Transgenic expression of *Wolbachia* cytoplasmic incompatibility factors in *Aedes aegypti* mosquitoes.

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Abstract

Many strains of the maternally transmitted intracellular bacteria *Wolbachia* can invade arthropod populations and remain at high frequency through modifications of host reproduction. Most commonly this manipulation occurs via a mechanism known as cytoplasmic incompatibility (CI), whereby *Wolbachia*-mediated modification of the paternal chromatin renders infected males incompatible with uninfected females. If the females carry a compatible *Wolbachia* strain this incompatibility is rescued which provides *Wolbachia*-carrying females with a relative fitness advantage. The basis for incompatibility stems from two *Wolbachia* encoded CI factors (Cifs), CifA and CifB, which interact in a cognate-specific manner. If *Wolbachia* strains possess divergent copies of the *cif* genes, then incompatibility cannot be rescued. The modifications associated with CI induction and rescue are largely attributed to CifB and CifA respectively. However, prior research in transgenic insect species has revealed that CifA might play a role in the induction phenotype which has led to diverging hypotheses on the mechanism of CI. Although the conditional sterility associated with *Wolbachia*-mediated CI has been exploited to control populations of the primary arbovirus vector *Ae. aegypti*, an understanding of how the Cifs manipulate reproduction in this species remained unknown. In this study, the phenotypes of CI induction and rescue were recapitulated through the transgenic expression of *cif* genes in *Ae. aegypti* mosquitoes and revealed that the Cifs likely interact in a toxin-antidote mechanism. Interchanging Cif homologues from alternate strains revealed different levels of CifB toxicity in the male reproductive tissues, which might explain the discrepancies concerning the need for paternal *cifA*-antidote expression in sterility induction between alternate studies of *cif* variants. As *Wolbachia*-mediated *Ae. aegypti* control strategies have proven effective at reducing disease transmission, the transgenic mediation of CI in this species provides an opportunity to utilise this reproductive manipulation for vector control in the absence of the bacterium. It is believed that the findings of this study could be used to generate different transgenic systems that either replace or suppress *Ae. aegypti* populations and thus reduce the burden of arboviral diseases.

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List of Abbreviations

ADE – antibody-dependent enhancement

***Ael*DNV – *Aedes albopictus* densovirus**

BSA – bovine serum alumin

CHIKV – chikungunya virus

CI – cytoplasmic incompatibility

Cif – cytoplasmic incompatibility factor

DCV – *Drosophila* C virus

ddH_2O – double-distilled water

DENV – dengue virus

DHF – dengue haemorrhagic fever

Dox – doxycycline

dsRNA - double stranded RNA

DSS- dengue shock syndrome

DUB – deubiquitinase

exu – exuperantia

FHV – Flock house virus

fsRIDL – female specific RIDL

GM – genetically modified

HDR – homology directed repair

HGD – homing-based gene drive

HM – host-modification

IIT – incompatible insect technique

ISF – insect specific flavivirus

ISV – insect specific virus

miRNA – micro RNA

NHEJ – non-homologous end joining

NLS – nuclear localisation signal

PBS – phosphate buffered saline

***prot* – protamine**

PUb* – *polyubiquitin

PUbt* – truncated *polyubiquitin

qPCR – quantitative PCR

RIDL – release of insects carrying a dominant lethal gene

RNAi – RNA interference

ROS – reactive oxygen species

RT-qPCR – quantitative reverse transcription PCR

ScFv – single-chain variable fragment antibodies

SIT – sterile insect technique

TA – toxin-antidote

tetO – tetracycline operator

topi* – *matotopetli

TRE – tetracycline response element

tTAV – transcriptional transactivator

TUNEL – Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling

VBD – vector borne disease

ZIKV – Zika virus

***zpg* – zero population growth**

β2t* – *β2 tubulin

β2t** – optimised *β2 tubulin

(-)RNA – negative sense RNA

(+)RNA – positive sense RNA

(s)gRNA – (single)guide RNA

+ssRNA – positive sense single stranded RNA

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Firstly, I would like to dedicate this thesis to my parents Michael and Morag McNamara, who have nurtured and supported me throughout my life. Although my father did not live to see this moment, he instilled in me all the qualities and traits that have led me to this point and for that I will be forever grateful.

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Author's declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Cameron James McNamara

Chapter 1:
Introduction

1. Introduction

1.1 Disease vectors and vector control methods

1.1.1 Vector-borne diseases

Vector-borne diseases (VBDs) result from an infection (e.g. viral, bacterial, parasitic) transmitted by a range of arthropod vectors. It is projected that 80% of the global human population are at risk of contracting one or more VBD[1]. More than 17% of infectious disease cases are VBDs, of which more than a million cases per year result in death[2]. Notable vectors are sandflies (leishmaniasis), ticks (Lyme disease, Crimean-Congo haemorrhagic fever, tick-borne encephalitis), tsetse flies (African trypanosomiasis), and the mosquito genera *Aedes* (dengue, chikungunya, Zika, yellow fever viruses, lymphatic filariasis), *Anopheles* (malaria, lymphatic filariasis, o'nyong-nyong), and *Culex* (West Nile fever, Japanese encephalitis, lymphatic filariasis)[2]. As many of the most important vector species are confined to tropical and subtropical regions, these areas are the most affected by VBDs. Unfortunately, these areas are also disproportionately affected by poverty and insufficient healthcare services which exacerbates the burden of disease[1]. Increases in i) the global temperature, ii) the size of human populations and their settlements, and iii) the trade and travel links between these populations have not only increased the vector habitat but also the proximity of these vectors to human populations[3]. Due to vector competency, many VBDs can co-circulate and mutations in the transmitted pathogens can cause rapid emergence or re-emergence of disease[3].

Due to i) the cost of developing and administering effective disease treatments or prophylactics, ii) the ability for pathogens to develop resistance to these treatments, iii) the rapid emergence of vector borne disease epidemics, and iv) the fact that vector species can transmit several pathogens - it is often more (cost-)effective to control the vector populations than to target the pathogen directly. Many types of vector control strategies exist and can comprise of either chemical, physical, biological, and genetic methods. Chemical control methods involve the use of insecticides as larvicides or against adults and are widely used to suppress vector population size. The use of insecticide-treated/long-lasting insecticidal nets are a form of physical as well as chemical control. Another form of physical control involves

the removal of vector breeding sites which includes the removal of any man-made container that may collect rainwater.

Biological methods include the introduction of either predator species[4,5], toxin releasing bacteria[6] and entomopathogenic fungi[7], or *Wolbachia*-infected individuals (discussed in 1.3) [8–10]. There are numerous ways in which genetic manipulation can be used to either reduce or modify vector populations (discussed in 1.4). Genetics based population suppression methods include the sterile insect technique (SIT) and release of insects carrying a dominant lethal gene (RIDL)[11–13]. Gene drive systems (discussed in 1.4.2) could also be used in the future for population suppression however they could additionally be used to spread alleles into vector populations that make them refractory to pathogen infections. Although each of these control methods can be effective, when used alone they run the risk of promoting escape mutations which can lead to the re-emergence of the disease being challenged. For example, many vector species have developed resistance to routinely used insecticides. Therefore, the consensus from global health initiatives such as the World Health Organisation is to use a range of control methods (preferably ones that target different life stages of a vector species) which have been selected and tailored to function optimally in the specific region of deployment. Novel vector control tools are continually sought to be incorporated into this integrated vector management approach. Recent trials of population suppression and replacement strategies using either *Wolbachia*-infected[8–10] or genetically modified vector species[13,14] have successfully demonstrated why these techniques should be incorporated into current vector control endeavours.

1.1.2 The vector species *Aedes aegypti*

The mosquito species *Ae. aegypti* originated in Africa and is thought to have been introduced to the Americas via the transatlantic slave trade[15]. Now, *Ae. aegypti* can be found in tropical, subtropical, and warm temperate regions throughout the world. The lifecycle of *Ae. aegypti* is complex consisting of both aquatic and terrestrial stages: adult females will lay eggs above the water line, once submerged the eggs will hatch and go through four stages (instars) of larval development and then pupate before the adult mosquitoes eclose. Female *Ae. aegypti* mosquitoes are anautogenous and therefore must consume a bloodmeal in order to produce eggs. It is believed that that the African subspecies *Ae. aegypti formosus*, which primarily

resides in forest regions and is zoophilic in nature (preferring to feed on animals opposed to humans) evolved into the subspecies *Ae. aegypti aegypti* which primarily reside in urban areas and preferentially feed on the human inhabitants (anthropophilic) before the subspecies migrated out of the continent[16]. This adaptation to urbanisation is likely what has led to *Ae. aegypti* becoming one of the most notable vectors of disease worldwide. Often referred to as the yellow fever mosquito due to its key role in yellow fever virus transmission, this species remains the primary vector for a range of other arthropod-borne viral (arboviral) diseases such as chikungunya, dengue, and Zika.

Chikungunya virus (CHIKV) is a single-stranded positive sense RNA (+ssRNA) virus of the Togaviridae family, and infections can cause severe fever as well as debilitating polyarthralgia[17]. Derived from the Makonde language, chikungunya means "that which bends up" on account of the posture patients suffering from the disease-associated joint pain would adopt - in some cases, this joint pain can last for several years[17,18].

Dengue is the fastest spreading arboviral disease and cases occur in over 120 countries with an estimated 3.9 billion people (around half the world's population) at risk of contracting the disease[19,20]. Although over 390 million infections are estimated to occur annually, most cases are asymptomatic or consist of a characteristic fever known colloquially as "breakbone fever"[21]. However, some patients can develop dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS) which are life threatening conditions. Dengue is caused by dengue virus (DENV) a +ssRNA virus of the Flaviviridae family. There are four distinct but closely related serotypes of the virus (DENV-1, DENV-2, DENV-3, and DENV-4). Recovery from an infection with one serotype does not confer full immunity to the other three, in fact subsequent infection from a different serotype increases the risk of developing severe dengue. This occurs via a phenomenon known as antibody-dependent enhancement (ADE) where antibodies from the primary infection fail to neutralise the virus in the secondary infection and promote the uptake of the virus into immune cells from where the virus can freely replicate[22].

Zika virus (ZIKV) is closely related to DENV and also belongs to the Flaviviridae family. An infection with ZIKV is usually asymptomatic or consists of a mild fever, however ZIKV can also be known to cause congenital Zika syndrome (which can include conditions like microcephaly) and Guillain-Barré syndrome[23]. Due to the closely related nature of DENV and ZIKV, prior

infection with one before the other is thought to increase the risk of severe disease symptoms[24–26]. As well as increasing the severity of disease in regions where the two viruses co-circulate, ADE also presents a problem in creating effective vaccines[22,27–29]. The only licensed dengue vaccine, Dengvaxia, has been shown to increase the risk of severe disease on subsequent infection in vaccinated patients that were previously seronegative[30]. As there are currently no approved antiviral treatments for DENV, ZIKV and CHIKV infections, and the use of Dengvaxia is somewhat controversial, vector control methods remain the primary public health intervention to these arboviruses.

1.2 *Wolbachia* and cytoplasmic incompatibility

1.2.1 *Wolbachia* overview

Members of the genus *Wolbachia* are intracellular α-protobacteria in the order Rickettsiales. First discovered in the reproductive tissues of *Culex pipiens* mosquitoes, the type species was named *Wolbachia pipientis*[31,32]. Although this remains the only species described, the species is highly diverse and currently divided into 18 phylogenetic supergroups (named A-F,H-Q,S, and T)[33–35]. Some *Wolbachia* supergroups can be found in a wide array of invertebrate species (e.g. supergroups A and B found in multiple arthropod taxa), whereas some are restricted to certain host lineages (e.g. supergroup H found in termites)[36]. *Wolbachia* are thought to infect around half of all arthropod and several filarial nematode species[37,38] making it the most abundant endosymbiont in existence. This abundance can be attributed to two contributing factors: firstly, *Wolbachia* can rapidly spread through a host population by manipulation of reproductive fitness[39]; and secondly, occurrences of horizontal transfer allow for host shifts[40–44]. Horizontal transmission can arise from hybrid introgression[40,41], predation or parasitism of infected individuals[42,43], and possibly plant-mediated transmission to species that share ecological niches[44]. However, *Wolbachia* are primarily maintained in a host population through vertical transmission from mother to offspring in a transovarial manner. To assist this vertical transmission, as well as replicating to high densities in the reproductive tissues, many strains of *Wolbachia* can increase the reproductive fitness of an infected host either through nutrient provision[45], increasing host fecundity[46], protection from pathogens [47–51], or reproductive parasitism[39]. There are four well documented reproductive parasitism

phenotypes mediated by *Wolbachia*; male-killing, parthenogenesis, feminisation, and cytoplasmic incompatibility (CI).

1.2.2 Male-Killing

When vertically transmitted from an infected female host, some *Wolbachia* strains are known to selectively kill male offspring during development resulting in female-biased sex ratios. These ratios are conducive to spread as the fitness of females increases as a result of the death of their male siblings through reduced larval competition, and only infected females can pass on a *Wolbachia* infection. This male-killing phenotype can be seen in several insect orders e.g. Lepidoptera[52], Coleoptera[53], and Diptera[54] as well as pseudoscorpion arachnids[55]. Some strains of *Wolbachia* such as *w*Rec (native to *D. recens*) can cause both male-killing or CI phenotypes depending on the host species it infects[56]. It is unknown how *Wolbachia* mediates the male-killing phenotype however some genes associated with this phenotype have been identified. Comparative genomics between male-killing *Wolbachia* strains that infect *Drosophila* identified a candidate gene termed *wmk* (*WO-mediated killing*)[57]. Expression of *wmk* in transgenic *Drosophila melanogaster* induced several cytological defects resulting in embryonic death and a significant female bias[57]. However, the exact mechanism which resulted in this phenotype remains unknown. In an independent study of the male-killing phenotype in lepidopteran insects, a factor termed Oscar identified in the *Wolbachia* strain *w*Fur (native to *Ostrinia furnacalis*) was found to target an essential protein for masculinisation and dosage compensation in lepidopteran insects[58]. *Oscar* was found to share some homology with the *Wolbachia cifB* gene (associated with CI induction) which might suggest that a common ancestor of this gene could induce both types of reproductive manipulations. Furthermore, although multiple homologs of the *wmk* gene were encoded in the *w*Fur genome, none were found to elicit similar male-killing interactions to *Oscar* in lepidopteran cells which might suggest they play an alternative role in lepidopteran insects[58].

1.2.3 Parthenogenesis

Parthenogenesis induced by *Wolbachia* is well documented in species belonging to the order Hymenoptera, but also occurs in thrips and mites[59]. These species typically exhibit arrhenotokous parthenogenesis in which fertilised eggs all develop into females and unfertilised eggs develop into males[60]. When virgin mothers are infected with a

parthenogenesis-inducing *Wolbachia* strain, the unfertilised eggs develop into females instead which represents thelytokous parthenogenesis. As *Wolbachia* are maternally-inherited the production of mostly female offspring is beneficial for its spread. One mechanism in which *Wolbachia*-induced parthenogenesis can occur is through chromosomal endoduplication in the host – where the haploid chromosome set is duplicated but fails to separate during the first or second rounds of mitosis which results in the formation of a diploid nucleus[61]. However, it is unknown how parthenogenesis inducing *Wolbachia* strains mediate this.

1.2.4 Feminisation

Wolbachia-induced feminisation results in the conversion of genetic males into females and is most common in isopod crustaceans[62–64]. In some instances, *Wolbachia*-induced feminisation can result in the development of low-fertility intersex individuals[65] and the degree of feminisation can be shown to correlate with *Wolbachia* density in several systems[66]. Interestingly, a feminising "f element" in the genome of the isopod *Armadillidium vulgare* was found to have significant similarity to a region of the native *Wolbachia* strain *w*VulC which indicated an instance of horizontal gene transfer between *Wolbachia* and its host[67]. As with the other reproductive mechanisms that alter the sex-bias of offspring, feminisation increases the number of host progeny that are able to transmit the bacterial infection. However, the specific factors responsible for feminisation induced by *Wolbachia* remain unknown.

1.2.5 Cytoplasmic incompatibility

CI is the most common reproductive manipulation observed in *Wolbachia*-infected arthropods. First described in *C. pipiens*[68], CI remains the only *Wolbachia*-mediated reproductive manipulation observed in mosquitoes. Defined as a disruption of karyogamy, CI leads to embryonic lethality in crosses between infected males and either i) uninfected females (unidirectional incompatibility), or ii) females infected with non-compatible strains of *Wolbachia* (unidirectional and bidirectional incompatibility) (Figure 1.1).

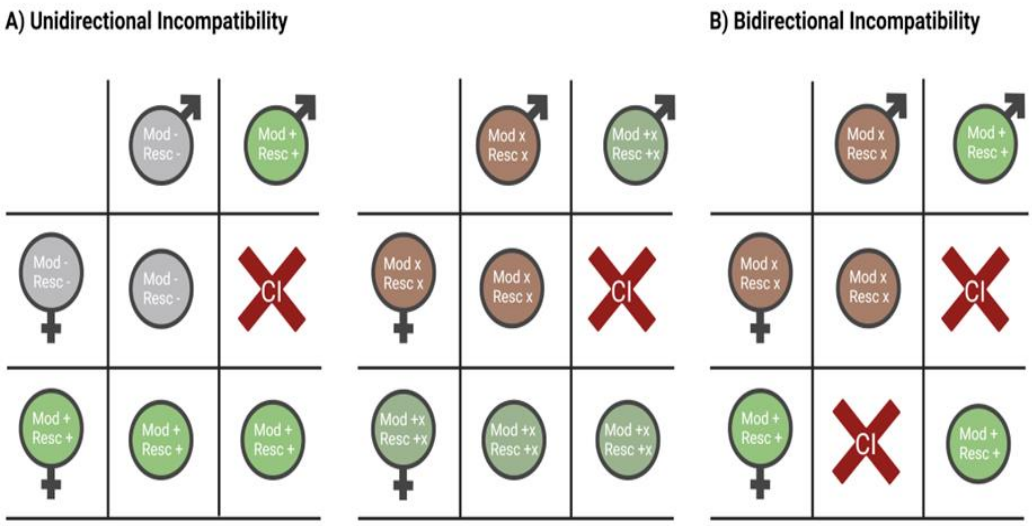


Figure 1.1: Illustration of uni-and bidirectional incompatibility. A) Unidirectional CI can be observed when a *Wolbachia*-infected male (mod +/resc +) mates with an uninfected (mod -/resc -) female**.** Alternatively, it is observed when one strain (mod +x/resc +x) can rescue CI induced from an another (mod x/resc x), but not vice versa. **B)** Bidirectional CI can be observed when two strains possess different mod and resc factors and are therefore incompatible. Note: "-"means no mod or resc capacity, and "+" and "x" denote diverging mod and resc factors. The colour grey indicates uninfected individuals whilst the other colours indicate infections with varying strains of *Wolbachia*.

Cytological studies have revealed that CI usually results from a delay in the paternal pronuclei's mitotic progression which leads to abnormal or failed chromosome segregation during the anaphase of early embryonic mitosis[69]. This delay is characterised by a decrease in the rates of histone H3.3/H4 complex recruitment, chromatin condensation, cyclin-dependent kinase 1 (Cdk1) activation and nuclear envelope breakdown[70,71]. *Wolbachia*-mediated modifications of either chromatin or mitotic factors are expected to be at the root of these aberrant phenotypes and the extent of modification is expected to affect the severity of mitotic delay. In cases, where the asynchronicity of paternal and maternal pronuclei condensation and alignment are marginal, abnormal segregation can lead to chromatin bridges (a hallmark of CI) and the production of inviable aneuploid progeny. In severe cases, failure of paternal pronuclei segregation results in haploid daughter nuclei, which in some haplodiploid insect species such as the parasitic wasp *Nasonia vitripennis* results in healthy haploid male production[72]. In diploid species such as *C. pipiens* haploid development can lead to a later stage of developmental arrest[73]. Interestingly, as *Wolbachia* are excluded from

mature sperm during individualisation, the CI observed in embryos is not a direct consequence of *Wolbachia*'s presence in the cell. Modifications that induce CI, must therefore occur either before endosymbiont exclusion or via secreted factors that persist in mature sperm. The presence of secreted CI factors was supported by the induction of CI by sperm produced from uninfected spermatid cysts of infected *Drosophila simulans* males[74]. However, the presence of these factors does not identify at what stage CI modifications occur. Although, the exact mechanisms of *Wolbachia* modification/*mod* remained unknown, what was established was that maternally-inherited *Wolbachia* possessed the ability to rescue/*resc* the CI phenotype.

