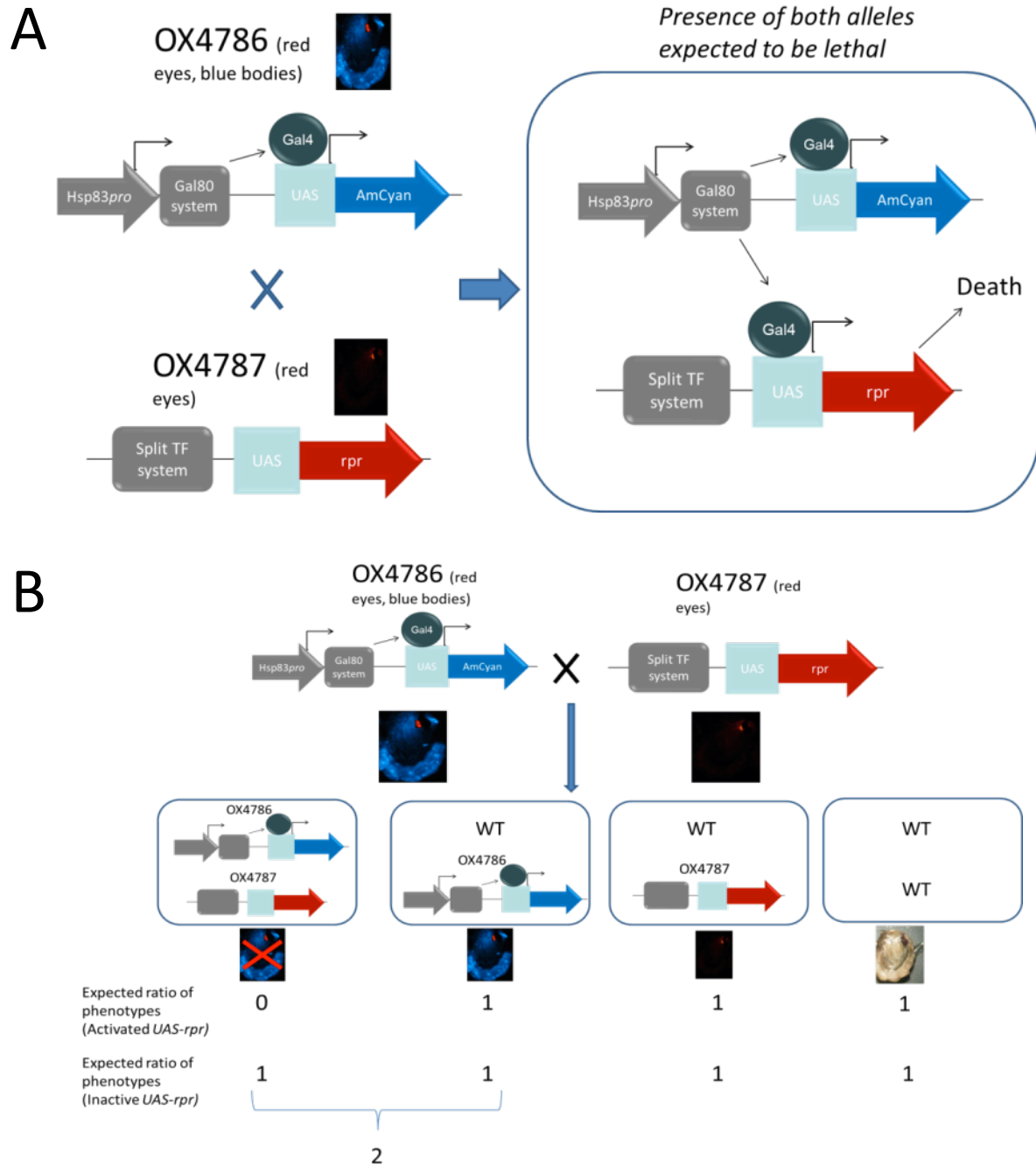
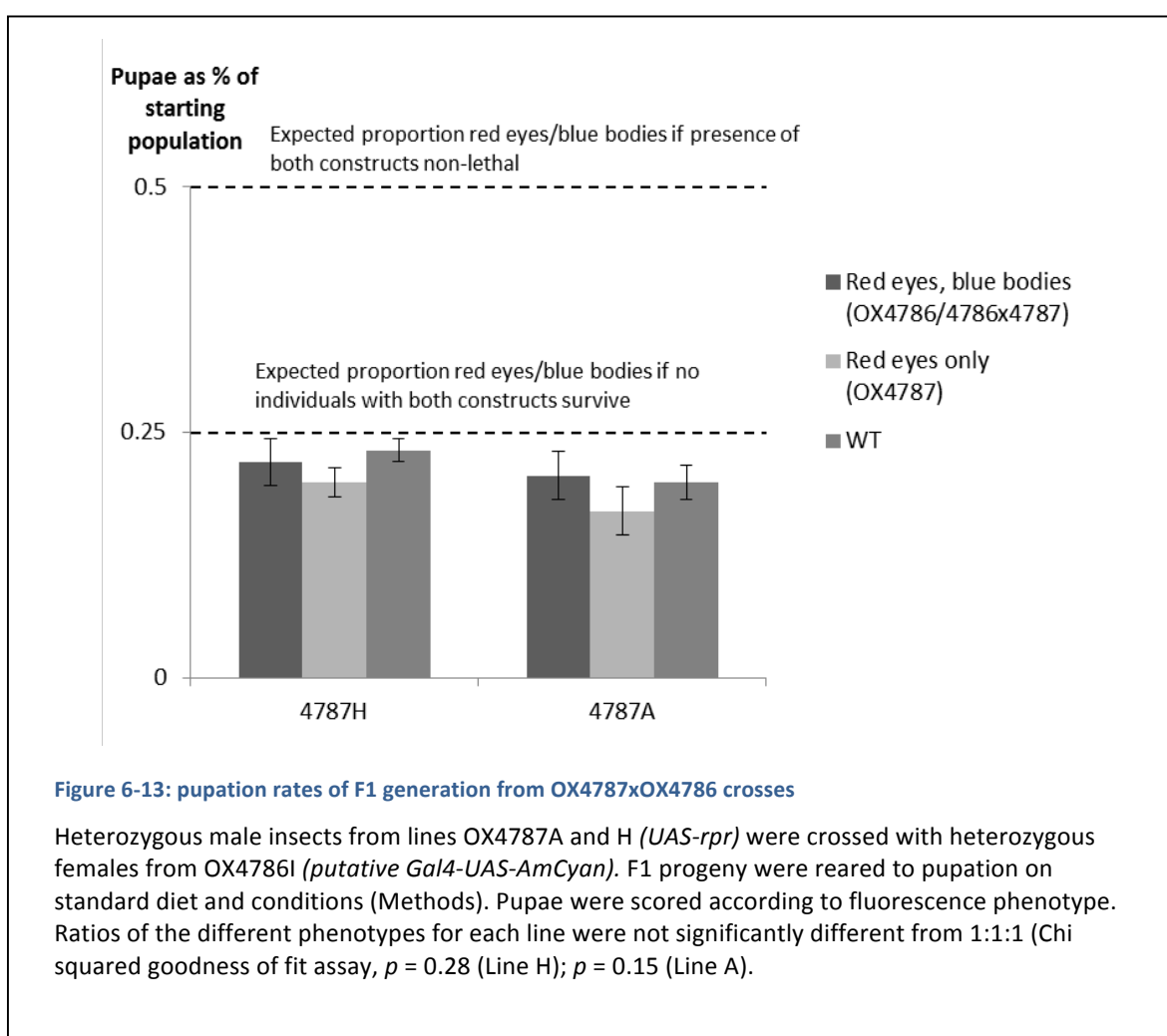