1.2.5.1 Preliminary models of cytoplasmic incompatibility

Since the *mod/resc* mechanism of CI was first conceptualised[75], several models were formulated to explain how the bacterium mediates these two functions. These included the i) lock-and-key, ii) mistiming, and iii) goalkeeper models. Briefly, the lock-and-key model postulated that *Wolbachia* places "locks" (modifications) on the paternal chromatin which only a compatible strain can unlock (rescue). An initial criticism to this model stated that variation of CI induction and directionality between *Wolbachia* strains would require the bacteria to possess a great number of "lock" and "key" variations[76]. This abundance of encrypted lock and key sets was accounted for by the discovery of the two CI factors (Cifs) responsible for CI mediation[77,78] (discussed in detail in 1.2.5.2). Alternatively, the mistiming model proposed that compensatory modifications in infected females would restore the synchronicity of pronuclei mitotic progression[70]. If the *mod* and *resc* functions are the same, then this model fails to appropriately address why some strains display bidirectional incompatibility. The goalkeeper model was regarded as a refinement of the mistiming model[76]. It assumed that two factors are responsible for modification and that slight variations between the modification of the sperm and those imposed on the female chromatin result in CI. The main conceptual difference between the lock-and-key model and the mistiming/goalkeeper models was that the former distinguished independent roles of *mod* and *resc*, whereas the latter denoted the *resc* function as a result of compensatory actions of the *mod* factor(s). Although these models provided a framework to visualise different mechanisms of CI, without experimental evidence none of the models could be validated.

1.2.5.2 Discovery of cytoplasmic incompatibility factors

Due to the obligatory intracellular nature of *Wolbachia*, the discovery of factors associated with CI *mod* and *resc* functions was severely hindered. Detection of a *Wolbachia* peptide in *Culex* spermathecae from females mated with infected males led to the hypothesis that this factor may be involved in CI[79]. A subsequent comparison of *Wolbachia* genomes led to the discovery of two co-diverging genes only found in strains capable of inducing CI[77,78]. One of these genes encoded the factor identified in the prior proteomics study[79]. Transgenic expression of these gene products in *D. melanogaster* revealed that they were capable of recapitulating CI phenotypes[77,78,80,81]. These cytoplasmic incompatibility factor (*cif*) genes are repeatedly found in the same orientation[82] and are expected to be transcribed as a single operon[78] (although this is contested[81,83]), *cifA* and *cifB* denote the upstream and downstream genes respectively. There is a significant degree of *cif* gene variation between *Wolbachia* strains (with some strains containing multiple and divergent *cif* gene pairs), and these *cifA* and *cifB* variants can be sorted into five distinct phylogenetic groups (Types I-V)[82]. The presence/absence of *cif* genes and variations in *cif* gene sequences correlate with the CI crossing patterns observed in nature. Many of the *cif* genes can be found in the *Wolbachia* prophage WO region, which might explain the variability of *cif* genes in *Wolbachia* genomes, however other mobile elements such as transposons and plasmids, as well as recombination and duplication events could also be contributing factors[82]. All five *cifB* types have two PD-(D/E)XK nuclease domains, however Type I *cifB* genes lack the residues attributed to the nuclease activity and have instead a functional deubiquitinase domain. Some researchers refer to Type I Cifs as CidA-CidB and Type II-IV Cifs as CinA-CinB on account of their respective deuibiquitinase and nuclease activity. However, a recent study has shown that the Type I *cifB* gene from *w*Mel may possess some nuclease activity[84]. Furthermore, some Type V *cif* genes can possess both the active nuclease domains and a deubiquitinase domain[82]. Therefore, in this thesis the format $cifA/B_{\text{strain(Type)}}$ will be used when discussing CI genes e.g. the Type I *cifB* gene from the wMel strain will be written as $cifB_{w\text{Mel(TI)}}$.

1.2.5.3 Contemporary models of cytoplasmic incompatibility

The molecular mechanisms that result in CI induction and rescue remain to be fully resolved however it is hypothesised that CifA-CifB binding is integral. Initial pull-down studies showed

that CifA and CifB proteins interact, and that this interaction only occurs between cognate *cif* pairs[78]. Resolution of CifA-CifB complex structures, have revealed a large interface which can be divided into three regions[85,86]. Mutation of residues in these interface regions prevents binding[85,86], whilst substitution of these regions with residues from another CifA homolog permits binding of this chimeric protein with the CifB homolog[85]. Transgenic expression of *cifB* in the yeast species *Saccharomyces cerevisiae* results in inhibited cell growth, which can be rescued when the cognate *cifA* is also expressed[78,85–87]. Mutations in the CifA-CifB binding interface prevents rescue of growth inhibition in yeast[85,86], whilst chimeric CifA proteins can rescue the toxic effects of the non-cognate CifB[85]. A study in *Drosophila* S2R+ cells has mirrored these results in yeast whereby *cifB* expression causes apoptosis, which is not observed when *cifA* is either co-expressed or solely expressed[88]. Additionally, mutations in the catalytic residues of *cifB* have been shown to prevent CifB growth inhibition in yeast[78,80] and CI induction in transgenic *Drosophila*[77,78,80,89]. Comparison of *Wolbachia* genomes has revealed that strains that possess *cif* genes but are not known to induce CI either lack the syntenic *cifB* gene or it has been pseudogenised[82]. Together these data point to CifB being responsible for the *mod* function associated with CI induction and led some researchers to believe that the *cif* genes function in a toxin-antidote (TA) system[90]. TA systems are widespread throughout bacterial genomes and plasmids and are generally encoded in a single operon where the "antidote" is located directly upstream from the "toxin"[91]. The *cif* genes share this genetic architecture and are closely associated with mobile genetic elements (homologues have even been found in a plasmid of related *Rickettsia* species)[82]. In the context of CI, the toxin-antidote model posits that CifB catalytic activity will result in CI unless rescued by CifA expressed in the oocyte[90]. The TA model can be seen to conform to the lock-and-key model (described in 1.2.5.1) where the CifB toxin represents a lock and the CifA antidote the key. In concordance with the TA model of CI, transgenic expression of $cifB_{w\mathrm{No(TIII)}}$ in the male germline of *D. melanogaster* flies was found to induce CI, which could be rescued via female germline expression of $cifA_{w\mathrm{No(TIII)}}$[92]. This has also been demonstrated in transgenic *Anopheles gambiae* mosquitoes using Type I genes from *w*Pip[93].

Interestingly, prior studies in *D. melanogaster* using this pair of *cif* genes suggested that both $cifA_{w\mathrm{Pip(TI)}}$ and $cifB_{w\mathrm{Pip(TI)}}$ needed to be expressed in the male germline in order to induce CI[78]. This was also found to be true when using the Type IV *cif* genes from the same strain and the

Type I *cif* genes from *w*Mel, however in both cases maternal expression of *cifA* alone was sufficient for rescue[80,81]. This led to the formulation of a "two-by-one" model[81]. Mutations in *cifA* have resulted in loss of CI induction phenotypes as well as rescue in transgenic *Drosophila*, which suggested a role of CifA in both CI phenotypes[89]. Initial hypotheses about the role of CifA in induction and rescue of CI posited that CifA was the primary *mod* factor in both the male and female germlines, whilst CifB assisted CifA in its role in the male germline. This model of compensatory modifications in the oocyte would conform to the prior mistiming/goalkeeping models of CI (described in 1.2.5.1). However, this mechanism does not explain how rescuable CI has been demonstrated through the expression of only *cifB* in male germlines[92,93]. Therefore, the more likely role of CifA in CI induction is assisting CifB in its *mod* function. A potential method in which CifA might assist CifB's function is through changing its localisation within the host cell. Studies have shown that CifA and CifB can have different cellular localisations when expressed independently or in tandem[88,94]. Further evidence for CifA having a role in cellular localisation came from the discovery of a bipartite nuclear localisation signal (NLS) found in $cifA_{w\text{Mel(TI)}}$ which reduces CI penetrance in males when deleted [94].

The key difference between the TA model and the competing host-modification (HM) model (which encompasses the mistiming/goalkeeper models amongst others[95]) is that the TA model suggests that the modification of paternal genetic material occurs in the oocyte, whereas the HM model suggests that modifications of the paternal chromatin occurs before fertilisation and that rescue results from either the reversal of these modifications or a likewise modification in the fertilised oocyte (Figure 1.2). In the TA model the CifB toxin is expected to be packaged into mature sperm, upon fertilisation the CifB toxin would disrupt the paternal chromatin unless the CifA antidote is expressed in the oocyte. Evidence for CifB being packaged into sperm nuclei has been demonstrated in transgenic *D. melanogaster* studies using either the Type I *cif* genes from both *w*Mel and *w*Pip[88,94]. However, only the study focused on *w*Pip Cifs showed CifB transfer to the egg[88]. In the context of this model, CifA's roles might include attenuating the toxicity of CifB and/or localising the toxin to chromatin during spermatogenesis. As CI induction has been recapitulated through the expression of *cifB* only, the role of CifA-mediated CifB localisation does not seem integral to CI induction[92,93].

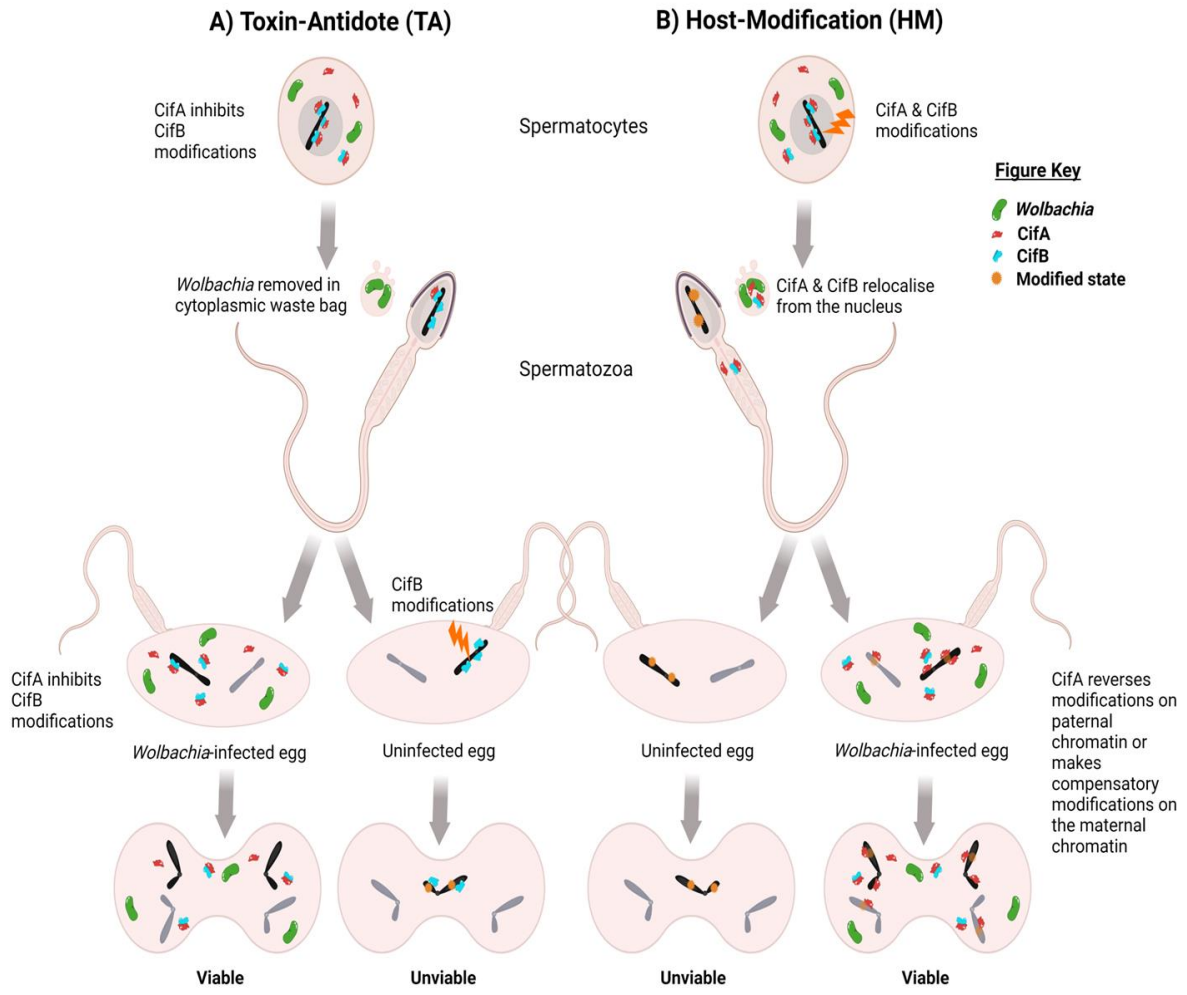


Figure 1.2: Illustration of the two diverging models of CI. A) The toxin-antidote(TA) model of CI posits that CifA binds to CifB during spermatogenesis which inhibits the "toxicity" of CifB, both CifA and CifB are localised in the nucleus during sperm maturation, CifA is either lost before mature sperm are produced or transferred to the egg and subsequently degraded. CifA in the oocyte/embryo supplied by a *Wolbachia* infection can bind to CifB preventing its toxicity, however, in the absence of CifA (uninfected eggs) CifB is free to modify the paternal chromatin leading to CI phenotypes. **B)** The host-modification (HM) model posits that CifA and CifB temporarily localise in the nucleus during spermatogenesis and modify the paternal chromatin. Both Cifs dissociate from the nucleus and are not transferred to the egg. CifA expressed by a *Wolbachia* infection either reverses the modifications on the paternal chromatin or makes compensatory modifications on the maternal chromatin. Without CifA in the egg, the modifications on the paternal chromatin leads to CI phenotypes.

The reason for why *cifA*$_{w\text{Pip(TI)}}$ is needed in CI induction in transgenic *Drosophila* but not in *Anopheles* may reflect differences in i) relative toxin expression levels based on promoter selection, and ii) host sensitivities to the toxin which would highlight a role of CifA in attenuating CifB toxicity. Supporting evidence for CifA attenuation comes from the *Anopheles* study, where it was found that when CifA was expressed at much higher levels than CifB, the CI phenotype was inhibited[93].

1.3 *Wolbachia* as a vector control method

1.3.1 *Wolbachia*-mediated pathogen inhibition

Wolbachia-mediated pathogen inhibition was first discovered in the fruit-fly *D. melanogaster*[47]. Although native infections of the *Wolbachia* strain *w*Mel can manipulate host reproduction, the spread of the bacterium is still threshold-dependent. Yet *w*Mel can spread efficiently through wild populations which suggested that the infection must confer some alternative fitness benefit for it to be maintained. Indeed, it was found that flies carrying *w*Mel had an increased survival rate after being challenged with natural pathogens *Drosophila* C virus (DCV) and Nora virus, as well as non-natural pathogen Flock House virus (FHV). It was found that *Wolbachia* reduced the viral titre of these pathogens (except FHV), which suggested *Wolbachia* may inhibit viral replication[47]. The fact that *Wolbachia* increased the survival rate but did not decrease the viral titre of FHV indicated that a *Wolbachia* infection may allow the host to tolerate some viral infections if it did not directly inhibit viral replication[47]. Since then, many strains of the bacterium have been shown to protect the host from a wide range of positive sense RNA viruses [(+)RNA], as well as filarial nematodes[48], and *Plasmodium* parasites[50,51]. The mechanisms behind this broad pathogen inhibition are not completely understood, however it likely involves a combination of factors mediated by *Wolbachia*[96–98]. Furthermore, inhibition is most often seen when a *Wolbachia* strain is transferred to a non-native host[99]. The initial hypothesis was that the bacterium primed the host innate immune system to be antagonistic to a pathogenic infection, however some *Wolbachia* infections in their native hosts do not activate the innate immune system yet still inhibit pathogen replication[100,101]. In transinfections, *Wolbachia*'s presence in cells is often linked to an increase in reactive oxygen species (ROS), which can activate the host's Toll innate immune pathway and antimicrobial peptide production[102]. Additionally, there is some

evidence that *Wolbachia* may suppress cell autophagy, which may be antagonistic to some viruses that induce autophagy to create cellular environments more conducive to replication[103–105]. An interesting observation is that *Wolbachia* are known to inhibit an array of (+)RNA viruses, but not negative sense RNA viruses [(-)RNA] or DNA viruses. A conserved feature of (+)RNA virus replication is the creation of sub-cellular compartments through the rearrangement of organelle membranes[106]. These compartments provide a scaffold for viral replication and protect the replication complexes from host immune factors. The membrane-lipid composition seems integral to the creation of these viral replication organelles, and some viruses are known to recruit the host lipid synthesis machinery to provide the right conditions for replication[106]. As *Wolbachia* are located in cytoplasmic vacuoles derived from host organelle membranes and are known to modulate cholesterol and lipid homeostasis, it has been suggested that the seemingly selective viral inhibition is based on *Wolbachia*-mediated perturbations of lipid pathways needed for (+)RNA virus replication [which DNA and (-)RNA viruses do not rely on][98]. Indeed, *Ae. aegypti* cells infected with *Wolbachia* were found to have a higher abundance of cholesterol stored in lipid droplets in comparison to uninfected cells, treatment with 2-hydroxypropyl-β-cyclodextrin released the trapped cholesterol which resulted in a rescue of DENV replication[98].

1.3.2 Transinfected *Ae. aegypti* releases

The idea to exploit CI to control mosquito vector populations was proposed before even the discovery of the causative agent, *Wolbachia*[107]. In the last decade there has been major progress in demonstrating the benefit *Wolbachia* based vector control strategies can have on reducing the disease burden worldwide. These strategies can consist of either population suppression or replacement. In *Ae. aegypti* populations, suppression can be achieved through the release of *Wolbachia* infected males only, whereas population replacement can occur when both infected males and females are released (Figure 1.3). Establishing and maintaining a *Wolbachia* infection in *Ae. aegypti* populations functions as a vector control intervention due to the bacterium's ability to broadly inhibit +ssRNA viruses (discussed in 1.3.1), a group of viruses which encompasses most of the notable arboviruses spread by this vector. The release of *Wolbachia*-infected males only is often referred to as the incompatible insect technique (IIT) and can successfully reduce vector population sizes by reducing reproductive capacity through sterile matings.

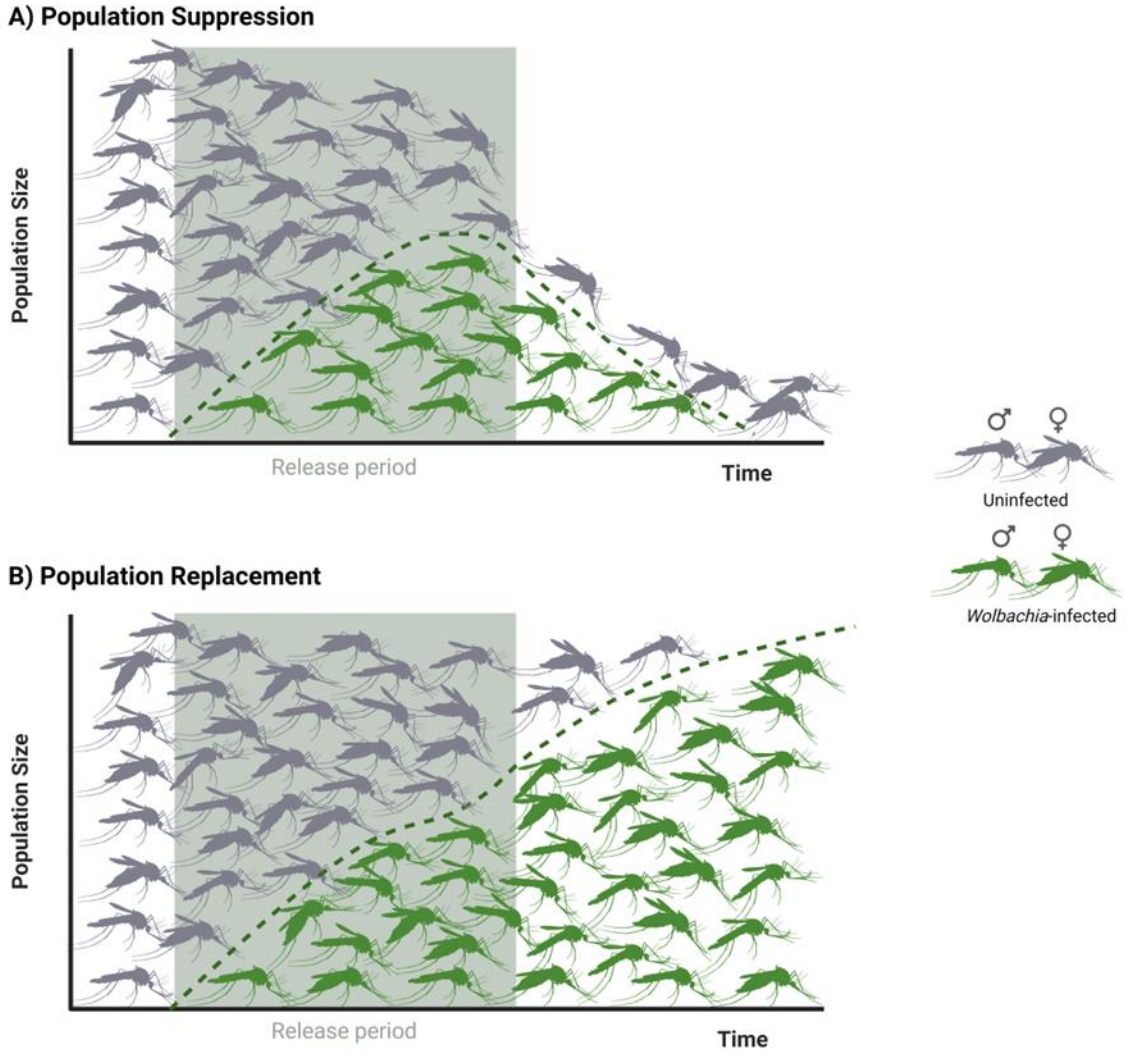


Figure 1.3: Illustration of *Wolbachia*-mediated *Ae. aegypti* population suppression and replacement strategies. A) Population suppression: Release of *Wolbachia*-infected males only leads to a reduction of the population size. **B)** Population replacement: Release of both *Wolbachia*-infected males and females leads to an increase in the *Wolbachia* infection frequency (dashed line) until fixation.

There are no known native *Wolbachia* infections of *Ae. aegypti*[108], however transfer of infected embryo cytoplasm from alternative species using embryonic microinjection has permitted the creation of *Ae. aegypti* lines carrying different strains of the bacterium[99,109–112]. Different strains can display variable levels of i) bacterial density, ii) tissue tropism, iii) pathogen inhibition, iv) temperature sensitivity, v) host fitness and fecundity effects, and vi) CI penetrance[109,113–115]. Therefore, the selection of the appropriate strain for transinfection is crucial for the long-term efficacy of *Wolbachia* as a vector control tool in release regions. The

first *Wolbachia*-infected *Ae. aegypti* mosquitoes involved *w*MelPop, a strain found in laboratory reared *D. melanogaster*. This strain is considered pathogenic and can shorten the lifespan of the infected host[116]. It was hoped that the *Wolbachia* infection would prevent adults reaching transmission age without crashing the population. However, it was found that this strategy was not self-sustaining, and it would be preferential to use a non-pathogenic strain like the closely related *w*Mel[117,118]. In 2011, *w*Mel-carrying *Ae. aegypti* were released in Cairns, Australia, and have since established an infected population that has significantly reduced the incidence of dengue by 96%[10]. Subsequent releases of mosquitoes carrying this strain in both Yogyakarta, Indonesia, and Rio de Janiero, Brazil, have met with similar success with an average decrease in dengue cases of 77% and 69% in their respective release sites[119,120]. Due to this success, the release of *Ae. aegypti* infected with *w*Mel is ongoing and several new countries including Columbia, Fiji, Mexico, New Caledonia, and Sri Lanka have started trial releases.

However, not all releases have been successful in establishing populations of infected mosquitoes. In Nha Trang City, Vietnam, the prevalence of the bacterium fluctuated in response to seasonal-temperature changes and was subsequently lost in two release site areas[121]. Recently, it has been found that *w*Mel is more susceptible to heat stress during rearing than alternative strains[121–123]. This susceptibility results in decreased *Wolbachia* densities which can reduce the ability to both inhibit pathogens and manipulate the host's reproduction[113,115]. The *w*AlbB strain naturally found in *Aedes albopictus*, is less susceptible to heat stress and provides an alternative to *w*Mel when considering releases in regions that experience extreme heat fluctuations[113,124]. This strain is also efficient at blocking arboviral transmission and releases of *w*AlbB-carrying *Ae. aegypti* in urban sites of Greater Kuala Lumpur, Malaysia, have led to a decrease in dengue cases of around 40-85%[9]. Releases of males carrying this strain have also been used to reduce the size of *Ae. aegypti* populations in the United States, Australia, Mexico, and Taiwan[8,125–127]. One limitation to consider when selecting this strain for future releases is that prolonged egg desiccation can result in infertile infected females[114]. This effect could be problematic in regions that experience long dry seasons yet preliminary data suggests that the effect of egg quiescence on infected female fertility is largely dependent on the host's susceptibility to ROS which varies between host backgrounds (Unpublished data).

1.3.3 Concerns and limitations surrounding the use of *Wolbachia.*

As both the heat sensitivity of *w*Mel and the quiescence associated fertility costs of *w*AlbB were found after releases had commenced, it remains unknown what other negative effects or limitations to the use of *Wolbachia* as a vector control will be discovered for these or any other *Wolbachia* strain. Transinfected *Wolbachia* strains may co-evolve with their new host, and this might attenuate the bacterium's ability to inhibit arboviruses. For instance, *Ae. albopictus* is naturally infected by two strains (*w*AlbA and *w*AlbB) yet is still capable of transmitting several notable arboviruses. As *Wolbachia*-mediated pathogen inhibition is believed to be cell-autonomous, relatively high densities of *Wolbachia* in the mosquito's midgut and salivary glands should be maintained to efficiently reduce arboviral transmission[109,128]. However, the somatic tissue densities of *w*AlbA and *w*AlbB in *Ae. albopictus* are relatively low and both are mostly restricted to germline tissues[129]. Interestingly, when *w*AlbB is transferred to *Ae. aegypti* the relative density and range of infected somatic tissues increases leading to efficient pathogen blocking in this host[130,131]. This can also be observed when a non-native strain such as *w*Mel is introduced into *Ae. albopictus* which suggests that the restricted tissue tropism of native infections is strain-specific[132]. Therefore, if this phenomenon occurs in transinfected *Ae. aegypti* it may be rectified by replacing one strain with another. Interestingly, one study reported that *w*Mel-infected *Ae. aegypti* host lineages that were selected for a weaker blocking phenotype (based on natural host genetic variation) tended to have lower fitness for various life-history traits, which suggests that mosquitoes with strong blocking phenotypes may be selected for in the wild[133].

Another concern surrounding the use of *Wolbachia*-mediated population replacement strategies, is that the bacterium may increase the susceptibility of the host to certain viruses or promote viral escape mutations. A study in *D. melanogaster* showed that *Wolbachia* infection selected for certain viral populations when challenged with a heterogenous population of DCV[134]. Although this suggests *Wolbachia* imposes a selection pressure for viral evolution, DCV was unable to evade the antiviral properties a *Wolbachia* infection provides[134]. As *Wolbachia*-mediated viral inhibition is hypothesised to be multifaceted, this might explain why DCV (a natural pathogen) has been unable to evolve resistance to *Wolbachia* strains native to this host species. This multifaceted antiviral effect likely makes the use of *Wolbachia*

more ‘evolution-proof’ than more direct methods of tackling arboviruses such as insecticides or antiviral drugs. This remains to be proven, yet *Wolbachia*-carrying mosquitoes have been released and monitored for over a decade and no cases of arboviral evolution in response to the endosymbiont have arisen. There are some reports, however, which suggest a *Wolbachia* infection might increase the susceptibility of *Ae. aegypti* to certain viruses[135–137]. For example, a study suggested that *w*Mel-carrying mosquitoes in Cairns, Australia, were more susceptible to insect-specific flaviviruses (ISFs)[136]. ISFs do not replicate in humans so do not pose a direct health threat, however as they belong to the same virus family as major arboviruses, DENV and ZIKV, it may suggest *Wolbachia*’s blocking ability is not conserved for all flaviviruses. However, this study did not provide adequate controls such as *Wolbachia*-free mosquitoes from the wMel release sites, so the supposed susceptibility of *Wolbachia*-infected mosquitoes to ISFs may actually reflect natural geographical differences in ISF abundance and/or the genetic background of the sampled populations[136].

Co-infecting viruses are known to either enhance or hinder the replication success of one another - which in the context of mosquitoes results in differences in vector competence. For example, a correlation between infection with *Culex* flavivirus (an ISF) and a reduction of West Nile virus (an arbovirus) dissemination was observed in *C. pipiens*[138]*.* Additionally, *Ae. albopictus* cells persistently infected with a DNA insect-specific virus (ISV), *Aedes albopictus* densovirus (*Aal*DNV), were more refractory to DENV-2 infection[139]. Interestingly, it has been found that *Wolbachia* infection can enhance the replication of *Aal*DNV in *Ae. aegypti* cells[137]. Therefore, *Wolbachia*-mediated enhancement of the host’s susceptibility to certain ISVs might actually further reduce vector competency through this superinfection exclusion phenomenon[140]. That being said the dynamics associated with superinfections in mosquitoes can be highly complex, and it is not fully understood how interactions between *Wolbachia* and the host microbiota will ultimately affect arbovirus transmission. Although *Wolbachia* has proven an effective vector control tool for several years now, to alleviate the concerns of viral enhancement, research should be conducted on potential ways this method can be edited or enhanced once a *Wolbachia* infected population has already been established.

1.4 Genetic control of *Ae. aegypti* populations

1.4.1 Current methods

SIT has been used to suppress population sizes of agricultural pests for more than 65 years, and this technique can be applied to vector populations[11,141,142]. SIT traditionally involves the release of males that have been sterilised through radiation or chemical treatments, which when mated with wild females results in a reduction of viable offspring. A limitation to this technique is that the sterilised males often have a reduced fitness and mating competitiveness in comparison to wild-type males. A variant of SIT known as RIDL works through the creation of vector populations carrying a dominant lethal transgene under the control of a repressible system[12,13]. The transgenic OX513A line of *Ae. aegypti* expresses a tetracycline-repressible transcriptional transactivator (tTAV) that binds to an upstream tetracycline response element (TRE) which further increases the expression of tTAV[13]. In the wild, this positive feedback loop results in lethality at later stages of development, however when mosquitoes are reared in the presence of tetracycline the expression of tTAV is repressed which permits the mass-rearing of these mosquitoes. Because the lethality does not occur until later stages of development, once transgenic males have been released the resulting offspring can compete with the wild-type larvae for resources. This reduces the survival of non-transgenic offspring and therefore this strategy requires a lower overflooding ratio (sterile:wild males) than alternative SIT/IIT methods to sufficiently supress a population[13]. Trial releases of the OX513A line has been shown to successfully reduce wild *Ae. aegypti* population sizes in the Caymen Islands, Brazil, and Panama[14,147,148]. A limitation of these population suppression techniques was that releases relied on a labour-intensive process of sex-sorting to ensure only adult males are released. Introduction of a sex-specific intron in the gene encoding tTAV results in a lethal phenotype in females only, half of the viable male offspring will carry the transgenes and mate with the wild-type females and this cycle will continue in a self-limiting manner[149]. This female-specific RIDL (fsRIDL) approach negates the need for sex-sorting and allows for the release of both larvae and adults.

1.4.2 Gene Drive Systems

Gene drives are selfish genetic elements that bias their inheritance in offspring over the expected 50%. Synthetic recapitulation of this super-mendelian inheritance allows drive of

desirable transgenes through vector populations and can be utilised to either supress or replace said populations. There are several types of gene drive systems which can be split into categories based on their temporal dynamics (self-propagating, majority wins, and self-limiting) as well as their spatial dynamics (localised and non-localised)[150]. Self-propagating gene drives (e.g. autonomous homing-based gene drives (HGDs), and *Medea*) have a low threshold frequency for invasion. The threshold is determined by the number of individuals with a gene drive element that need to be released relative to the target population in order for the gene drive to spread. Low threshold gene drives can spread rapidly through a population at low release numbers and are non-localised in their spread meaning they can invade neighbouring populations. Whereas high-threshold drives (e.g. underdominance, and toxin-antidote systems) require a higher proportion of the population to carry the drive components for the system to spread through a population, and therefore need a large initial release number (which exceeds the threshold frequency) or rely on repeated releases. Self-limiting gene drives (e.g. split HGDs, daisy drive and killer-rescue systems) can have either high or low release thresholds but can only spread/persist in a population for a short period of time, both self-limiting and high-threshold gene drives are localised in their spread and can be restricted to a target population.

The discovery and subsequent exploitation of the CRISPR/Cas system has accelerated the design of gene drive systems and has led to the creation of the only systems to be tested in caged *Ae. aegypti* populations[151,152]. These systems represent a self-limiting split HGD, where the Cas9 endonuclease and the guide RNA (gRNA) are located on different genomic loci. When a mosquito possesses both components the gRNA guides the Cas protein to a target site which is then cleaved and repaired by homology-directed repair (HDR) leading to the generation of homozygous germline cells and therefore super-mendelian inheritance of the transgene cassette. In these studies the gRNA was linked to a fluorescent reporter gene however this could be replaced with an effector gene which would transform this gene drive into a vector control tool. For example, the reporter gene could be replaced with the male-determining factor *Nix*, which can masculinise transgenic females resulting in population suppression[153]. Alternatively, if these systems were used for population replacement, there are several types of effector genes that have been shown to be effective at targeting arboviruses transmitted by *Ae. aegypti*. For instance, double-stranded RNAs (dsRNAs) or micro RNAs (miRNAs) can

activate the host RNA interference (RNAi) pathway to target and cleave the genomes of key arboviruses such as DENV and ZIKV[154–157]. Alternatively, hammerhead ribozymes can be designed to target and cleave arboviruses without the need for any endogenous host machinery[158,159]. Another promising group of antiviral effectors are single-chain variable fragment antibodies (scFv), one scFV has been shown to render transgenic mosquitoes refractory to all four serotypes of DENV[160]. Many of these effector types, especially miRNAs and hammerhead ribozymes show the potential to be multiplexed and therefore gene drives linked to these effectors can be used to target multiple arboviruses simultaneously[161]. However, like with *Wolbachia* based replacement strategies there are concerns that the targeted viruses can evolve to evade these effectors rendering the released gene drive defunct. Furthermore, there is a real concern that the gene drive components themselves may over time lose functionality. For example, strategies that rely on endonucleases, such as the split HGD described above, have inherent problems that will impact the efficacy of the drive e.g. cleavage-resistant alleles can occur either through natural sequence variation in the target site between wild populations or when the cut site is repaired by nonhomologous end-joining (NHEJ) instead of HDR[151]. Recent work has focused on methods to recall or override gene drives in order to address the concerns and limitations surrounding the efficacy and confinement of gene drives.

1.4.3 Exploitation of Cifs in the design of gene drive systems

Several synthetic gene drive systems have been adapted from natural drive systems. For example, *Medea* elements are widespread in wild populations of the red flour beetle *Tribolium castaneum*[162]. *Medea* elements consist of two linked genes: a maternally expressed toxin and a zygotically expressed antidote. Synthetic recapitulation of this system in *Drosophila* was achieved using maternally expressed miRNAs that targeted a gene necessary for embryo development and a miRNA-insensitive version of that gene which was expressed in the zygote[163]. Although the application of this *Medea* drive system to vector control strategies is appealing, the non-localised spread of the drive poses the threat of an unrestricted spread of the drive elements to neighbouring populations. Therefore, more localised forms of toxin-antidote based drive systems are being considered for future trial releases. One such method is called a *Semele* drive system which comprises of a toxin expressed by transgenic males which upon mating either kills or renders wild-type females

infertile unless they express the required antidote[164]. Unlike the *Medea* system this drive would require a higher introduction frequency threshold and can therefore be confined to select populations. This system closely resembles *Wolbachia*-mediated CI where a *mod* factor expressed by infected males renders wild-type females infertile unless they are likewise infected and the fertilised oocytes express a *resc* factor. Now that the genes associated with the *mod* and *resc* functions of CI have been discovered gene drive systems incorporating these elements can be envisioned and adapted for use in *Ae. aegypti* population replacement and suppression strategies.

One benefit to creating a gene drive system using Cifs is that *Wolbachia*-mediated CI can occur in a wide range of arthropod hosts meaning the strategy could be readily adapted to any given vector species. This is especially useful in vector species that have proven harder to transinfect with *Wolbachia* strains such as *An. gambiae.* Furthermore, there are a vast array of naturally occurring *cif* types and variants that can either be swapped if the host develops resistance to one of the *cif* "toxins" or combined in underdominance-like systems to further confine the spread of the drive. The use of *cif* genes to build gene-drive systems was recently modelled and showed promising results[165]. However, as the proposed mechanism of CI seems to differ depending on which transgenic model and *cif* variants are used in a given study, to further inform future gene drive designs involving *cif* genes in *Ae. aegypti* a detailed study on how they function in this species must be undertaken.

1.5 Aims of this study

The aims of this study were to establish transgenic lines of *Ae. aegypti* mosquitoes that express *Wolbachia cif* genes in either the male or female reproductive tissues and to characterise their effect on fertility. Different promoter and *cif* sequences were utilised to provide comparison between sterility phenotypes based on either expression level/ localisation or Cif type respectively. It was expected that the data gathered from this study could be used to inform the generation of gene drive systems utilising *Wolbachia cif* genes.

Chapter 2:

Materials and Methods

2. Materials and methods

2.1 Molecular methods

2.1.1 Nucleic acid quantification

Nucleic acids were quantified using a NanoDrop 1000 or NanoDrop One spectrophotometer (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Double distilled H_2O(ddH_2O) was used as a blank whilst 1ul of nucleic acid solution was used for measurement. Purity of a sample was determined by A_{260}/A_{280} and A_{260}/A_{230} ratios.

2.1.2 Gene synthesis

The coding sequences of the Type I *w*Pip(Pel) *cifA* (CAQ54390.1) and *cifB* (CAQ54391.1) genes, and the Type IV *w*AlbB cifA (QBB83746.1) and *cifB* (QBB83745.1) were codon optimised for expression in *Ae. aegypti*, synthesised, and cloned into pUC-GW-Amp plasmids using the GENEWIZ PriorityGENE service (Azenta Life Sciences, Germany). The *cifA* genes were His-tagged whilst the *cifB* genes were Flag-tagged and flanked by an upstream SacII and downstream ApaI restriction site.

2.1.3 Polymerase chain reaction (PCR) amplification

Q5 High Fidelity DNA polymerase was used as per the manufacturer's recommendation to amplify DNA fragments for cloning/sequencing. Both genomic and plasmid DNA were used as templates for PCR amplification. Primer sequences were designed using the Primer3 programme (https://primer3.ut.ee/) and synthesised as oligonucleotides (Sigma-Aldrich, USA).

2.1.4 Agarose gel electrophoresis and purification

Agarose gel electrophoresis was used to separate DNA fragments of different sizes. Samples were loaded into a 1% agarose gel made with 1X TAE buffer (stained with ethidium bromide) and ran in a gel tank containing 1x TAE buffer at 120V. When a loading dye was needed, samples were combined with 6X Purple Gel Loading Dye (NEB, USA) and 1kb Plus DNA Ladder (NEB, USA) was used to estimate DNA fragment size. Gels were visualised using a UV light transilluminator and desired DNA fragments were excised from the gel and DNA was purified using the Monarch® DNA Gel Extraction Kit (NEB, USA) according to the manufacturer's instructions.