Figure 6-12: Schematic of expected outcomes from OX4786 x OX4787 cross

A: Construct OX4786 was designed such that Gal4 activity would be suppressed in the absence of auxin by co-expression of the Gal4 repressor, Gal80, as discussed above. However, the observed non-induced expression of *AmCyan* in OX4786 lines A and I could result from active Gal4 binding the UAS enhancer as shown. When crossed to OX4787 lines containing a UAS-*rpr* cassette, this Gal4 would also activate *rpr* expression, resulting in lethality in insects carrying both constructs. B: expected observations resulting from OX4786 x OX4787 crosses. Since both constructs have red eye markers, the fluorescence phenotype exhibited by insects carrying both constructs is expected to be identical to that of insects carrying the 4786 construct only – i.e. red eyes and blue bodies. Hence, if inheritance of both constructs proves a lethal condition as expected, the three possible phenotypes (red eyes, blue bodies; red eyes only; wild-type) will be present in equal proportions in F1 progeny, as shown. A non-lethal phenotype would result in twice the number of F1 progeny exhibiting red eyes and blue bodies as either of the other possible phenotypes (H^0).

Counts of pupae in the F1 generation resulting from the OX4787 x OX4786 crosses indicated that no individuals carrying both constructs survived to pupation, resulting in the expected 1:1:1 ratio of the three different possible phenotypes (Figure 6-13). For crosses with each of the OX4787 lines (A and H), the ratio of phenotypes (red eyes, blue bodies; red eyes only; wild-type) was found not to be significantly different from the 1:1:1 ratio expected (Chi squared goodness of fit assay, $\chi^2 = 2.57$, $p = 0.28$ (Line H); $\chi^2 = 3.80$, $p = 0.15$ (Line A)).



These results indicated that active Gal4 was indeed present in OX4786 line I, where it was able to bind the UAS enhancer to activate expression of *AmCyan* and, in trans, activate expression of the lethal *rpr* gene in insects also carrying a *UAS-rpr* cassette. Furthermore, the results indicate that, in the presence of a functional transcriptional activator, *rpr* expression can be induced in OX4787 lines via the UAS enhancer.

6.4 CONCLUSIONS: IMPLICATIONS OF THIS STUDY FOR FUTURE DEVELOPMENT OF NOVEL INDUCIBLE SYSTEMS

Development of an inducible expression system would be a major step towards establishing an improved second-generation dominant lethal system for mosquito population control. An orthogonal mechanism to the tetracycline expression system would enable a late-acting bi-sex lethal system to be combined with earlier acting, female-specific lethality. This would add to the efficiency of late-acting lethality a mechanism for facile removal of unwanted females from the release generation. An inducible (rather than repressible) mechanism would moreover facilitate finely-tuned activation of such a system.