2.1.5 Restriction enzyme cloning

Type II restriction enzymes were selected based on restriction sites flanking a desired DNA sequence, which were identified using the SnapGene software (https://snapgene.com/). 1µg of plasmid DNA/PCR product were digested with the selected restriction enzymes and the recommended enzyme buffers at a temperature specified by the manufacturer for a minimum of an hour. The restriction enzymes/ primers used to generate donor plasmids using the restriction cloning method are listed in Figure A1. Digested fragments were analysed and purified using the methods stated in section 2.1.4 Purified DNA fragments were mixed with T4 DNA ligase and 10x T4 DNA ligase buffer (NEB, USA) and incubated overnight at cycling temperatures (16°C for 2 minutes and 22°C for 2 minutes). The resulting ligation product was transformed into *E. coli* bacterial cells (Section 2.1.7).

2.1.6 HiFi DNA assembly cloning

When restriction enzyme cloning was not possible, the HiFi DNA assembly protocol (NEB, USA) was used. Primers were designed using the NEBuilder Assembly Tool (https:// https://nebuilder.neb.com/) and used to amplify DNA sequences with a minimum overlap of 20bp. The primers used for HiFi cloning are listed in Figure A1. Purified DNA fragments (0.05pmols each) were added to a NEBuilder® HiFi DNA Assembly Master Mix (NEB, USA), diluted to 10µl with ddH_2O, and incubated for an hour at 50°C. The resulting ligation product was transformed into *E. coli* bacterial cells (Section 2.1.7)

2.1.7 Bacterial transformation

2µl of the ligation product was added to a 50µl vial of NEB® 5-alpha Competent *E. coli* (NEB, USA) cells that were thawed for 10 minutes on ice. The mix was incubated a further 30 minutes on ice before being heat shocked for 30 secs at 42°C, and then placed back on ice for a further 5 minutes. 950µl of S.O.C. medium (NEB, USA) was added to the vials, which were then incubated and shaken at 37°C for an hour. The transformed cells were then plated on LB Agar plates containing 100µg/ml ampicillin.

2.1.8 Colony PCR

25µl reaction mixes were prepared using 2X DreamTaq Green Buffer, DreamTaq DNA polymerase (Thermo Fisher Scientific, USA) and selected primers. Bacterial colonies were

picked using a pipette tip and dipped into the PCR reaction mixes before being ejected into Universal tubes containing 3ml of LB broth (containing 100µg/ml ampicillin). The extension time and annealing temperature were adjusted based on PCR product size and primer annealing temperatures respectively, and the initial denaturing step was extended to 5 minutes. Agarose gel electrophoresis was used to analyse the PCR products and identify bacterial colonies containing the transformed plasmid DNA. Universal tubes containing the desired colonies were selected for plasmid DNA extraction (Section 2.1.9).

2.1.9 Plasmid DNA extraction

For minipreparation of plasmid DNA, 7ml of LB broth (containing 100µg/ml ampicillin) was added to the 3ml of LB broth (containing 100µg/ml ampicillin) containing the selected bacterial colony and left to shake overnight at 180rpm and 37°C. A QIAprep® Spin Miniprep Kit (QIAGEN, USA) was then used to extract and purify the plasmid DNA according to the manufacturer's instructions. Plasmid DNA was eluted in 12µl of ddH_2O. For midipreperations, the 3ml of LB broth (containing 100µg/ml ampicillin) containing the selected bacterial colonies was added to a conical flask containing 97ml of LB broth (containing 100µg/ml ampicillin) and left to shake overnight at 180rpm and 37°C. 50ml of the mix was aliquoted to a 50ml falcon tube and spun at 6800xg for 30mins, the supernatant was removed and the remaining 50ml of bacterial culture was added and the spin repeated. A QIAGEN® Plasmid Midi Kit (QIAGEN, USA) was then used to extract and purify the plasmid DNA according to the manufacturer's instructions. Plasmid DNA was resuspended in 40µl of ddH_2O.

2.1.10 Sanger Sequencing

PCR products and isolated plasmid DNA were Sanger sequenced either by Source BioScience, UK or GENEWIZ (Azenta Life Sciences, Germany) using the sample submission guidelines provided.

2.1.11 Genomic DNA extraction

An adult male/female mosquito was anaesthetised on ice, before being homogenised in 100µl of STE buffer (Sigma-Aldrich, USA) using a hand-held pestle. The homogenate was boiled at 95°C for 10 minutes, placed on ice for 10 minutes and then spun at 12,000xg for 15 minutes. The supernatant was diluted with ddH_2O in a 1:10 ratio.

2.1.12 Quantitative PCR (qPCR)

The relative density of *Wolbachia* was quantified through qPCR using the Rotor-Gene Q (QIAGEN, USA) machine. 10µl reaction mixes comprised of 2µl genomic DNA, 5µl 2x QuantiNova® SYBR® Green (QIAGEN, USA), 2µl of ddH_2O, and 0.5µl of both the forward and reverse primers. The following primers were used to target the *Wolbachia* 16S (q16S-F: GAAAGCCTGATCCAGCCATG and q16S-F: CGGAGTTAGCCAGGACTTCT and host HTH gene (qHTH-F:TGGTCCTATATTGGCGAGCTA and qHTH-R:TCGTTTTTGCAAGAAGGTCA). Cycle conditions are listed in Table 2.1.

Table 2.1: Cycling conditions used on the Rotor-Gene Q machine

Stage	Temperature (°C)	Time
Hold	95	15 minutes
Cycling (40x)	95	15 secs
	60	20 secs
Melt curve	65	1 minute 30 secs
	65-95	5 secs

2.1.13 RNA extraction

Biological samples were added to a 2ml screw-cap microtube containing 500µl of TRIzol (Sigma-Aldrich, USA) and 1mm borosilicate glass beads (Sigma-Aldrich, USA) and homogenised using a Precellys 24 tissue homogenizer (Bertin Technologies, France). Homogenates could be stored at -80°C for a considerable time. After thawing, 120µl of chloroform was added and the mix was vortexed and left to stand at room temperature (RT) for 3 minutes before centrifugation at 13,500xg for 15 minutes at 4°C. The top aqueous phase was then transferred to an Eppendorf containing 250µl of isopropanol, mixed and left to stand for 10 minutes at RT. The tubes were then centrifuged at 9,500xg for 10 minutes at 4°C to pellet the RNA. The pellet was rinsed twice with 70% ethanol solution before being resuspended in 12µl of ddH_2O.

2.1.14 Reverse transcription qPCR (RT-qPCR)

Quantification of gene expression was performed using RT-qPCR. Firstly, RNA samples were converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher

Scientific, USA) using the manufacturer's instructions in 20µl reactions. The cDNA was diluted to 100µl with ddH_2O. Experiments comparing the expression of genes in the ovaries and remaining carcasses of transgenic females were conducted using the Rotor-Gene Q (QIAGEN, USA) machine, and 10µl reaction mixes comprised of 2µl cDNA, 5µl 2x QuantiNova® SYBR® Green (QIAGEN, USA), 2µl of ddH_2O, and 0.5µl of both the forward and reverse primers. Cycle conditions are the same as those listed in Table 2.1. Experiments comparing the expression of genes in transgenic males were conducted using the QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific, USA). 10µl reaction mixes comprised of 2µl cDNA, 5µl Fast SYBR™ Green Master Mix (Thermo Fisher Scientific, USA), 2µl ddH_2O, and 0.5µl of both the forward and reverse primers (sequences listed in Table 2.2). Cycle conditions are listed in Table 2.3.

Table 2.2: Primers used to determine the relative expression of *cif* genes.

Target gene	Primer sequence (5'-3')
$cifA_{w\text{Mel(TI)}}$	GACCCCAATCGAAACCAAGC
	GTTTCCACCCAACGCTTGAT
$cifB_{w\text{Mel(TI)}}$	GATCAACACCATCCTGCGTC
	GTTTGCAGCCAGTAACGGAA
$cifA_{w\text{AlbB(TIV)}}$	GCGAACGATACACCACCTTC
	TTCCCACACGTTCATCATGC
$cifB_{w\text{AlbB(TIV)}}$	AAGATCGCCATCCTGACCAA
	GCGATTTTCTCCAGCTCTCC
$cifB_{w\text{Pip(TI)}}$	CCGAACGATCTGGGACTGTA
	CTTGGTTCAGGGTGTTGTGG
rps17	CACTCCCAGGTCCGTGGTAT
	GGACACTTCCGGCACGTAGT

Table 2.3: Cycling conditions used on the QuantStudio™ 3 Real-Time PCR System

Stage	Temperature (°C)	Time
Hold	95	20 secs
Cycling (40x)	95	1 sec
	60	20 secs
Melt curve	95	1 sec
	60	20 secs
	95	1 sec

2.1.15 Sequencing of the *cifA*$_{w\text{Pro(TI)}}$ gene

DNA from *w*Pro-infected adult mosquitoes was conducted as stated in section 2.1.11. Primers designed to target the expected flanking regions of *cifA* based on the genome sequence of *w*Mel were used to amplify a DNA sequence through PCR (Table 2.4). The PCR product and selected primers (Table 2.3) were sent to Source BioScience, UK, for Sanger sequencing. A consensus sequence was generated using the sequencing reads (using the SnapGene software) and compared to the *cifA*$_{w\text{Mel(TI)}}$ gene sequence using the BLASTN program (NCBI, USA).

Table 2.4: Primers used to sequence the *cifA*$_{w\text{Pro(TI)}}$ gene

Primer Name	Primer sequence (5'-3')
cifA Flanking F	GCTGAACAGAACTGAAGGGC
cifA Flanking R	ACCATCAAGATCTCCATCCACT
cifA Internal F	TGACCAGAACGTTTGGGTATCA
cifA Internal R	ACACAAAGCACGTCTCTATTTGC

2.2 Mosquito maintenance and experimental procedures

2.2.1 Mosquito strains and rearing

All mosquito colonies were maintained at 27°C and 70% humidity with 12-hour light/dark cycles. Larvae were fed tropical fish pellets (Tetramin, Tetra, Melle, Germany) whilst adults had access to a 5% sucrose meal ad libitum. Blood meals were provided using a Hemotek artificial blood-feeding system (Hemotek, UK) and human blood (Scottish National Blood Bank, UK). Damp filter-paper (Grade 1 filter paper, Whatman plc, GE Healthcare, Coventry, UK) was provided as an oviposition source for egg collection. Eggs were desiccated for 5-10

days before hatching in water containing 1g/l bovine liver powder (MP Biomedicals, Santa Ana, California, USA). The *w*Mel, *w*AlbB, and *w*Pro *Ae. aegypti* lines used in this study were generated previously through embryo microinjection, the method of which is described in Blagrove *et al.,* 2012.

For the Tet-Off experiments, when doxycycline is needed to repress the system a stock solution of doxycycline hydrochloride (Sigma) (300µg/ml) is prepared. Adults have access to a 5% sucrose meal with doxycycline (Dox) stock solution added to a final concentration of 3µg/ml. Egg cones during oviposition are soaked in 3µg/ml Dox and ddH_2O solution and are hatched after drying in 3µg/ml Dox solution and ddH_2O.

2.2.2 Generation of transgenic lines

The *piggyBac* transposon system was used in this study to transform *Ae. aegypti* mosquitoes with *cif* genes from different *Wolbachia* strains. This system is comprised of two components, a transposase and a non-autonomous transposon: the *piggyBac* transposase facilitates the specific insertion of the donor transposon (carrying the genes of interest) at random 'TTAA' target sites found within the genome. The pseudo-random nature of *piggyBac* insertion can result in differences in transgene expression based on the insertion site (positional effect). So, when possible, multiple lines consisting of different insertional sites were generated and characterised to ensure any phenotype observed was based on the transgene's expression and not an artefact of the transgene insertion. However, positional effects on transgene expression can be advantageous to a study when comparing the effects of expression levels on an expected phenotype. *Ae. aegypti* embryos were microinjected using the method described in Morris, Eggleston, and Crampton (1989) using a Nikon Eclipse TS100 microscope and air pump (Jun-Air, Denmark). Injection mix consisted of a final concentration of 500ng/µl donor plasmid and 300ng/µl helper plasmid in 1x injection buffer. Oil was dabbed away from injected embryos using a paper towel, and embryos were placed using a fine-tip paintbrush onto a damp filter paper contained within a petri dish. The petri dish was sealed using Parafilm M and embryos were left for 4 days before hatching. Microinjection survivors (G_0) were screened for marker gene expression at the 4^{th} instar larval stage using the Leica M165 FC fluorescent microscope and appropriate filter setting, those transiently expressing the marker gene were individualised and mated with 3 wild-type mosquitoes. To identify transgenic lines the resultant G_1 generation from these crosses were likewise screened at the 4^{th} instar.

Transgenic lines were maintained by mating virgin transgenic females with an excess of wild type males, offspring were screened at 4th instar stage. A complete list of all donor plasmids and how they were created is listed in Figure A1.

2.2.3 Viability and hatch rate assays

Virgin females were mated with an excess of males in small (15x15x15cm) Bugdorm cages (Megaview Science Co., Taiwan). After blood-feeding, non-bloodfed females were removed and bloodfed individuals were left 3 days to become gravid. Females were then individualised onto small damp filter-paper disks (Grade 1 filter paper, Whatman plc, GE Healthcare, Coventry, UK). Females were left for 2 days to lay eggs, after which the females were removed and spermathecae were dissected to confirm mating. Egg cones were left for 5-10 days before counting the percentage viability of embryos on each cone. The viability (ie. the ability to hatch) of *Ae. aegypti* embryos can be observed easily due to morphological differences using a light microscope (Figure A2). To ensure the percentage viability count was reliable selected cones were floated, and after 2 days the egg cones were removed and dried, and the percentage hatch rate was calculated.

2.2.4 Dissection of *Ae. aegypti* tissue

Adult mosquitoes were anaesthetised on ice before dissection. Tissues were dissected in a drop of phosphate-buffered saline (PBS) using a dissection microscope. For maternal gene expression experiments ovaries were dissected from individual females and the remaining carcasses were kept. For paternal gene expression experiments, testes were extracted from pools of 3 males and the remaining carcasses were kept. To determine if females were inseminated, spermathecae were dissected and placed in a drop of PBS on a glass slide before a cover slip was placed on-top. The slide was then viewed under a Nikon Eclipse TS100 microscope to look for the presence of sperm. A similar method was used to analyse sperm production; however it was the testes and seminal vesicles that were dissected, and the coverslip was lightly pressed to break the tissue and release the sperm if any was produced. Brightfield images of testes and seminal vesicles were taken using the Leica DMi8 (Leica Microsystems, Germany) using a 10x objective lens.

2.3 Immunostaining and fluorescent microscopy

2.3.1 Protein extraction

For extraction of protein from insect cell lines: *Ae. albopictus* Aa23 cells were plated into 6 well plates and either transfected with a *cif* gene plasmid or mock transfected using Lipofectamine™ 3000 Reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Cells were left for 3 days and then lysed using Passive Lysis Buffer (Promega, USA). Prior to plating, the Aa23 cells were cultured in 25 cm^2 flasks containing 5 ml of Schneider's media (supplemented with 10% foetal bovine serum) and kept at 27°C.

2.3.2 Polyacrylamide gel electrophoresis (PAGE) and western blotting

Protein samples were first quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA) as per the kit's instructions and the results read on a plate reader. Protein sample mixes were composed of 2.5µl 4X NuPAGE™ LDS Sample Buffer (Thermo Fisher Scientific, USA), and extracted protein (the concentration of protein was equilibrated between samples through dilution with RIPA Lysis and Extraction Buffer and made up to 10µl). The samples were heated to 70°C for 10 minutes before loading into 15-well Mini-PROTEAN® TGX Stain-Free™ Precast gels (Bio-Rad Laboratories, USA). PageRuler™ Plus Prestained Protein Ladder (Thermo Fisher Scientific, USA) was used as a size standard. The gels were run in a Bio-Ras gel tank with 1x Tris/Glycine/SDS (TGS) buffer (Bio-Rad Laboratories, USA) at 100V until the samples had migrated to the bottom. Proteins were transferred to PVDF membranes using the TransBlot® SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, USA). Membranes were blocked in 5% bovine serum albumin (BSA) and PBS solution for 1 hour before adding the primary antibodies (diluted in a 1% BSA-PBST solution) and left to rock overnight at 4°C. The primary antibodies used in these experiments were the Anti-DYKDDDDK Tag Mouse Monoclonal Antibody (FG4R), HRP (Product # MA1-91878-HRP, 1:500 dilution) and 6x-His Tag Recombinant Rabbit Monoclonal Antibody (RM146) (Product # MA5-33032, 0.5µg/ml dilution). Membranes were rinsed 3x in 1% BSA-PBST solution and Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP conjugate (Product # 31460, 1:10,000 dilution) was added to the membrane previously probed with the 6x-His Tag Recombinant Rabbit Monoclonal Antibody. After an hour incubation at RT the membrane was washed 3x in 1% BSA-PBST solution. Both membranes were then rinsed in PBS, and a working solution of ECL substrate prepared

according to the Pierce™ ECL Western Blotting Substrate kit (Thermo Fisher Scientific, USA) was added to the membranes for 1 minute, before the membranes were removed and placed in plastic sheet protectors. Chemiluminescence was imaged using the ChemiDoc™ MP Imaging system (Bio-Rad Laboratories, USA).

2.3.3 Immunofluorescence

Testes were dissected from either transgenic or wild-type males into PBST and transferred onto glass slides. The tissues were then fixed with 4% formaldehyde for 10 minutes, washed 3x in PBST and then left to permeabilise in PBST for 15 minutes. The tissue samples were then blocked in 2% BSA in PBST solution for 1 hour and then incubated with the primary antibody overnight at 4°C. The primary antibody used in these sets of experiments was the V5 Tag Monoclonal Antibody (Product # R960-25) at a 1:500 dilution in 1% BSA. After removing the primary antibody solution, the tissues were washed 3x with PBST and the secondary antibody was added in 1% BSA (PBS) solution and left at RT for 1 hour. The secondary antibody used was the Rabbit anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 647 (Product # A-21239) at a concentration of 2µg/mL. After incubation with the secondary antibody the tissues were washed twice with PBST solution, a drop of NucBlue™ Live ReadyProbes™ Reagent (Hoechst 33342) (Thermo Fisher Scientific, USA) was diluted in 500µl of PBS and 20µl was dropped onto the slide and left for 15 minutes before being completely removed. Vectashield mounting media was added to the slides and a coverslip was laid on top and sealed. Images were acquired using a Zeiss LSM 880 confocal microscope (Zeiss, Germany), V5-tagged CifA was imaged using a 633nm laser with GaAsP detector. Nuclei stained with Hoechst 33342 were imaged using a 405nm laser with GaAsP detector. Image exposure settings were kept constant throughout the groups.

2.3.4 TUNEL assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assays were used to detect single- and double-stranded DNA nicks and fragmentation via the ApopTag® Fluorescein In Situ Apoptosis Detection Kit (Merck Group, Germany). Testes were dissected from either wild-type, *w*AlbB-infected or transgenic males. The samples were then fixed with 4% formaldehyde for 10 minutes, washed 3x in PBST and then left to permeabilise in PBST for 15 minutes. 15µl of equilibration buffer was added to the samples for at least 10

seconds before being removed and replaced with working strength TdT enzyme solution and left to incubate for 1 hour at 37°C in the dark. Stop/Wash buffer was then added to the samples which were agitated and left for 10 minutes at RT before being replaced with PBS containing NucBlue™ Live ReadyProbes™ Reagent (Hoechst 33342) (Thermo Fisher Scientific, USA). Vectashield mounting media was added to the slides and a coverslip was laid on top and sealed. Images were acquired using a Zeiss LSM 880 confocal microscope (Zeiss, Germany), Fluorescein (DNA breaks) was imaged using a 488nm laser with GaAsP detectors. Nuclei stained with Hoechst 33342 were imaged using a 405nm laser with GaAsP detector. Image exposure settings were kept constant throughout the groups.

2.4 Statistical analysis

Statistical analysis was performed using the GraphPad Prism software. A Shapiro-Wilk normality test was used to determine if the data was normally distributed. In each experiment one or more of the groups were not normally distributed so non-parametric Mann-Whitney U tests were performed.