The auxin-dependent protein degradation system developed by Nishimura, Fukagawa *et al* (2009) offered the potential to develop a highly sensitive and rapidly-induced gene expression mechanism, which would meet the requirements of safety, cost and specificity outlined in the introduction to this chapter. Development of an inducible system is a key goal for Oxitec Ltd. As a highly business-driven research programme – albeit one at a very early stage – the project was conceived as a fully collaborative one, wherein the Oxitec molecular team led on the design and synthesis of DNA constructs to establish an auxin-inducible expression system in *Aedes* mosquitoes. The aim of the work presented here was therefore to evaluate fully the *in vivo* efficacy of these constructs and, if necessary, to derive recommendations for Oxitec pertaining to the development of inducible systems going forward, whether based on auxins or alternative mechanisms.

6.4.1 AUXINS DO NOT APPEAR TO BE TOXIC TO WILD-TYPE MOSQUITOES

Results reported in this chapter demonstrate that auxins can be administered to wild-type *Ae. aegypti* mosquitoes either via blood meal or in larval rearing water, apparently without major effects across the survival parameters measured. Although the toxicity of the EtOH solvent was

found to be a limiting factor in achieving very high concentrations of auxin in larval rearing water, no apparent toxicity was observed at 50 μ M, which is 2.5 times greater than the concentration found by Nishimura *et al* to result in 95% reduction of the target degran.

6.4.2 THE AUXIN-BASED SYSTEMS TESTED DO NOT CONFER INDUCIBLE GENE EXPRESSION IN TRANSGENIC INSECTS

Two putative auxin-dependent systems are described in this chapter: the degradation-dependent 'Gal80 system' and a split transcription factor system. Both systems are based upon auxin dependent binding of the TIR protein and an auxin-inducible degran (AID) peptide. However none of the constructs designed to test these systems appeared to confer the expected auxin-induced gene expression in transformed insects. In all lines tested, RT-PCR results showed that at least some of the components of the auxin-responsive systems were expressed as expected. However, both RT-PCR and phenotypic analysis showed that the auxin-dependent component (the effector gene under control of the UAS enhancer) was not induced in the presence of auxin.

All of the constructs tested in this chapter were complex, multi-component systems, incorporating a number of elements which were hitherto un-tested in the context of ectopic expression in *Ae. aegypti*. Consequently, many of the potential causes for the failure of these systems were not amenable to direct testing. Two elements which could be tested, however, were the functionality of the UAS-effector cassettes present in both systems, as well as functionality of the Gal4 transcription factor present in the Gal80 system (OX4786) (and correspondingly, failure of Gal80 to repress it). The observation of strong *AmCyan* expression in two of the OX4786 (Gal80 system-UAS-*AmCyan*) lines suggested one of two possible explanations: either there was a high level of 'leaky' expression from the minimal promoter, or a functional Gal4 transcription factor was present in these lines, which was competent to activate gene expression from the UAS enhancer. The second of these possibilities appears

likely, following the observation that the OX4786 construct from one of these lines was able to induce lethality when crossed to OX4787 transgenic individuals carrying *UAS-rpr*. It therefore appears that transgenic insects tested in this assay possessed a UAS-effector cassette that could be induced in the presence of the Gal4 transcriptional activator; hence, the lack of auxin-inducible gene expression observed in these lines likely resides in the lack of an auxin-dependent transcriptional activator. In the split transcription factor lines, this may indicate that the expected split-transcription factor does not assemble in the presence of auxin. In the case of AmCyan-expressing OX4786 (Gal80 system) lines, it appears that Gal4 is not repressed or only part repressed by Gal80; hence the presence of a transcriptional activator regardless of whether the addition of auxin induces Gal80 degradation.

6.4.3 THE PRESENCE OF C-TERMINAL UBIQUITIN MOIETIES MAY CONTRIBUTE TO LOSS OF FUNCTION

Having discounted the possibility of a non-functional UAS-effector cassette, there remain a number of factors which could explain the lack of auxin-inducible gene expression observed in transgenic insects.

A key explanatory factor in the non-functionality of both systems may be the presence of a ubiquityl (Ub^{K48R}) moiety on the C-terminus of some of the protein components. Use of Ub at the N-terminus of transgenic proteins is a well-established mechanism of expressing proteins of interest with the desired N-terminus (Bachmair et al., 1986). Indeed, it has been shown to enhance the abundance of transgenic proteins, possibly due to increased stability conferred by the rapidly-folded Ub (Hondred et al., 1999, Baker, 1996). However, for C-terminal fusions, where the Ub moiety remains attached to the protein of interest, the presence of Ub has the potential to affect both the stability of the resulting fusion proteins and their functionality.

The potential for the attached Ub to serve as a substrate for ubiquitin ligases, and therefore render the fusion protein unstable through triggering 26S proteasome-mediated degradation, has been addressed by a Lysine-Arginine (K-R) mutation at residue at position 48 in the native Ub moiety, which serves as a major site of isopeptide bond formation in poly-ubiquitin chains (Suzuki and Varshavsky, 1999, Lévy et al., 1996). Arginine cannot serve as a substrate for isopeptide bonds, and fusion proteins containing Ub^{K48R} were found to be stable in protein degradation assays (Suzuki and Varshavsky, 1999). Indeed, the stability of such configurations enables them to serve as reference proteins in Ub-based split-protein degradation assays (reviewed in Varshavsky (2005)). It is therefore unlikely that the presence of a C-terminal Ub^{K48R} moiety on some elements of the auxin systems described renders these fusions unstable *per se*.

A separate question is the likely effect of the C-terminal Ub^{K48R} moiety on the functionality of the fusion proteins. Both auxin systems described here rely on functional fusion proteins. However, the presence of the Ub^{K48R} moiety at the C-terminus of Gal80, TIR (Gal80 system) and TIRΔ (split-transcription factor system) is a further complicating factor that inevitably increases the likelihood of impairment of function. There is precedence in the literature for functional N- and C-terminal fusions with both Gal80 and TIR proteins (Leuther and Johnston, 1992, Melcher and Xu, 2001, Pilauri et al., 2005, Yu et al., 2013, Gray et al., 1999) while VP16 and Gal4 fusions of the kind used here are well known (Zhu et al., 2002, Brückner et al., 2009). Furthermore, flexible SG(4)S linker elements were inserted between the fused elements with the aim of minimising the possibility of the Ub moiety interfering with correct folding of the fused proteins. It was therefore hoped that the various fusions would not impair function in either system. Nonetheless, this possibility cannot be discounted; in particular, the likely impact of both N- and C-terminal fusions, which would effectively 'sandwich' Gal80 in the Gal80 system and TIRΔ in the split-transcription factor system, was unknown.

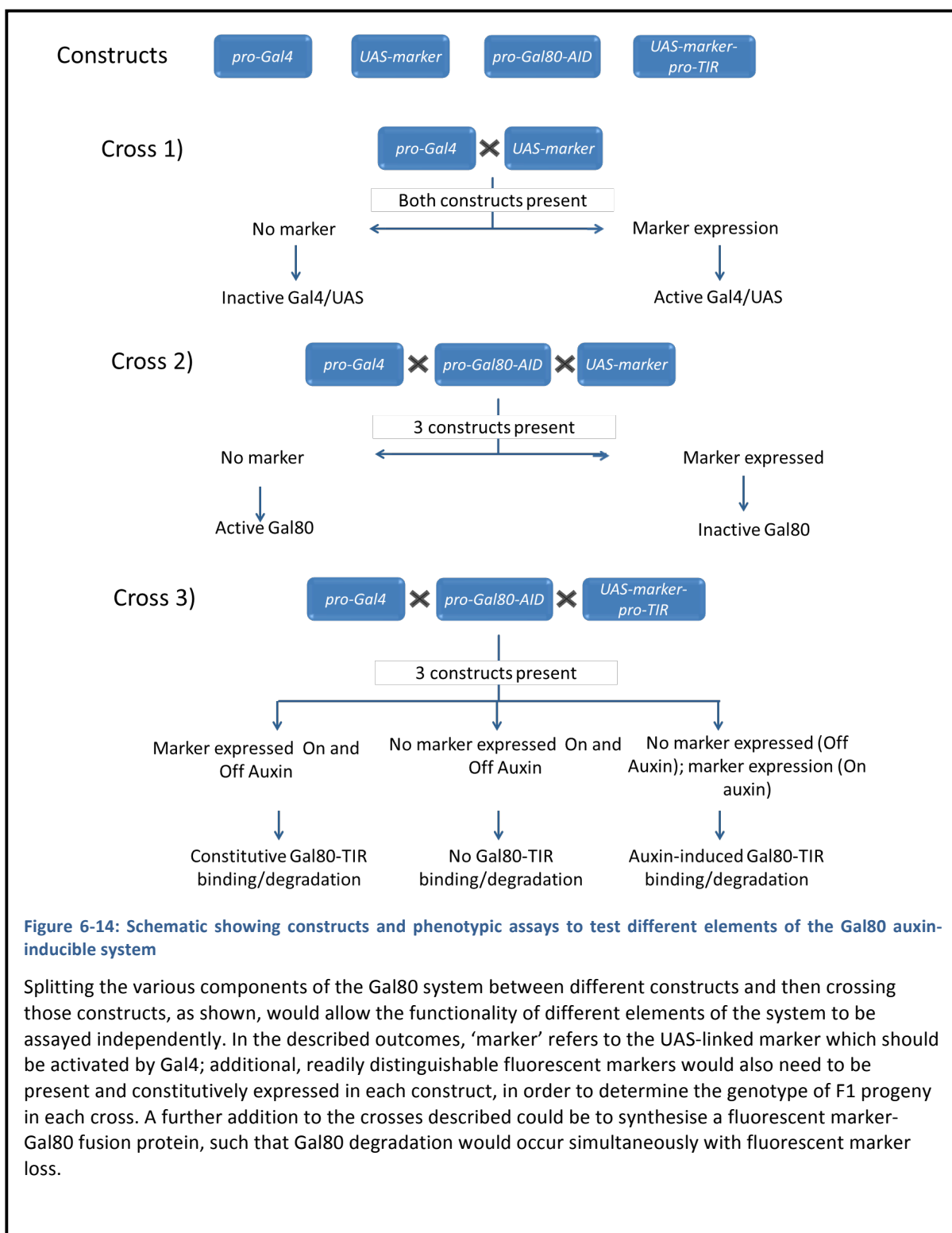
In summary, while these results cannot provide clear evidence as to the impact of the C-terminal Ub^{K48R} moiety, they at least call into question the utility of the ubiquitin cleavage approach for the expression of multiple transgenic proteins from a single promoter. For the future design of multi-component systems, alternative cleavage mechanisms which result in free-form proteins should be explored. One such is the use of “2A-like” peptides to induce ribosomal skipping (Donnelly et al., 2001), which has been recently demonstrated in *D. melanogaster* cells (Diao and White, 2012). Another possible alternative is the use of a modified ubiquitin fusion approach, wherein only a small 14 aa fragment of the Ub C-terminus remains fused to the C-terminal protein (Walker and Vierstra, 2007).