Chapter 3:

Investigating CI induction and rescue in *Ae. aegypti* using *cif* genes from the *w*Mel strain.

3. Investigating CI induction and rescue in *Ae. aegypti* using *cif* genes from the *w*Mel strain.

3.1 Introduction

The mediation of CI by strain *w*Mel is an important focus of study due to the continued exploitation of this strain in *Wolbachia*-mediated vector replacement strategies in *Aedes aegypti* mosquitoes, and its natural occurrence in the model host, *Drosophila melanogaster*. In *D. melanogaster* the CI induced by *w*Mel is both weak and transient[166–168], however the inverse is observed in transinfected *Ae. aegypti*[117]. This strain possesses one set of Type I *cif* genes[77,78]. Prior transgenic expression studies (in *D. melanogaster*) suggested that $cifA_{w\text{Mel(TI)}}$ and $cifB_{w\text{Mel(TI)}}$ co-expression was needed in the male germline in order to induce CI whilst only $cifA_{w\text{Mel(TI)}}$ expression in the female germline was sufficient for CI rescue[81]. Unlike in native *w*Mel infections, the strength of CI induction was strong in the transgenic *D. melanogaster* system and does not decrease with age[77,81,169]. This 'two-by-one' model of CI was also observed in transgenic *D. melanogaster* when using the Type I *cif* genes encoded by the strain *w*Pip[78], however in transgenic *An. gambiae* $cifA_{w\text{Pip(TI)}}$ expression was not found to be necessary for CI induction[93]. Therefore, the mechanism of CI induction mediated by the *w*Mel *cif* genes might not necessarily adhere to the two-by-one model when tested in transgenic *Ae. aegypti*. Investigating whether the two-by-one model can be observed in *Ae. aegypti* may help to illuminate the function(s) of CifA whilst providing supporting evidence to either the HM or TA models (see Section 1.2.5.3) both of which can incorporate the two-by-one model into their mechanisms of CI mediation.

Whilst $CifA_{w\text{Mel(TI)}}$ is hypothesised to play a role in CI induction, $CifB_{w\text{Mel(TI)}}$ is expected to be the primary *mod* factor - although the exact mechanism(s) behind this function remains unknown. Characteristically Type I *cifB* genes encode two putative PD-(D/E)XK-like nuclease domains and a deubiquitinase (DUB) domain[82]. The nuclease domains do not have the catalytic residues predicted to be essential for nuclease activity, however, $CifB_{wMel(TI)}$ displays nuclease activity *in vitro* and *in situ*[84]. The presence of a QxxxY motif encoded in $cifB_{w\text{Mel(TI)}}$ which is also found in $cifB_{w\text{Pip(TIV)}}$ (demonstrated to have nuclease activity) has been suggested as a possible reason for this discrepancy in enzymatic activity[84]. This motif is associated with PD-(D/E)XK domains in Type I restriction endonucleases and is thought to play an auxiliary role in DNA cleavage[170]. Unlike the PD-(D/E)XK-like domains, the DUB domain is restricted to all Type I

cifB genes and some Type V genes, which might indicate that it was either acquired independently by the ancestor of these genes or was present in the common ancestor of all *cif* types and subsequently lost multiple times[82]. The DUB domain of $CifB_{wMel(TI)}$ has demonstrated catalytic activity, cleaving poly-ubiquitin chains *in vitro*[78]. Furthermore, substitution of a catalytic residue in the DUB domain of $CifB_{wMel(TI)}$ resulted in the ablation of CI induction in transgenic *D. melanogaster* which supported the hypothesis that the DUB domain was primarily responsible for the modifications associated with CI involving Type I *cif* genes[78,171].

The ability to rescue CI induced by the *Wolbachia* strain *w*Mel is attributed to the expression of $cifA_{wMel(TI)}$ in infected oocytes[81,172]. It remains unknown how $CifA_{wMel(TI)}$ facilitates this rescue function, however CifA:CifB binding is expected to play an important role[78,80,86,90]. Interestingly, the DUB domain of $CifB_{wMel(TI)}$ remained functional when bound to $CifA_{wMel(TI)}$ *in vitro*[78] and therefore, rescue likely results from either a change in CifB localisation or access to its enzymatic targets mediated by CifA binding. Utilisation of the Gal4-UAS system in *D. melanogaster* showed that $cifA_{wMel(TI)}$ expression in female reproductive tissues recapitulated the CI rescue phenotype when transgenic females were mated with either *w*Mel-infected males or transgenic males expressing both $cifA_{wMel(TI)}$ and $cifB_{wMel(TI)}$[81,172]. Comparison between different Gal4 driver lines revealed that rescue was dependent on maternal $cifA_{wMel(TI)}$ expression levels[81,172].

The initial aim of this chapter was to generate transgenic *Ae. aegypti* lines which express the Type I *cif* genes from *w*Mel in the testes of adult male mosquitoes and characterise the effect of their expression on male fertility. It was expected that expression of both transgenes would result in a sterility phenotype which represented CI induction. To ensure that any sterility phenotype induced by the expression of the $cif_{wMel(TI)}$ genes represented canonical CI induction, the ability of *w*Mel-infected females to rescue embryonic lethality would be tested. A secondary aim of this chapter was to generate transgenic *Ae. aegypti* lines which express either $cifA_{wMel(TI)}$ or $cifB_{wMel(TI)}$ independently in the testes. Uncoupling the expression of the *cif* genes to different genomic loci would permit investigation of the effect of sole $cifA/B_{wMel(TI)}$ expression on male fertility whilst enabling a study comparing the effects of variable relative expression levels of either $cifA_{wMel(TI)}$ or $cifB_{wMel(TI)}$ on male fertility. It was expected that information gathered from this study would confirm whether the two-by-one model of CI

induction characterised in *D. melanogaster* is conserved in *Ae. aegypti* and whether *cifA*$_{w\text{Mel(TI)}}$ expression either attenuates or enhances CI penetrance. This information would be beneficial in resolving whether CI-mediation conforms to a TA or HM model.

Another aim of this chapter was to generate transgenic *Ae. aegypti* lines which could rescue CI through the expression of *cifA*$_{w\text{Mel(TI)}}$ in the ovaries of adult female mosquitoes. Utilisation of different promoters throughout this study would allow for the selection of regulatory sequences which resulted in robust induction and rescue phenotypes and would therefore inform the potential construction of gene drive systems which utilised the *cif*$_{w\text{Mel(TI)}}$ genes. In the context of gene drive construction, another aim of this chapter was to investigate the effect of early embryonic *cifB*$_{w\text{Mel(TI)}}$ expression on viability, and whether *cifA*$_{w\text{Mel(TI)}}$ expression could repress the expected lethality phenotype. It was hypothesised that if this phenomenon occurred, it could form the basis of constructing a two-locus underdominance gene drive system. To investigate, an aim was to generate transgenic lines of *Ae. aegypti* mosquitoes that expressed either *cif*$_{w\text{Mel(TI)}}$ gene in early embryo development in a tetracycline repressible manner.

3.2 Results and Discussion

3.2.1 Co-expression of *cifA*$_{wMel(TI)}$ and *cifB*$_{wMel(TI)}$ from a single construct does not induce CI

3.2.1.1 Basic transposon design

The *piggyBac* donor transposon used to study the effect of dual *cif*$_{wMel}$ gene expression on male fertility consisted of a nuclear-localised fluorescent transformation marker (NLS-DsRed2) under the control of the ubiquitous IE1-Hr5 promoter-enhancer sequence, and a *cif* gene expression cassette consisting of both *cifA*$_{wMel(TI)}$ and *cifB*$_{wMel(TI)}$ regulated by variable promoters that drive expression in the testes (Figure 3.1). The syntenic *cif* gene pairs in *Wolbachia* are thought to be expressed as a single operon[78], with *cifA* located directly upstream of *cif*B. Insertion of the 2A peptide sequence from the Thosea asigna virus (T2A) between the two transgenes promotes ribosomal skipping, and results in the translation of both peptides at near equimolar quantities. Prior studies have shown that CI induction is robust when using a 2A-mediated "self-cleavage" mechanism[77,78,80,81,92,93], which might mirror how the genes are transcribed and processed in *Wolbachia.* Therefore, for this study both *cifA*$_{wMel(TI)}$ and *cifB*$_{wMel(TI)}$ were linked with a T2A peptide sequence. To allow for future localisation and/or pulldown studies epitope tags were attached to the N-terminus of the *cif* genes: a His-tag comprising of six histidine residues or Flag-tag (DYKDDDDK) was fused to *cifA*$_{wMel(TI)}$ and *cifB*$_{wMel(TI)}$ respectively. The *cif* genes were codon optimised for efficient translation in *Ae. aegypti* cells and a downstream simian virus 40 polyadenylation (SV40 polyA) signal was inserted to stabilise mRNA transcripts and enhance translation efficiency.

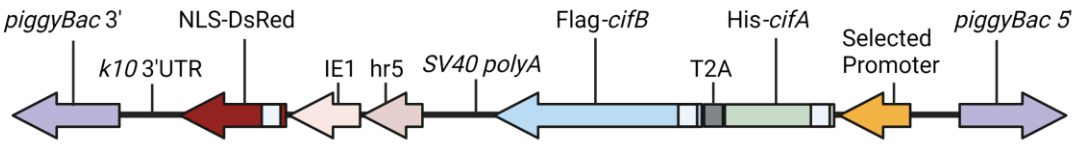


Figure 3.1: Schematic representation of the transposon used to investigate the effect of *cifA*$_{wMel(TI)}$ and *cifB*$_{wMel(TI)}$ co-expression on male fertility.

3.2.1.2 Generation of *ß2t-cifA*$_{w\text{Mel(TI)}}$-T2A-*cifB*$_{w\text{Mel(TI)}}$ lines

The expression of the *Ae. aegypti ß2 tubulin* (*ß2t*) gene is restricted to the primary spermatocyte region of testes, starting weakly at later larval stages and increasing throughout adulthood[173]. Prior studies have shown that *ß2t* driven transgene products can loaded into mature spermatozoa and have been shown to function after deposition in fertilised embryos[173,174]. Therefore, the *ß2t* promoter was selected to drive expression of the *cifA*$_{w\text{Mel(TI)}}$-*T2A*-*cifB*$_{w\text{Mel(TI)}}$ cassette.

CI induced by *w*Mel-carrying males crossed with wild-type females is observed as a near complete loss of embryo viability. To test for transgenic CI induction an excess of heterozygous males from each of the four *ß2t-cifA*$_{w\text{Mel(TI)}}$-*T2A*-*cifB*$_{w\text{Mel(TI)}}$ lines (see Table A1) were crossed with wild-type females and the viability rates of the resulting embryos were recorded. There were no significant decreases in the median viability rates in any of the crosses involving heterozygous transgenic males from each of the four independent lines compared to a wild-type x wild-type control cross (Figure 3.2a). To confirm that the *cif* transgenes were being actively transcribed in the testes, RNA from either dissected testes or the remaining carcasses of males used in the experimental crosses was extracted and the relative expression of the bicistronic transcript was determined by RT-qPCR (Figure 3.2b). Of the four *ß2t-cifA*$_{w\text{Mel(TI)}}$-*T2A*-*cifB*$_{w\text{Mel(TI)}}$ insertion lines, three (L1-L3) had significantly higher expression of the *cif* transcript in pooled testes in comparison to the remaining carcasses (L1: 12-fold, L2: 15-fold, and L3: 6 fold difference). Although, the fourth line (L4) had a 2-fold higher relative expression of *cifA/B*$_{w\text{Mel(TI)}}$ in the testes versus the carcass, the difference was not significant (Figure 3.2b). As the promoter appeared to be driving expression of the *cif* transgenes in the testes yet no sterility phenotype was observed, it was hypothesised that alternative promoters should be tested.

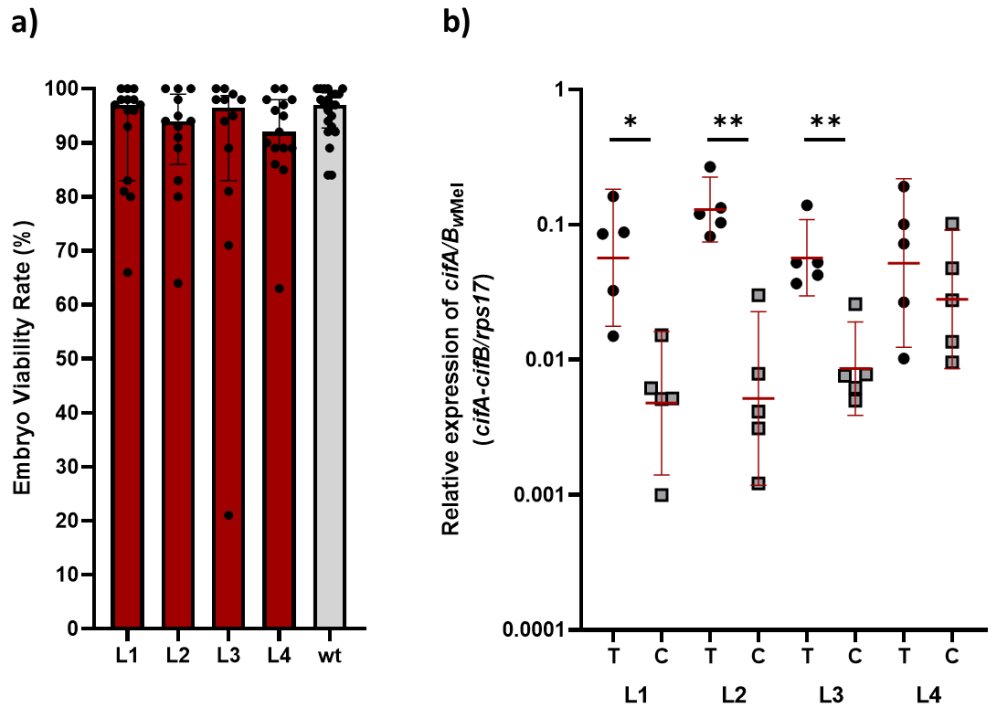


Figure 3.2: Co-expression of *cifA*$_{wMel(TI)}$ and *cifB*$_{wMel(TI)}$ under the control of testes-specific *β2t* promoter does not induce CI. a) Heterozygous males from four different genomic insertion *β2t-cifA*$_{wMel(TI)}$-T2A- *cifB*$_{wMel(TI)}$ lines (L1-L4) did not significantly reduce the viability rate of the progeny when crossed with wild-type (wt) females. Dots represent the percentage of viable embryos laid by a single female, bars represent the median and error bars denote interquartile ranges. **b)** The expression of *cifA/B*$_{wMel(TI)}$ relative to housekeeping gene *rps17* in pooled adult testes (T) and remaining carcasses (C). Lines denote geometric mean, error bars denote 95% CI. Significance calculated by Mann-Whitney U-test *=p<0.05, **=p<0.01

3.2.1.3 Generation of *topi-cifA*$_{wMel(TI)}$-T2A-*cifB*$_{wMel(TI)}$ lines

The expression of the meiotic arrest gene *matotopetli* (*topi*) is found to be restricted to primary spermatocytes in *D. melanogaster*[175]. The *Ae. aegypti topi* promoter is expected to promote transcription at an earlier stage of spermatogenesis than the *β2t* promoter, however the expression is also expected to be weaker (Dr Tim Harvey-Samuel pers. comm.). Because the *cif*$_{wMel}$ genes had failed to induce CI when under the control of the *β2t* promoter, it was hypothesised that this earlier expression might result in a different phenotype. Three independent genomic insertion *topi-cifA*$_{wMel(TI)}$-*T2A-cifB*$_{wMel(TI)}$ lines were generated (Table A1). However, similar to the findings for *β2t*, heterozygous males from each of the three lines failed to significantly reduce the median viability rate of progeny when crossed with wild-type

females (as expected for the induction of CI) in comparison to the control cross (Figure 3.3a). The relative expression of the bicistronic transcript was determined by RT-qPCR as described previously for the *β2t-cifA*$_{w\text{Mel(TI)}}$*-T2A-cifB*$_{w\text{Mel(TI)}}$ lines. As expected, the relative expression was lower than the *β2t-cifA*$_{w\text{Mel(TI)}}$*-T2A-cifB*$_{w\text{Mel(TI)}}$ lines (Figure 3.6), however although expression in the testes was detected this expression was not found to be testes-specific (Figure 3.3b).

The expression of the meiotic arrest gene *matotopetli* (*topi*) is found to be restricted to primary spermatocytes in *D. melanogaster*[175]. The *Ae. aegypti topi* promoter is expected to promote transcription at an earlier stage of spermatogenesis than the *β2t* promoter, however the expression is also expected to be weaker (Dr Tim Harvey-Samuel pers. comm.). Because the *cif*$_{w\text{Mel}}$ genes had failed to induce CI when under the control of the *β2t* promoter, it was hypothesised that this earlier expression might result in a different phenotype. Three independent genomic insertion *topi-cifA*$_{w\text{Mel(TI)}}$*-T2A-cifB*$_{w\text{Mel(TI)}}$ lines were generated (Table A1). However, similar to the findings for *β2t*, heterozygous males from each of the three lines failed to significantly reduce the median viability rate of progeny when crossed with wild-type females (as expected for the induction of CI) in comparison to the control cross (Figure 3.3a). The relative expression of the bicistronic transcript was determined by RT-qPCR as described previously for the *β2t-cifA*$_{w\text{Mel(TI)}}$*-T2A-cifB*$_{w\text{Mel(TI)}}$ lines. As expected, the relative expression was lower than the *β2t-cifA*$_{w\text{Mel(TI)}}$*-T2A-cifB*$_{w\text{Mel(TI)}}$ lines (Figure 3.6), however although expression in the testes was detected this expression was not found to be testes-specific (Figure 3.3b).

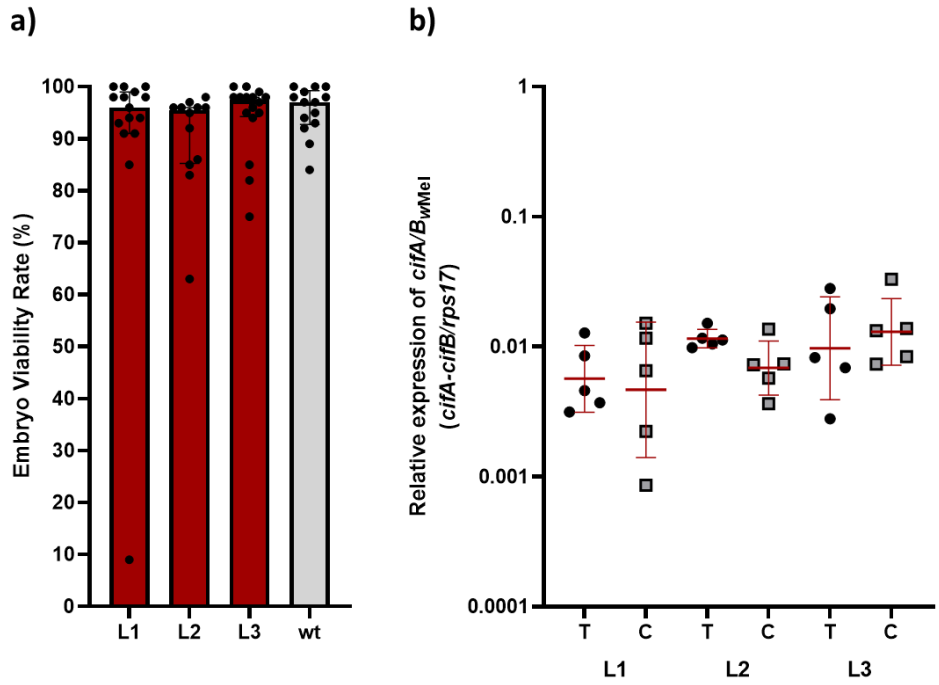


Figure 3.3: Co-expression of *cifA*$_{wMel(TI)}$ and *cifB*$_{wMel(TI)}$ under the control of the *topi* promoter does not induce CI. a) Heterozygous males from three different genomic insertion *topi-cifA*$_{wMel(TI)}$-T2A- *cifB*$_{wMel(TI)}$ lines (L1-L3) did not significantly reduce the viability rate of the progeny when crossed with wild-type (wt) females. Dots represent the percentage of viable embryos laid by one female, bars represent the median and error bars denote interquartile ranges. **b)** The expression of *cifA/B*$_{wMel(TI)}$ relative to housekeeping gene *rps17* in pooled adult testes (T) and remaining carcasses (C). Lines denote geometric mean, error bars denote 95% CI.

3.2.1.4 Generation of *Ø2t*-cifA*$_{wMel(TI)}$-T2A-*cifB*$_{wMel(TI)}$ lines

An addition of the native sequence to the *Ø2t* promoter was found to increase the levels of transgene expression regulated by this sequence (Dr Tim Harvey-Samuel pers. comm.). To determine if a higher co-expression of *cifA*$_{wMel(TI)}$ and *cifB*$_{wMel(TI)}$ is needed to induce a sterility phenotype in transgenic males, the original *Ø2t* promoter in the donor transposon plasmid was replaced with this optimised *Ø2t* promoter (*Ø2t**), and 5 independent genomic insertion lines were established (see Table A1). However, similar to the findings for *Ø2t*, heterozygous males from each of the five *Ø2t*-cifA*$_{wMel(TI)}$*-T2A-cifB*$_{wMel(TI)}$ lines failed to significantly reduce the median viability rate of progeny when crossed with wild-type females (Figure 3.4a). Unexpectedly, the relative expression of the *cif* gene operon in the testes was not found to

be higher than that recorded for the *β2t-cifA*$_{wMel(TI)}$*-T2A-cifB*$_{wMel(TI)}$ lines (Figure 3.6), which may explain why the *β2t** lines showed similar phenotypic profiles to the *β2t* lines.

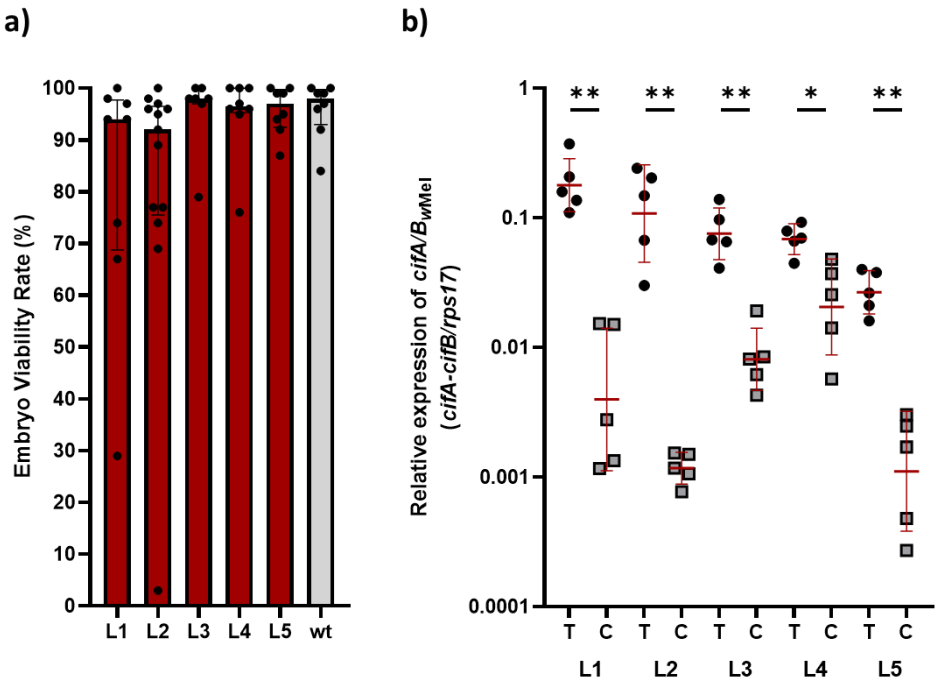


Figure 3.4: Co-expression of *cifA*$_{wMel(TI)}$ and *cifB*$_{wMel(TI)}$ under the control of the optimised testes-specific *β2t* promoter (*β2t) does not induce sterility. a)** Heterozygous males from five different genomic insertion *β2t**-*cifA*$_{wMel(TI)}$-T2A-*cifB*$_{wMel(TI)}$ lines (L1-L5) did not significantly reduce the viability rate of the progeny when crossed with wild-type (wt) females. Dots represent the percentage of viable embryos laid by a single female, bars represent the median and error bars denote interquartile ranges. **b)** The expression of *cifA/B*$_{wMel(TI)}$ relative to housekeeping gene *rps17* in pooled adult testes (T) and remaining carcasses (C). Lines denote geometric mean, error bars denote 95% CI. Significance calculated by Mann-Whitney U-test *=p<0.05, **=p<0.01

3.2.1.5 Generation of *prot-cifA*$_{wMel(TI)}$-T2A-*cifB*$_{wMel(TI)}$ lines

During the post-meiotic stage of spermatogenesis, histones are replaced with protamines which ensures the correct level of chromatin condensation for packaging into sperm heads. According to the TA model of CI, the *cif* gene products are expected to be packaged into mature sperm. Prior transgenic experiments where DsRed2 expression was driven by an *Ae. aegypti* protamine (*prot*) promoter resulted in high levels of fluorescence observed in mature

sperm (Dr Tim Harvey-Samuel pers. comm.) and therefore provides a potential alternative to the *β2t* promoter. Two independent genomic insertion *prot-cifA*$_{wMel(TI)}$*-T2A-cifB*$_{wMel(TI)}$ lines were generated in this study (see Table A1). Expression of the transgenes was restricted to the testes in line 1 (Figure 3.5b) and comparable to the relative expression levels observed when utilising the *β2t* promoter (Figure 3.6). Heterozygous males from the two *prot-cifA*$_{wMel(TI)}$*-T2A-cifB*$_{wMel(TI)}$ lines were crossed with wild-type females and failed to significantly decrease the median embryonic viability rate in comparison to the control wild-type cross (Figure 3.5a).

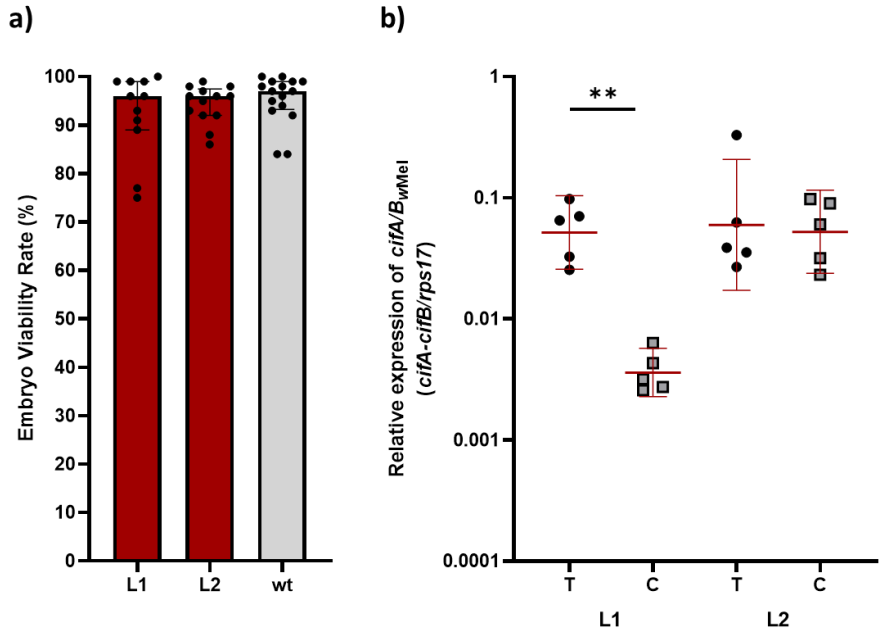


Figure 3.5: Co-expression of *cifA*$_{wMel(TI)}$ and *cifB*$_{wMel(TI)}$ under the control of the testes-specific protamine promoter (*prot)* does not induce sterility. a) Heterozygous males from two different genomic insertion *prot-cifA*$_{wMel(TI)}$*-T2A-cifB*$_{wMel(TI)}$ lines (L1 and L2) did not significantly reduce the viability rate of the progeny when crossed with wild-type (wt) females. Dots represent the percentage of viable embryos laid by a single female, bars represent the median and error bars denote interquartile ranges. **b)** The expression of *cifA/B*$_{wMel(TI)}$ relative to housekeeping gene *rps17* in pooled adult testes (T) and remaining carcasses (C). Lines denote geometric mean, error bars denote 95% CI. Significance calculated by Mann-Whitney U-test **=p<0.01

3.2.1.6 Generation of PUb-cifA$_{wMel(TI)}$-T2A-cifB$_{wMel(TI)}$ lines

As co-expression of *cifA*$_{wMel(TI)}$ and *cifB*$_{wMel(TI)}$ under the control of the testes-specific *β2t, β2t*, topi, and prot* promoters had failed to produce a sterility phenotype in heterozygous males it was hypothesised that a more active and temporally ubiquitous promoter might be required to generate sterility. The *Ae. aegypti polyubiquitin* (*PUb)* regulatory sequence is a strong constitutive promoter and was therefore selected to drive *cif* gene expression[176]. Five independent genomic insertion *PUb-cifA*$_{wMel(TI)}$*-T2A-cifB*$_{wMel(TI)}$ lines were generated (see Table A1) and the *PUb* promoter was found on average to drive a higher expression of the *cif* transcript in the testes in comparison to the previously tested promoters (Figure 3.6).

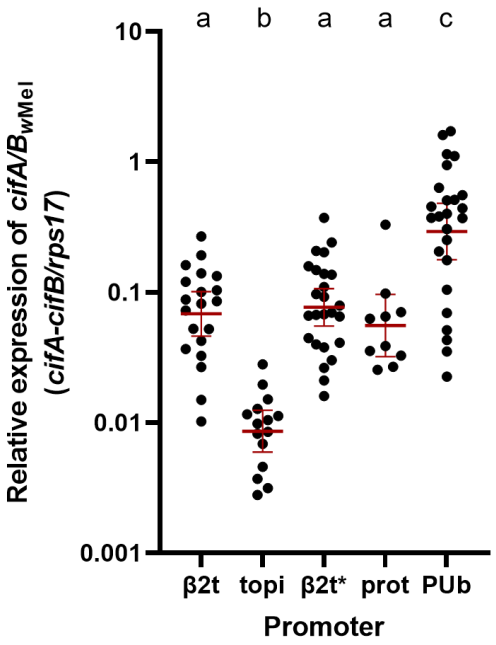


Figure 3.6: Comparison of the relative expression of the *cifA*$_{wMel(TI)}$ and *cifB*$_{wMel(TI)}$ transcript regulated by different promoter sequences. Lines denote geometric mean, error bars denote 95% CI. Significance between groups ($p<0.05$) calculated by Mann-Whitney U-tests is indicated by letters

Consistent with expectations for a constitutive promoter, the relative expression of the *PUb*-driven transgenes was high in both the testes and the remaining carcasses of transgenic males (Figure 3.7b). However, none of the *PUb-cifA*$_{wMel(TI)}$*-T2A-cifB*$_{wMel(TI)}$ lines reduced embryonic viability when heterozygous males were crossed with wild-type females (Figure 3.7a).

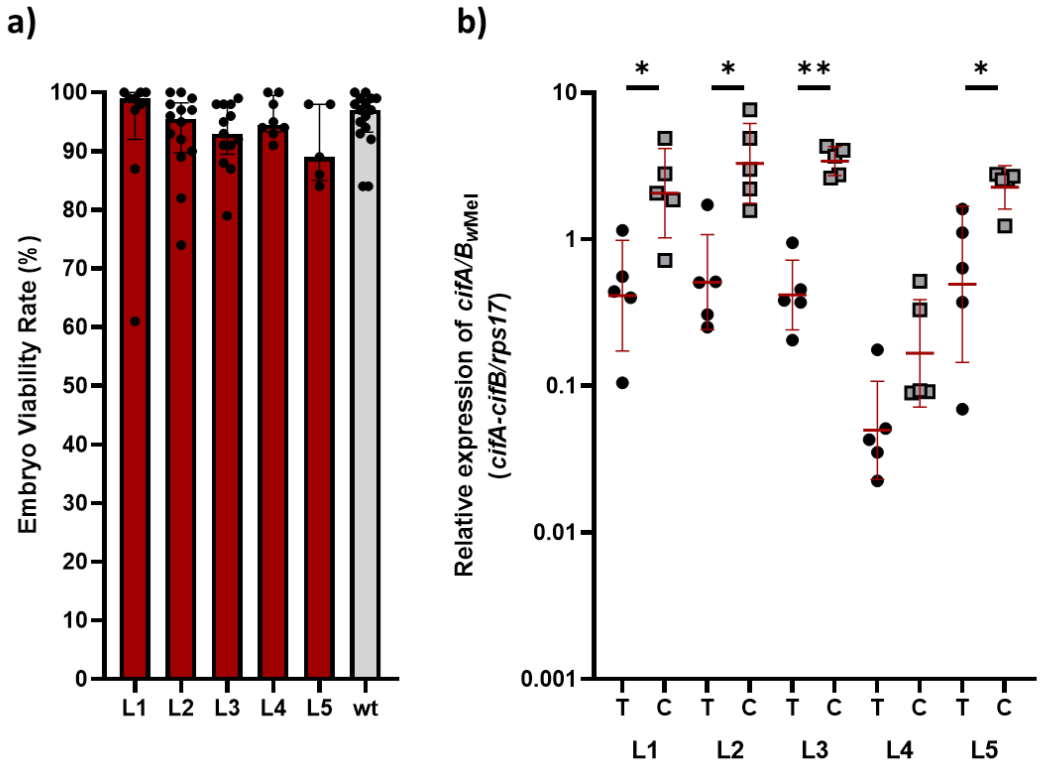


Figure 3.7: Co-expression of *cifA*$_{wMel(TI)}$ and *cifB*$_{wMel(TI)}$ under the control of a constitutive promoter (*PUb)* does not induce sterility. a) Heterozygous males from five different genomic insertion *PUb-cifA*$_{wMel(TI)}$*-T2A-cifB*$_{wMel(TI)}$ lines (L1-L5) did not significantly reduce the viability rate of the progeny when crossed with wild-type (wt) females. Dots represent the percentage of viable embryos laid by a single female, bars represent the median and error bars denote interquartile ranges. **b)** The expression of *cifA/B*$_{wMel(TI)}$ relative to housekeeping gene *rps17* in pooled adult testes (T) and remaining carcasses (C). Lines denote geometric mean, error bars denote 95% CI. Significance calculated by Mann-Whitney U-test *=p<0.05,**=p<0.01

3.2.2 Expression of *cifB*$_{w\text{Mel(TI)}}$ alone does not induce CI

3.2.2.1 Basic transposon design

Expression of only *cifB*$_{w\text{Mel(TI)}}$ in the male germline had failed to induce CI in *D. melanogaster* studies. However, a recent study in *An. gambiae* had demonstrated conditional male-sterility upon *cifB*$_{w\text{Pip(TI)}}$ expression which was inconsistent with the two-by-one model of CI implied by prior CI studies (using the *cif*$_{w\text{Pip(TI)}}$ genes) in *D. melanogaster*[81,93]. Moreover in *An. gambiae* it was found that high levels of *cifA*$_{w\text{Pip(TI)}}$ could attenuate CI induced by *cifB*$_{w\text{Pip(TI)}}$[93]. We therefore hypothesised that the presence of *cifA*$_{w\text{Mel(TI)}}$ may be reducing *cifB*$_{w\text{Mel(TI)}}$ activity in *Ae. aegypti*. As prior attempts to induce CI through the co-expression of *cifA*$_{w\text{Mel(TI)}}$ and *cifB*$_{w\text{Mel(TI)}}$ had failed, lines of transgenic mosquitoes expressing only *cifB*$_{w\text{Mel(TI)}}$ under the control of male germline promoters were generated. The transposon consisted of a nuclear-localised fluorescent transformation marker (NLS-AmCyan1) regulated by the ubiquitous IE1-Hr5 promoter-enhancer sequence, and the codon optimised Flag-tagged *cifB*$_{w\text{Mel(TI)}}$ under the control of either the *prot* or *β2t** promoter to ensure testes-specific expression (Figure 3.8).

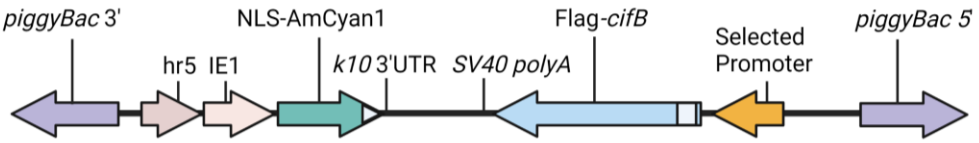


Figure 3.8: Schematic representation of the transposon used to investigate the effect of *cifB*$_{w\text{Mel(TI)}}$ expression on male fertility.

3.2.2.2 Generation of prot-cifB$_{w\text{Mel(TI)}}$ lines

Seven independent genomic *prot-cifB*$_{w\text{Mel(TI)}}$ insertion lines were generated (see Table A1) and the effect of the transgene on fertility was tested through mating heterozygous males with wild-type females. Males from each of the seven *prot-cifB*$_{w\text{Mel(TI)}}$ lines remained fully fertile, with none significantly reducing the median viability rate of the progeny in comparison to the control wild-type cross (Figure 3.9a). The relative expression *cifB*$_{w\text{Mel(TI)}}$ in the testes of heterozygous males from five of the seven lines was determined by RT-qPCR. Only two of the five lines tested displayed testes-specific expression (Figure 3.9b).

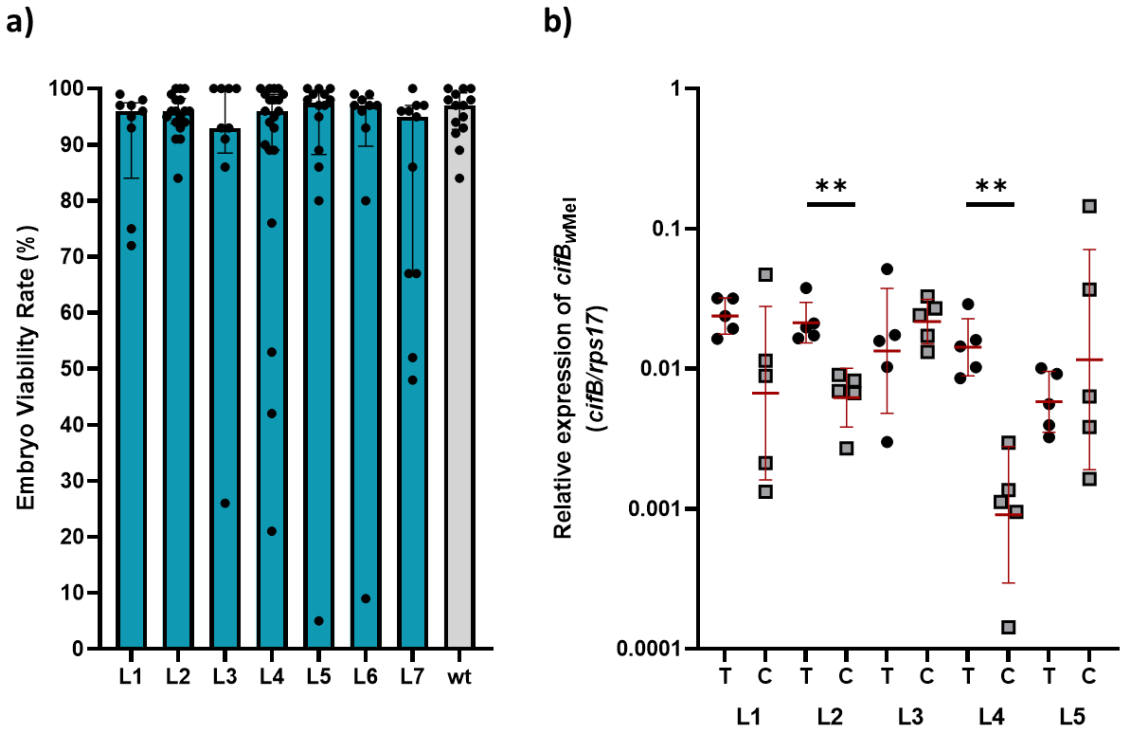


Figure 3.9: Expression of *cifB*$_{wMel(TI)}$ under the control of testes-specific promoter (*prot)* does not induce sterility. a) Heterozygous males from seven different genomic insertion *prot-cifB*$_{wMel(TI)}$ lines (L1-L7) did not significantly reduce the viability rate of the progeny when crossed with wild-type (wt) females. Dots represent the percentage of viable embryos laid by a single female, bars represent the median and error bars denote interquartile ranges. **b)** The expression of *cifB*$_{wMel(TI)}$ relative to housekeeping gene *rps17* in pooled adult testes (T) and remaining carcasses (C) from five of the seven *prot-cifB*$_{wMel(TI)}$ lines. Lines denote geometric mean, error bars denote 95% CI. Significance calculated by Mann-Whitney U-test. **=p<0.01

3.2.2.3 Generation of *ϐ2t*-cifB*$_{wMel(TI)}$ lines

The *ϐ2t** promoter resulted in comparable expression to the *prot* promoter, however it was found to be more testes-tissue specific. The *ϐ2t** promoter was therefore selected as an alternative to the *prot* promoter. However, the median embryo viability rate was not significantly decreased when heterozygous males from each of the nine independent genomic *ϐ2t*-cifB*$_{wMel(TI)}$ lines (see Table A1) were mated with wild-type females (Figure 3.10a). The relative expression of *cifB*$_{wMel(TI)}$ was determined by RT-qPCR for seven of the nine lines. Unlike when the *ϐ2t** promoter was utilised to drive both *cifA*$_{wMel(TI)}$ and *cifB*$_{wMel(TI)}$, the expression of *cifB*$_{wMel(TI)}$ was found to be much lower and in the majority of the lines the expression was not testes-specific (Figure 3.10b). This might suggest the expression of the transgene was the

result of random transcription events (baseline expression) and not driven by the promoter sequence. This could have been the result of insertion into less accessible chromatin regions, however as several lines have similar expression patterns, and the *prot-cifB*$_{wMel(TI)}$ lines also had lower expression it might suggest a problem with the transposon design or mutations gained during cloning that affected expression. As the expression of *cifB*$_{wMel(TI)}$ is very weak, it cannot be determined whether the lack of observed sterility is due to issues with the "toxicity" of CifB$_{wMel(TI)}$ or if the necessary expression level was not reached.