6.4.4 A MORE STEP-WISE APPROACH TO SYSTEM DEVELOPMENT WOULD ASSIST IN ‘TROUBLESHOOTING’ NOVEL EXPRESSION SYSTEMS.

Regardless of the impact of the protein fusions on function *per se*, there remain a number of loci at which loss of function for the overall system could occur. For the Gal80 system, the observed non-inducible marker expression could result from non-functional Gal80, or from constitutive Gal80-TIR association. For the split-transcription factor system, loss of function could result from lack of auxin-induced association of the transcription factor components, or alternatively loss of function of the DNA-binding or transcriptional activator components of the system.

Extensive additional testing would clearly be required in order to elucidate which if any of these factors may explain the observations made in transgenic lines. Western blots were carried out on samples from each of the auxin systems described. However, despite the availability of commercial antibodies for both Gal4 and VP16, no results could be obtained. Analysis was therefore limited to the level of gene expression and phenotypic analysis, without investigating the intermediate level of protein expression and activity in transgenic insects.

While protein analysis would have enabled additional questions to be addressed, such as whether the expected protein-protein interactions indeed occur, the analysis described in this chapter clearly shows that the auxin-inducible systems described do not function as expected. Nonetheless, the outcomes reported have highlighted the value of adopting a more step-wise approach to the development of novel systems, which in the present case might have facilitated more granular phenotypic assays. Figure 6-14 shows a schematic outlining how a more step-wise approach could shed light on which elements of the Gal80 system might be responsible for the observed non-functionality. The split-transcription factor system is less amenable to this more granular phenotypic assay; nonetheless, a construct expressing Gal4 alone would have at least provided a quick assay to determine whether the UAS-effector cassette was functional.



6.4.5 ALTERNATIVE MECHANISMS TO ACHIEVE AN INDUCIBLE EXPRESSION SYSTEM SHOULD BE EXPLORED

The sum of results reported in this chapter is that neither of the auxin-based degradation systems described represents a viable mechanism by which to introduce an inducible expression system in *Ae. aegypti*. While additional assays may shed light on the causes of the failure of these systems, or even suggest elements whose re-design could result in a functional system (for example, altering the location of the fusion join in chimeric proteins), both the necessary investigations and the resulting re-design would clearly involve significant amounts of additional time and labour, and the outcome of the process would nonetheless remain uncertain.

An inducible expression system remains an important goal of future RIDL programmes. However, further pursuit of an auxin-inducible system is unlikely to represent an optimal route by which to attain to this goal. Alternative systems should instead be explored. Based on experience with the auxin architectures, it would be a considerable advantage for candidate systems to involve fewer component parts, making them more amenable to downstream ‘troubleshooting’. In particular, more modular systems, whose components could be independently tested in a transgenic background before being combined in a single system, should be regarded favourably when identifying suitable candidates to take forward. Furthermore, as discussed above, systems which require the expression of multiple components from a single promoter may benefit from the use of alternative processing mechanisms to the ubiquitin cleavage approach employed here.

7 SUMMARY AND CONCLUSIONS

Mosquito-borne diseases are a significant and growing public health challenge. Of particular concern, the mosquito-borne arboviruses dengue fever and chikungunya are growing rapidly in global range, incidence and severity (Murray et al., 2013, Bhatt et al., 2013, Burt et al., 2012). Their expansion can be largely attributed to the recent widespread establishment and population growth of their primary vectors, the mosquitoes *Ae. aegypti* and *Ae. albopictus* (Gubler, 2011b, Lambrechts et al., 2010).

Effective approaches for the control of these species are therefore of paramount importance to meeting a global health challenge. Recently, a promising new technology has been developed based on the release of transgenic male insects carrying a lethal allele under the control of a tetracycline repressible ('Tet-Off') expression system (Thomas et al., 2000, Phuc et al., 2007, Alphey and Andreasen, 2002).

As discussed in Chapter 1, this approach offers considerable advantages over non-transgene based approaches to the Sterile Insect Technique (SIT). Moreover, an engineered dominant lethal strain of *Ae. aegypti*, developed has shown marked success in field experiments, reducing wild populations of *Ae. aegypti* in the target areas by between 80 and 96% (Harris et al., 2012, Danilo O. Carvalho, 2014, McKemey, 2013).