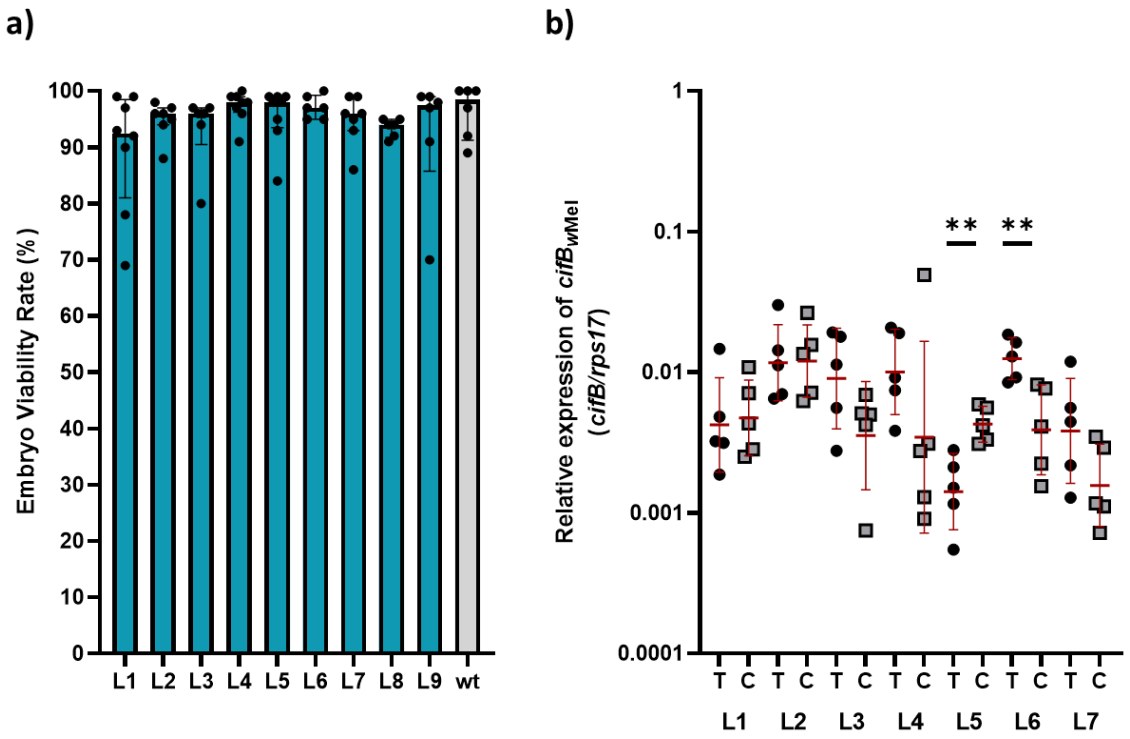


Figure 3.10: Expression of *cifB*$_{wMel(TI)}$ under the control of testes-specific promoter (*β2t) does not induce sterility. a)** Heterozygous males from nine different genomic insertion *β2t**-*cifB*$_{wMel(TI)}$ lines (L1-L9) did not significantly reduce the viability rate of the progeny when crossed with wild-type (wt) females. Dots represent the percentage of viable embryos laid by a single female, bars represent the median and error bars denote interquartile ranges. **b)** The expression of *cifB*$_{wMel(TI)}$ relative to housekeeping gene *rps17* in pooled adult testes (T) and the remaining carcasses (C) from seven *β2t**-*cifB*$_{wMel(TI)}$ lines. Lines denote geometric mean, error bars denote 95% CI. Significance calculated by Mann-Whitney U-test. **=p<0.01

3.2.3 Expression of *cifA*$_{wMel(TI)}$ and *cifB*$_{wMel(TI)}$ from independent insertions does not induce CI.

3.2.3.1 Generation of *β2t*-cifA*$_{wMel(TI)}$ lines

It was hypothesised that if *cifA*$_{wMel(TI)}$ was necessary for the induction of CI, then exploiting variable transgene expression between different genomic insertion lines might allow for the correct balancing of *cifA*$_{wMel(TI)}$ and *cifB*$_{wMel(TI)}$ expression - which bicistronic (and therefore theoretically equimolar) expression did not allow for. Consequently, three independent lines of mosquitoes expressing *cifA*$_{wMel(TI)}$ under the control of the *β2t** promoter (Figure 3.11) were generated through embryo micro-injection (see Table A1).

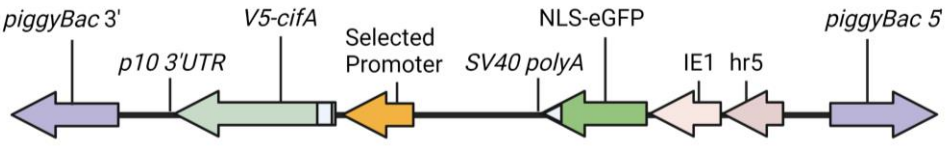


Figure 3.11: Schematic representation of the transposon used to investigate the effect of *cifA*$_{wMel(TI)}$ expression on male fertility.

The transposon insertion was found to be female-linked in *β2t*-cifA*$_{wMel(TI)}$ line 3, and therefore this line was not used in CI induction crosses. The remaining two *β2t*-cifA*$_{wMel(TI)}$ lines (L1 and L2) were crossed into two independent *β2t*-cifB*$_{wMel(TI)}$ lines (L1 and L5) and the resulting male progeny expressing either *β2t*-cifB*$_{wMel(TI)}$ and *cifA*$_{wMel(TI)}$ alone or independently together were selected and crossed to wild-type females (Figure 3.12a). *β2t*-cifB*$_{wMel(TI)}$ L5 was selected as it had the lowest mean relative expression of *cifB*$_{wMel(TI)}$ in the testes. As expected, paternal expression of *cifA*$_{wMel(TI)}$ alone did not affect male fertility, despite very high levels of *cifA*$_{wMel(TI)}$ expression regulated by the *β2t** promoter (Figure 3.12a, b). However, none of the *cifA/B*$_{wMel(TI)}$ combinations affected male fertility (Figure 3.12a). This is not unexpected as the expression of *cifB*$_{wMel(TI)}$ is very low and it is likely that a higher expression is needed before conclusions can be made about the role of CifA$_{wMel(TI)}$ in the CI induction phenotype.

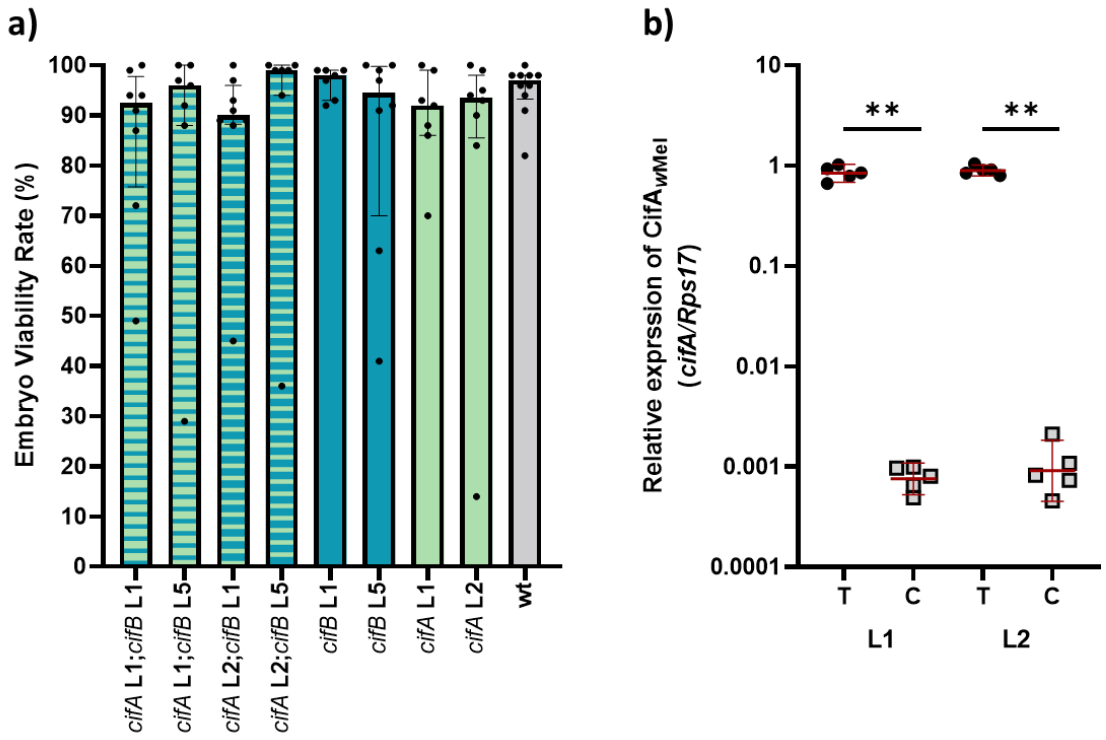


Figure 3.12: Co-expression of *cifA*$_{w\text{Mel(TI)}}$ and *cifB*$_{w\text{Mel(TI)}}$ in male testes from independent genomic loci under the control of testes-specific promoter (*ß2t) did not induce sterility. a)** Expression of *cifA*$_{w\text{Mel(TI)}}$ alone from two independent insertion *ß2t*-cifA*$_{w\text{Mel(TI)}}$ lines (L1 and L2) did not affect male fertility nor did co-expression of *cifA*$_{w\text{Mel(TI)}}$ and *cifB*$_{w\text{Mel(TI)}}$ when these lines were crossed into two independent insertion *ß2t*-cifB*$_{w\text{Mel(TI)}}$ lines (L1 and L5). Dots represent the percentage of viable embryos laid by one female, bars represent the median and error bars denote interquartile ranges. **b)** The expression of *cifA*$_{w\text{Mel(TI)}}$ relative to housekeeping gene *rps17* in pooled adult testes (T) and the remaining carcasses (C) from the two *ß2t*-cifA*$_{w\text{Mel(TI)}}$ lines. Lines denote geometric mean, error bars denote 95% CI. Significance calculated by Mann-Whitney U-test. **=p<0.01

3.2.4 Investigating the effect of *cifA*$_{w\text{Mel(TI)}}$ expression in the testes on CI penetrance.

3.2.4.1 Generation of *PUb-cifA*$_{w\text{Mel(TI)}}$ lines

The role of CifA in CI induction is not yet understood, however it is potentially needed to attenuate CifB toxicity during spermatogenesis. Previously, expression of *cifA*$_{w\text{Mel(TI)}}$ in transgenic/*w*Mel-infected male *D. melanogaster* fruit flies was found to increase the penetrance of CI rather than attenuate it[77]. This phenotype is harder to test in *w*Mel-infected *Ae. aegypti* males because there is no signal space to observe enhancement (CI induction results in a near complete loss of embryo viability). However, CI penetrance in *Ae. aegypti* is reduced when the eggs used to produce adults for crosses are quiesced for longer periods of time (extended egg quiescence results in reduced *Wolbachia* densities in the resulting adults, which in turn leads to lower CI penetrance)[114]. It was hypothesised that a high expression of *cifA*$_{w\text{Mel(TI)}}$ in the testes might attenuate the penetrance of a *w*Mel *Wolbachia* transinfection. As the *PUb* promoter was found to promote the highest transgene expression in the testes (Figure 3.6) and can activate transgene expression in very early embryos, this regulatory sequence was chosen for further study. After microinjection of the *PUb-cifA*$_{w\text{Mel(TI)}}$ donor plasmid (Figure 3.11) into embryos, it was noted that there was a large death rate of 1st instar injection survivors, and two of the three independent genomic *PUb-cifA*$_{w\text{Mel(TI)}}$ insertion lines generated (see Table A1) displayed a high mortality rate in transgenic progeny at early larval life stages - indicating that expression of *cifA*$_{w\text{Mel(TI)}}$ using a strong constitutive promoter might be toxic. This result was interesting as a high transgenic expression of *cifA* in other species (*D. melanogaster*, *An. gambiae*, and *S. cerevisae*) did not appear to produce a deleterious phenotype[77,78,85,88,93]. A recent paper has shown that *cifA*$_{w\text{Mel(TI)}}$ possesses both DNase and RNase activity *in vitro*, so it is possible that overexpression may cause this catalytic activity to become lethal[84]. It should be noted that 5 independent *PUb-cifA*$_{w\text{Mel(TI)}}$-*T2A-cifB*$_{w\text{Mel(TI)}}$ insertion lines were generated and none displayed any signs of transgene toxicity, suggesting that binding of cognate CifA$_{w\text{Mel(TI)}}$ to CifB$_{w\text{Mel(TI)}}$ attenuates CifA$_{w\text{Mel(TI)}}$ lethality.

3.2.4.2 Generation of *PUbt-cifA*$_{w\text{Mel(TI)}}$ lines

Due to the high mortality rate in the *PUb-cifA*$_{w\text{Mel(TI)}}$ lines, the regulatory sequence was replaced with a truncated form of the *PUb* promoter (*PUbt*) which was expected to reduce the expression of the transgene. However, as seen with the *PUb-cifA*$_{w\text{Mel(TI)}}$ injections, there

was a high mortality rate in 1st instar larvae. One of the three independent genomic *PUbt-cifA*$_{wMel(TI)}$ insertion lines (see Table A1) had comparable mortality rates to the two *PUb-cifA*$_{wMel(TI)}$ lines. However, no significant larval death was observed for the other two lines. It was hypothesised that a reduced expression of *cifA*$_{wMel(TI)}$ resulted in less mortality, however, the relative expression of the transgene in adult carcasses was not significantly different between these lines (Figure 3.13). Nevertheless, differences in expression might occur at early developmental stages (where mortality is observed).

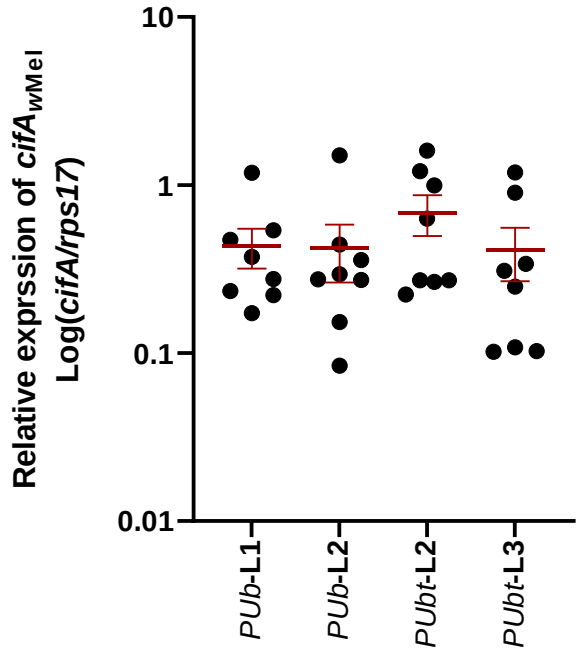


Figure 3.13: Comparison of the relative expression of *cifA*$_{wMel(TI)}$ in the carcasses of transgenic mosquitoes from different genomic insertion lines. The transgene was under the control of either the full-length *PUb* promoter or its truncated form (*PUbt*). Dots represent the expression of *cifA*$_{wMel(TI)}$ relative to the housekeeping gene *rps17* in the carcass of a single adult mosquito from one of two independent genomic insertion *PUb-cifA*$_{wMel(TI)}$ lines or two *PUbt-cifA*$_{wMel(TI)}$ lines. Red line is the mean, and error bars denote the SEM.

The *PUbt-cifA*$_{wMel(TI)}$ lines 2 and 3 were crossed into *w*Mel-infected females and the resulting transgenic/infected males were crossed to wild-type females to assess whether paternal transgenic *cifA*$_{wMel(TI)}$ expression affected CI strength. Paternal expression of *cifA*$_{wMel(TI)}$ was found to reduce CI penetrance in comparison to non-transgenic brothers in an expression dependent manner (Figure 3.14). As the relative *Wolbachia* density between siblings was

expected to be the same, this result suggests the presence of *cifA*$_{wMel(TI)}$ results in a reduction of CI strength. However, the *Wolbachia* densities of the males used in these crosses was not determined. It is possible that some transgenic males were uninfected; however, uninfected males would also have been expected to occur in the non-transgenic siblings, yet relatively strong CI was induced in each cross.