The release of insects carrying a dominant lethal gene has therefore gone some way to establishing itself as a new paradigm of pest control, and a much-needed new tool to control populations of the disease vectors *Ae. aegypti* and *Ae. albopictus*. Nonetheless, there remains considerable scope to improve on the current strain, in order to realise the full complement of benefits which a transgene-based approach can offer. To offer maximum efficiency of rearing and retain efficacy in the field, an 'ideal' strain would combine the following properties:

- 1) Late acting, bi-sex, repressible lethality, to retain density-dependent competition among larvae and achieve effective population suppression
- 2) An early-acting genetic sexing mechanism, enabling facile removal of females from the release generation and maximum rearing efficiency
- 3) A mechanism for regulating gene expression which would be orthogonal to (i.e. independent of) the 'Tet-off' mechanism, allowing the two fore-mentioned systems to be combined in a single strain.

Strategies for developing such an improved 'next-generation' strain in both *Ae. aegypti* and *Ae. albopictus* are the focus of work described in this thesis.

7.1 OSIRIS PROMOTERS HAVE SHOWN CLEAR POTENTIAL FOR USE IN ENGINEERING LATE-ACTING, BI-SEX LETHAL STRAINS.

Chapters 3 and 4 of this thesis discuss approaches to identify novel promoters for use in engineering late-acting (i.e. late adolescent) lethality in *Ae. aegypti* and *Ae. albopictus*.

7.1.1 A PUPAL-SPECIFIC, REPRESSIBLE LETHAL SYSTEM HAS BEEN DEVELOPED USING AN OSIRIS PROMOTER.

Work described in Chapters 3 and 4 has resulted in the identification of two promoters from *Ae. aegypti*, which show considerable promise for engineering late-acting expression systems.

Promoter regions isolated from the genes AAEL004275 and AAEL004298, characterised as putative *Osiris* genes (Dorer et al., 2003, Shah et al., 2012), were found to be capable of driving high levels of transgene expression in a pupal-specific manner. A putative repressible lethal construct, in which the second *Osiris* promoter (Os2) drove expression of the tetracycline-repressible trans-activator tTAF3, was found to result in repressible pupal-specific lethality in transgenic insects. *Prima facie*, this construct (OX5055) therefore shows promise as the basis for developing new late-acting, bi-sex lethal strains in both *Ae. aegypti* and *Ae. albopictus*.

7.1.2 NON-REPRESSIBLE TOXICITY MAY NEED TO BE ADDRESSED IN THE DEVELOPMENT OF FUTURE PRODUCT LINES

Results from testing repressible lethal systems with both *Osiris* promoters indicate a degree of mortality that was not consistently repressed in the presence of tetracycline. On balance, it appears likely that this results from over-expression of tTAV and consequent mortality from transcriptional ‘squenching’ (Gill and Ptashne, 1988).

A degree of latent toxicity may in part explain the mortality of populations homozygous for the OX5055 (Os2-*tTAF3-tetO-michelob_x*) construct, which was observed in preliminary assays of homozygous viability discussed in Chapter 4. The ability to make a strain homozygous is a prerequisite for its use in downstream population suppression applications. If non-repressible toxicity associated with the *Osiris*–trans-activator cassette causes lethality in the homozygous state, this is therefore a significant cause for concern. However, the observation that some homozygous individuals clearly survived to adulthood suggests that the lethality observed may in part have been an artefact of the preliminary assays performed. The process of counting out larvae at a very early developmental stage may be damaging to them. Although this is a standard experimental procedure and does not normally result in significant lethality, the fact that much higher survival rates were seen in a population which was not subjected to this procedure suggests that it may have had an impact on survival in this case; possibly it was a ‘tipping point’ for individuals whose fitness was already compromised by two copies of the transgene.

The Os2 promoter therefore remains, for the present, a viable prospect for the development of future late-acting lethal product strains. In contrast to the molecular approach underpinning the current Oxitec product strain, OX513A, the Os2 promoter confers late-pupal expression in a highly consistent manner across multiple different insertions, making it a useful molecular tool for conferring late-acting lethality in new product lines. Addressing the question of

whether dominant-lethal strains based on this promoter can be made into viable product strains should therefore be a priority. Rearing the current OX5055 (Os2-*tTAF3*-tetO-*micelob_x*) as a heterozygous population through a number of generations should be a first step. The process of outcrossing to wild-type strains and selection of healthy transgenic adults may over a number of generations result in a genetic background which is more robust to any non-repressible toxicity associated with the construct. Additional homozygous viability testing should then be conducted on all lines, before attempts made to create homozygous populations from any promising lines.

It should be noted that in the development of the OX5055 line, as with other lines developed in this thesis, no molecular characterisation of the insertion site was performed. This was because the majority of experiments performed have been designed to assess initial functionality of constructs in transgenic insects, for which detailed information on insertion sites was not required. However, this characterisation will need to be performed in order to facilitate PCR assays for homozygosity. In the absence of molecular characterisation, the presence of single insertions was assessed by simple genetics (i.e. counts of transgene frequency in heterozygous populations). However, it should be noted that such tests are unlikely to reveal the presence of 2 or more insertions which are very closely linked. This may not be of concern if such insertions were so closely linked as to be inherited essentially as a single allele. Nonetheless, if reported phenotypes result in some strains from the presence of more than one allele, this does raise the theoretical possibility of phenotypic changes arising in future generations. Molecular characterisation of the OX5055 lines prior to any attempts to develop a homozygous strain will shed light on this issue.

The success of attempts to attenuate the toxicity apparently associated with *Osiris*-driven trans-activator expression was unclear. In part, this was because the second *Osiris* promoter was not tested in an un-modified construct. The approaches described in Chapter 4 – transcript de-stabilisation using a *hsp70* 3'UTR and use of a less toxic trans-activator (tTAF3 in

place of *tTAV*) – may be of more general use in modulating the effect of promising constructs which show non-repressible toxicity. A phenotypic analysis of insects transformed with an unmodified construct (i.e., containing an *Os2-tTAV* cassette) may therefore be useful in elucidating the effect of measures taken to reduce toxicity.

7.1.3 THE PROMOTERS OF THE *OSIRIS* GENE FAMILY MAY PROVE A PROMISING ADDITIONAL SOURCE OF PROMOTERS FOR ENGINEERING LATE-ACTING EXPRESSION

In parallel to the continued development of the most promising *Os2* repressible lethal strains, alternative late-acting promoters should also be identified and tested. The two *Osiris* promoters analysed in Chapters 3 and 4 are part of a wider family of *Osiris* genes which in *D. melanogaster* number at least 24 (Shah et al., 2012). Many of these genes exhibit peak expression at developmental boundaries, including pupal development (Shah et al., 2012), while several *Ae. aegypti* *Osiris* promoters appear to share conserved binding sites for the pupal-determining transcription factors broad (*br*) and E74 (Molloy, 2014). In addition to the utility of the promoters investigated here, other members of the *Osiris* gene family may, therefore, also prove useful candidates for engineering late acting expression. Indeed, based on the *Ae. aegypti* developmental transcriptome database (Akbari et al., 2013), at least one further *Osiris* gene has been identified as a promising candidate for driving late acting expression (Molloy, 2014).

One concern raised by results reported in this thesis is that even very high levels of transgene expression at a late developmental stage may not be sufficient to achieve lethality if expression is concentrated in non-essential tissues. Following initial screens for further late-acting genes, it may therefore be advisable to conduct tissue-specific RT-PCRs at an early stage to identify candidates which have either a ubiquitous expression profile, or which are highly expressed in essential tissues, such as the brain.

More generally, the approaches pursued in Chapters 3 and 4, including the reporter constructs used to test the *Osiris* promoters, may be of further use in testing additional novel promoter candidates.

7.2 USE OF DOUBLESEX (DSX) SEX-SPECIFIC SPLICING REGIONS IS A PROMISING ROUTE TO DEVELOPING FEMALE SPECIFIC EXPRESSION IN *AE. ALBOPICTUS*

A key element of improved product strains will be an early-stage genetic sexing mechanism. Female-specific expression systems with the potential to be activated at an early developmental stage have been developed in *Ae. aegypti*, based on sex-specific splicing of the *doublesex* (*dsx*) gene (Collado, 2013). However, equivalent systems are lacking in *Ae. albopictus*. Work described in Chapter 5 of this thesis outlined parallel strategies undertaken in pursuit of this goal.

7.2.1 ISOLATION OF THE *AE. ALBOPICTUS* *DSX* GENE HAS FACILITATED DEVELOPMENT OF A FEMALE-SPECIFIC EXPRESSION SYSTEM IN THIS SPECIES

Results described in Chapter 5 show that the *Ae. aegypti* *dsx* mini-gene, which has been used to confer female-specific expression in *Ae. aegypti*, was not spliced in a sex-specific manner in *Ae. albopictus*. This result was in itself unexpected. However, it suggested that isolation of the *Ae. albopictus* *dsx* orthologue might provide the most likely means of establishing in *Ae. albopictus* the female-specific expression systems recently developed in *Ae. aegypti*.

As reported in Chapter 5, an orthologue of *dsx* has now been identified in *Ae. albopictus* (*Aaldsx*), and putative sex-specific splicing constructs based on sequences from this gene developed. Results reported in Chapter 5 show an *Aaldsx* mini-gene was indeed spliced in a

sex-specific manner in *Ae. albopictus*. However, they further show that splicing was ‘leaky’, with some female transcript present in male insects.

Further work should therefore be undertaken to address this ‘leaky’ splicing; clearly a necessary step if the *dsx* approach is to be successfully used as a female-specific expression system in *Ae. albopictus*. Additional sequencing of the native *dsx* gene, also reported in Chapter 5, identified a number of loci at which the ‘mini-gene’ used in these investigations might be modified to give closer equivalence to the native *dsx* gene. These findings have informed the design of a new construct containing a revised *Ae. albopictus dsx* mini-gene. The new construct will contain additional regions of the intron immediately upstream of the female-specific exons. In view of the finding that these regions were more extensively conserved than at first thought, including them in a revised construct will give more confidence that regions of importance in splicing regulation are included. The revised construct will also correct a number of discrepancies which were identified between the *dsx* sequence used in constructs OX5024 and OX5048, and the wild type sequence. Some of these occur at the 5’ end of the short intron which separates the female-specific intron, and so may possibly have affected splicing regulation in the *dsx* mini-gene used.

In summary, the findings reported in Chapter 5 have shown that a *dsx* splicing mechanism remains a promising approach to obtaining a female-specific expression system in *Ae. albopictus*. A final iteration of an *Aaldsx* construct is being developed as described in the previous paragraph. Once built, this should be tested for its ability to direct sex-specific splicing in *Ae. albopictus*. If the revised construct proves successful in this regard, experience using *dsx* in *Ae. aegypti* suggests that coupling such a construct to a lethal effector gene could result in female-specific lethal strains in *Ae. albopictus*.

7.2.2 ACTIN4 SPLICING MAY STILL PROVE AN ALTERNATIVE APPROACH TO DEVELOPING FEMALE-SPECIFIC EXPRESSION SYSTEMS.

The potential for sex-specifically spliced elements of the *Ae. aegypti Actin4* gene to be used in female-specific expression systems was also explored in Chapter 5. Constructs containing both the *Actin4* promoter and a male-specific exon have been used to confer female-specific expression in both *Ae. aegypti* and *Ae. albopictus* (Fu et al., 2010, Labbe et al., 2012). However, these constructs limit transgene expression primarily to the indirect flight muscles of developing females, and hence are unsuitable for implementing an early-acting genetic sexing mechanism. As reported in Chapter 5, attempting to circumvent the tissue- and stage-specific activity of the *Actin4* promoter by using only the sex-specific splicing regions of the *Actin4* gene results in loss of sex-specific splicing. Further attempts to divorce female-specific expression from the tissue and developmental stage-specificity of the *Actin4* regulatory elements were not pursued. However, additional strategies, such as promoter deletion analysis of the *Actin4* promoter, may prove an alternative route to developing a genetic sexing mechanism in *Ae. aegypti* and *Ae. albopictus*.

7.3 AUXIN-BASED EXPRESSION SYSTEMS WERE NOT SUCCESSFUL IN DEVELOPING INDUCIBLE EXPRESSION IN *AE. AEGYPTI*

Developing an expression system that functions independently of the “Tet-Off” system will be a third key component of a ‘next-generation’ product strain: it will allow late-acting bi-sex lethality to be combined with an early-acting female-killing system, activated in the release generation. Chapter 6 reported on the analysis in transgenic insects of two putative inducible systems developed by Oxitec Ltd, based on auxin-mediated protein binding (Nishimura et al., 2009).

Neither of the systems tested proved capable of directing transgene expression in an auxin-inducible manner. Further analysis suggested that at least the Gal4-UAS elements were functioning as expected (i.e. that functional Gal4 was produced in at least one transgenic line, and that it could induce expression of an effector gene through binding the UAS enhancer).

However, there remained a number of plausible explanations for the non-functionality of the systems tested. In particular, the impact on functionality of the various protein fusions used in the two systems was unclear.

Establishing an expression system capable of being activated independently of the tetracycline system remains a priority for future development of dominant lethal strains in *Aedes*. While the results presented in Chapter 6 do not rule out the use of auxin-induced systems, they at least suggest that a substantially different molecular approach would be necessary in order to be successful, and that even following such a re-design, outcomes would be far from certain. It is therefore recommended that Oxitec Ltd should pursue the development of alternative candidates for tetracycline-orthogonal, inducible expression systems. Such candidates include the vanillic acid system (Gitzinger et al., 2011), binary transcription-factor based systems such as the 'Q' system (Potter et al., 2010), light-activated transcription factors (Motta-Mena et al., 2014) or RNA-based expression control mechanisms, such as 'Aptazymes' (Ketzer et al., 2014). The Q system and a blue-light activating system have recently been tested in transgenic insects (the former in the moth *Plutella xylostella*, the latter in *Ae. aegypti*) without success (Harvey-Samuel, 2014, Nimmo, 2013) The vanillic acid system is currently in the early phases of testing in *Ae. aegypti*. However, in general, known operons which are responsive to small, low cost, non-toxic ligands (of which the vanillic acid system is one example) may represent the most simple, and perhaps therefore more promising, route to developing orthogonal expression control mechanisms.

In this regard, the results of testing the auxin-based systems reported on here have also highlighted the merits of adopting a more step-wise approach to developing novel expression systems. While the urgency of developing such novel systems might lend itself to testing multi-component systems, a more modular approach would allow for independent testing of each module, and thence more facile identification of non-functioning elements.

7.4 PROGRESS TOWARDS DEVELOPING ‘NEXT GENERATION’ DOMINANT LETHAL STRAINS

In summary, research reported in this thesis has laid important groundwork for developing ‘next generation’ dominant lethal strains in *Ae. aegypti* and *Ae. albopictus*: late-acting promoters have been identified and used to develop bi-sex repressible lethal strains; a promising approach to developing early-acting female specific expression in *Ae. albopictus* has been developed; and insights have been gained regarding the future development of inducible expression systems. These results will further the development of a highly promising approach to controlling two important global disease vectors.

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