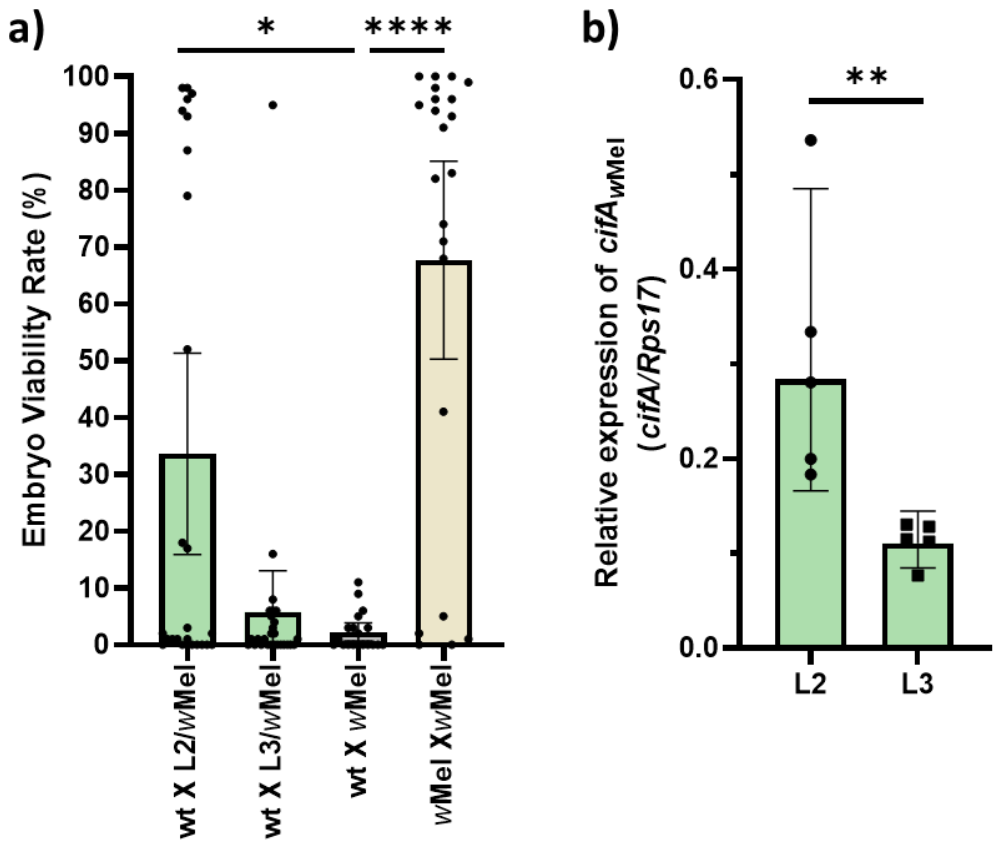


Figure 3.14: Constitutive expression of *cifA*$_{wMel(TI)}$ in males reduced the penetrance of CI in an expression-dependent manner. a) *w*Mel-infected heterozygous males from two independent insertion *PUbt-cifA*$_{wMel(TI)}$ lines (L2/*w*Mel & L3/*w*Mel) were crossed with wild-type (wt) males. Infected non-transgenic (*w*Mel) males were crossed with either infected female siblings or wt females. Dots represent the percentage of viable embryos laid by single females, bars represent the mean taken from 2 experimental repeats, and error bars denote 95% CI. **b)** The expression of *cifA*$_{wMel(TI)}$ relative to housekeeping gene *rps17* in pooled adult testes from the two *PUbt-cifA*$_{wMel(TI)}$ lines. Bars denote mean, error bars denote 95% CI. Significance calculated by Mann-Whitney U-test. *=p<0.05, **=p<0.01.

Interestingly, the expression of *cifA*$_{w\text{Mel(TI)}}$ was found to be higher in testes dissected from both *β2t*-cifA*$_{w\text{Mel(TI)}}$ lines when compared to the *PUbt-cifA*$_{w\text{Mel(TI)}}$ lines, and therefore might be able to attenuate CI penetrance (see Figures 3.12b and 3.14b). However, the *β2t** promoter is not expected to drive expression in the apical region of the testis which consists of spermatogonial stem cells; *Wolbachia* are known to infect these cells and therefore *β2t**-regulated expression of *cifA*$_{w\text{Mel(TI)}}$ may be too late to interfere with native *cif* expression/interactions. The *PUbt* promoter was expected to activate *cifA*$_{w\text{Mel(TI)}}$ in all cell types of the testes and was therefore deemed more appropriate to study the effect of CifA$_{w\text{Mel(TI)}}$ overexpression on CI induction in infected males. Immunofluorescence staining of testes from *PUbt-cifA*$_{w\text{Mel(TI)}}$ males confirmed that this promoter activated ubiquitous expression in the testes (Figure 3.15). In the apical region of the testis, CifA$_{w\text{Mel(TI)}}$ appears to be localised in the cytoplasm, however several condensed foci in the spermatocyte region of the testes resemble CifA$_{w\text{Mel(TI)}}$ localisation to chromatin undergoing meiosis (Figure 3.15F). This result would mirror the localisation studies conducted in insect cell lines, where it was found that CifA localised to the cytoplasm during interphase, however when cells underwent mitosis CifA accumulated on the chromatin[88]. However, as the Hoechst stain did not penetrate the whole tissue, firm conclusions on the localisation of CifA$_{w\text{Mel(TI)}}$ cannot be made.

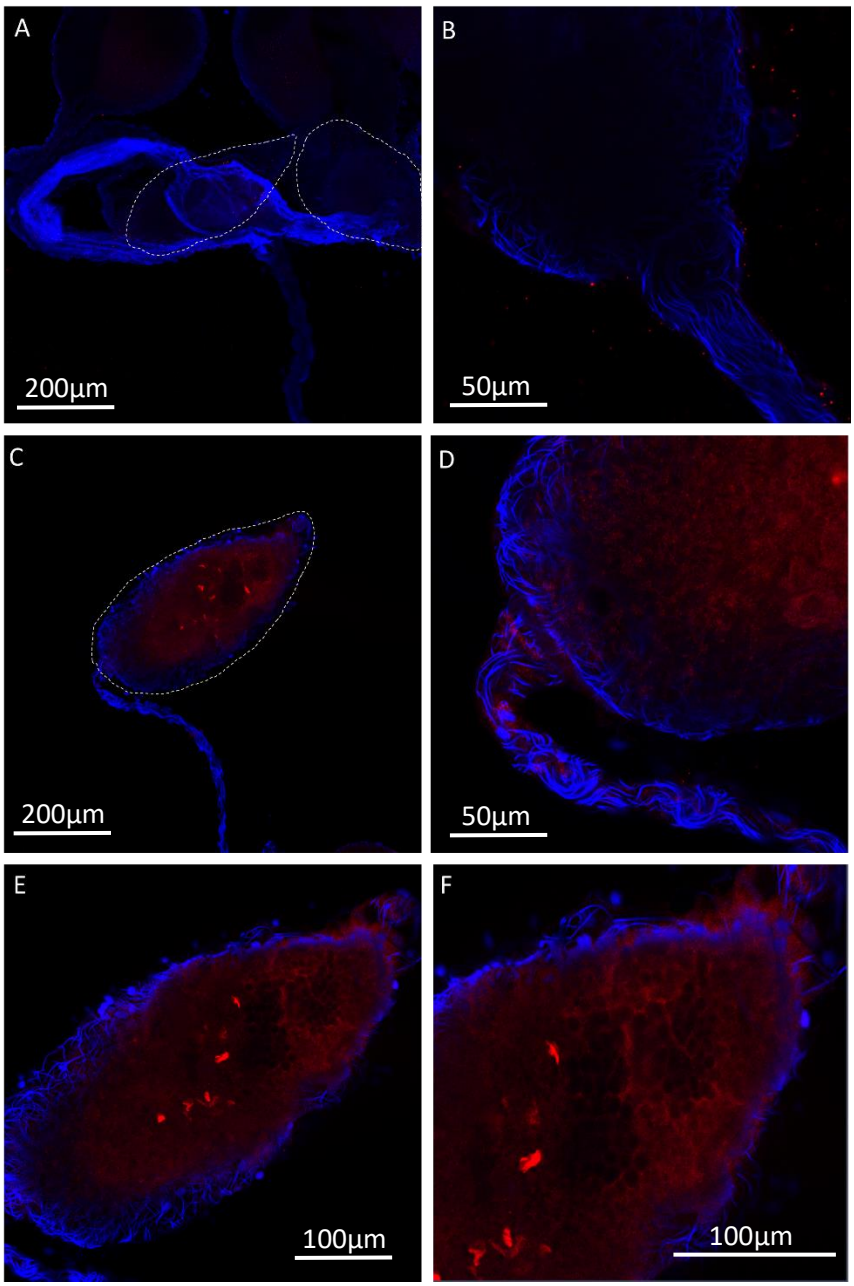


Figure 3.15: CifA$_{wMel(TI)}$ localisation in transgenic *Ae. aegypti* testes. Wild-type testes imaged at (A) 100x magnification (testes outlined in white dashes) and (B) 400x magnification. Nuclei were stained with Hoechst (Blue), as expected there was no signal of CifA$_{wMel(TI)}$ (red) in non-transgenic tissues. Teste dissected from a *PUbt-cifA*$_{wMel(TI)}$ male imaged at (C) 100x magnification (D) 400x magnification, and (E) 200x magnification. CifA$_{wMel(TI)}$ (red) is localised throughout the teste in all cell types when expression is regulated by the *PUbt* promoter. (F) Magnified view of image (E) showing the apical region of the testis. 3 sets of testes were visualised under the scope and these images were taken as representatives.

3.2.5 Maternal expression of *cifA*$_{wMel(TI)}$ driven by the *exu* promoter rescues *Wolbachia*-induced CI in *Ae. aegypti* mosquitoes.

To recapitulate the CI rescue phenotype in *Ae. aegypti* an appropriate promoter is needed to ensure the correct temporal and spatial expression of *cifA*$_{wMel(TI)}$. The *Ae. aegypti* orthologue (AAEL010097) of the *Drosophila exuperantia* (*exu*) gene is expressed at high quantities in the ovaries following a bloodmeal and its product is deposited in the oocytes resulting in a high abundance of the gene product in freshly laid embryos[177]. Therefore, the *exu* promoter was selected to drive expression of *cifA*$_{wMel(TI)}$ in this study (Figure 3.16).

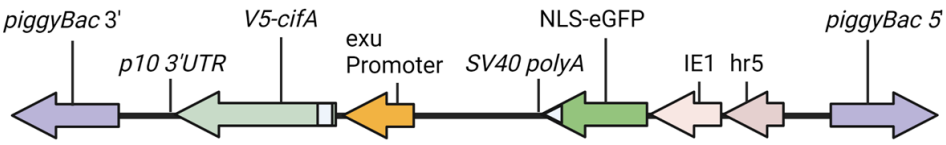


Figure 3.16: Schematic representation of the transposon construct used to investigate *w*Mel *Wolbachia* CI rescue through *exu*-mediated *cifA*$_{wMel(TI)}$ expression.

To test the ability of transgenic mosquitoes expressing *cifA*$_{wMel(TI)}$ to rescue CI, *w*Mel-infected males were crossed with females heterozygous for the transgene and the viability rate of their offspring was assessed. It should be noted that deposition of the CifA rescue factor through *exu* expression is maternal; therefore, those embryos that do not inherit a genomic copy of the transgene in a heterozygous x wild-type cross are still expected to display CI rescue. All four of the independent genomic *exu-cifA*$_{wMel(TI)}$ insertion lines generated (see Table A1) showed significant rescue of CI when crossed with *w*Mel-infected males (Figure 3.17b). The ability of each line to rescue corresponded to the average relative expression of *cifA*$_{wMel(TI)}$ in the ovaries (Figure 3.17a,b). Three of the 4 lines (L1-L3) had significantly higher expression of *cifA*$_{wMel(TI)}$ in the ovaries in comparison to the remaining carcasses, indicating that the promoter was functioning correctly (Figure 3.17c). Line 4 had significantly lower expression than the other lines and did not display ovary-specific expression (Figure 3.17a,c). This might reflect the insertion of the transposon in a genomic region less accessible to the transcription machinery in ovary tissues. This line also shows the weakest eGFP fluorescence, again suggesting reduced transcriptional accessibility.

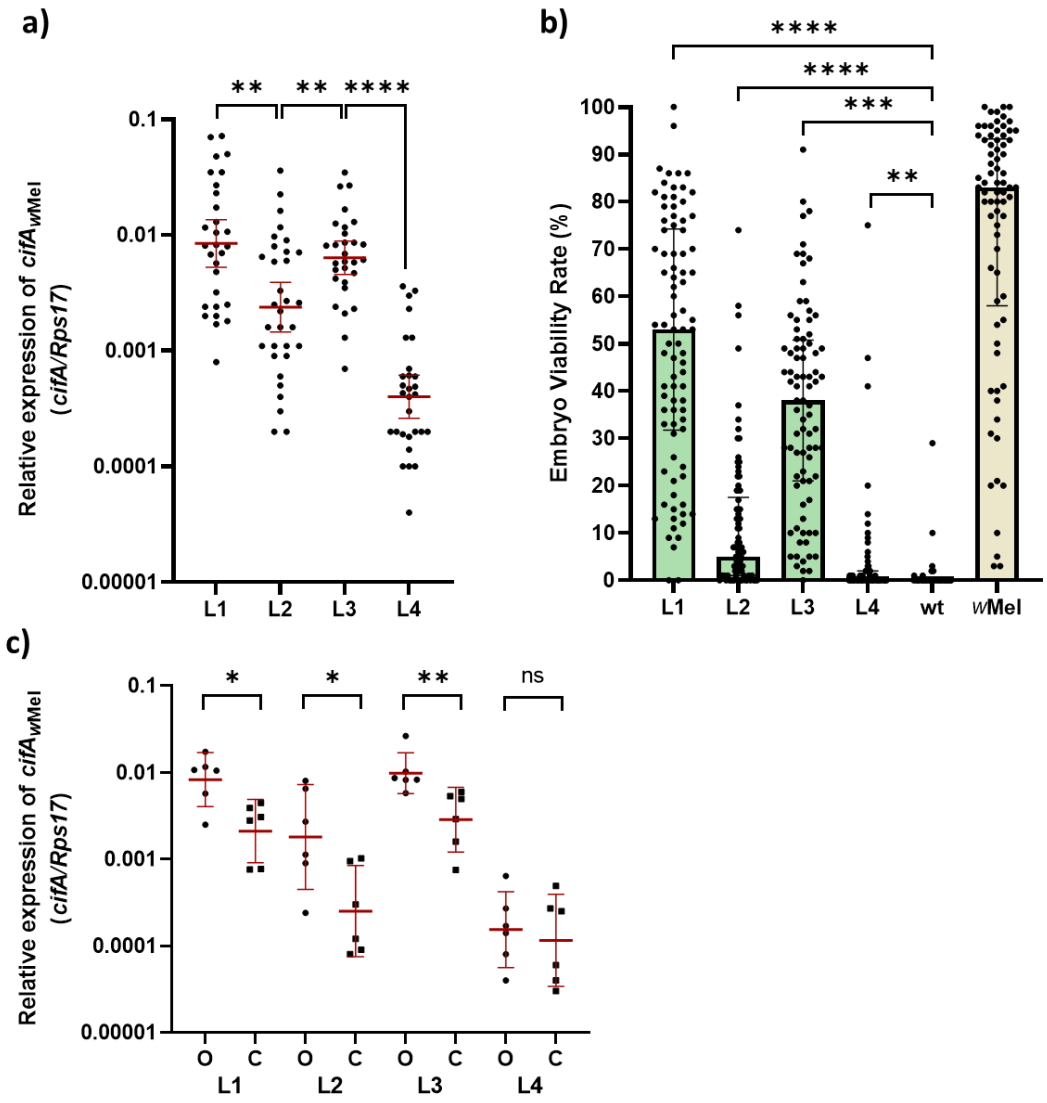


Figure 3.17: Expression of *cifA*$_{w\text{Mel(TI)}}$ under the *exu* promoter rescues CI induced by *w*Mel-carrying *Ae. aegypti* male mosquitoes. (a) The expression of *cifA*$_{w\text{Mel(TI)}}$ in the four *exu-cifA*$_{w\text{Mel(TI)}}$ lines (L1-L4) relative to housekeeping gene *rps17* in single pairs of ovaries 24hrs post oviposition. Red line is the geometric mean, error bars denote 95% confidence intervals, asterisks denote statistical significance. **(b)** Transgenic expression of *cifA*$_{w\text{Mel(TI)}}$ significantly rescues CI induced by *w*Mel-infected males. Dots represent the percentage of viable embryos laid by one female from each of the four *exu-cifA*$_{w\text{Mel(TI)}}$ mosquito lines, bars represent the median and error bars denote interquartile ranges. **(c)** The relative expression of *cifA*$_{w\text{Mel(TI)}}$ (*cifA*/*rps17*) in either the dissected ovaries (O) or remaining carcass (C) of individual females from each of the four lines. The red line is the geometric mean, error bars denote 95% confidence intervals, asterisks denote statistical significance. Statistical significance was determined by Mann-Whitney U-tests, ****=p<0.0001.

The surviving progeny from an *exu-cifA*$_{w\text{Mel(TI)}}$ female and *w*Mel-carrying male cross displayed a 1:1 ratio of transgene inheritance, indicating that rescue is dependent on maternal deposition of either *cifA*$_{w\text{Mel(TI)}}$ gene products or transcripts and not zygotic expression of the transgene – if zygotic expression contributed to rescue a greater proportion of transgenic progeny would be expected. The strength of CI rescue was also found to be related to the relative *Wolbachia* density of *w*Mel in infected males. At lower densities an increase of the median embryo viability rate was observed (Figure 3.18).

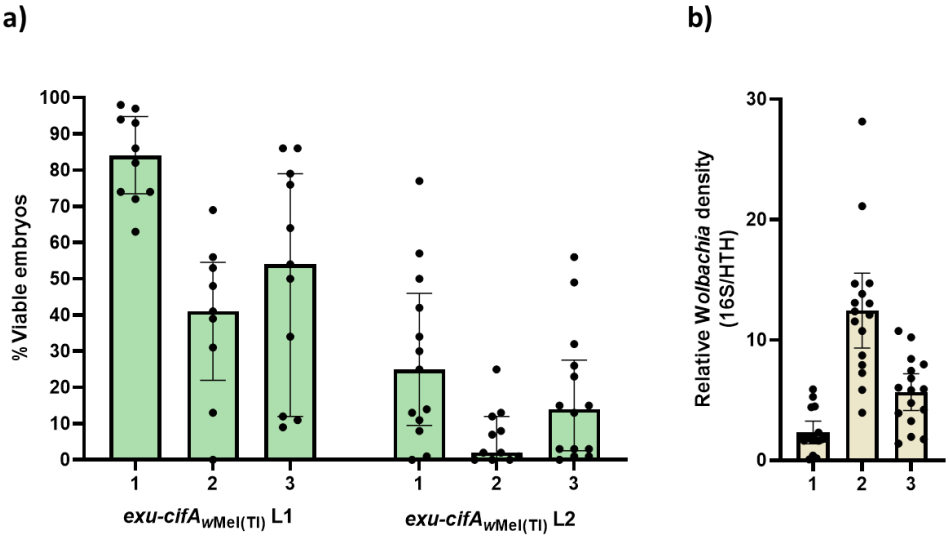


Figure 3.18: The ability of *cifA*$_{w\text{Mel(TI)}}$ expression to rescue is affected by the relative *Wolbachia* density in *w*Mel-carrying *Ae. aegypti* male mosquitoes. a) The median embryo viability rate resulting from crosses of heterozygous females from two different independent genomic *exu-cifA*$_{w\text{Mel(TI)}}$ insertion lines (L1 & 2) and *w*Mel-infected males varied between experimental repeats (1-3). Dots represent the percentage of viable embryos laid by one female, bars represent the median and error bars denote interquartile ranges. **b)** This variation could be attributed to the relative *Wolbachia* densities in the infected males measured by qPCR. The *Wolbachia 16S* gene was normalised to the host *HTH* gene. The dots represent the relative *w*Mel density in a single male mosquito, bars denote the mean and error bars 95% CI.

To ensure the rescue phenotype was specific to *w*Mel, females from each of the four *exu-cifA*$_{w\text{Mel(TI)}}$ lines were crossed with males infected with *w*AlbB, a strain that shows bidirectional incompatibility with *w*Mel (Figure 3.19a). *w*AlbB encodes two pairs of *cif* genes (Type III and Type IV), which show significant sequence divergence from one and other and that of the

Type I pair found in *w*Mel (Figure 3.19b). Consistent with the predicted interactional specificity of cognate *cif* gene pairs, *exu-cifA*$_{w\text{Mel(TI)}}$ females were unable to rescue *w*AlbB male-induced CI (Figure 3.19a).

Through embryonic cytoplasmic transfer from *D. simulans* to *Ae. aegypti*, a line carrying the *Wolbachia* strain *w*Pro was established by Dr. Thomas Ant in the laboratory group. *w*Pro belongs to *Wolbachia* supergroup A and is naturally found in the host species *Drosophila prosaltans*. CI crosses between *w*Pro-carrying males and wild-type females showed that CI penetrance is only partial with this strain in *Ae. aegypti* (Figure 3.19a: Mdn = 22.0%, IQR = 16.0-34.0%). Initial crosses revealed that *w*Mel-infected females could fully rescue CI when mated with *w*Pro-infected males. To determine if this cross-compatibility was reliant on *cifA*$_{w\text{Mel(TI)}}$ expression, *w*Pro-infected males were mated with females heterozygous for *cifA*$_{w\text{Mel(TI)}}$ from all four transgenic lines (Figure 3.19a). The ability for each *exu-cifA*$_{w\text{Mel(TI)}}$ line to rescue CI induced by *w*Pro was relative to the expression levels of the transgene in the ovaries as was the case with *w*Mel CI crosses. In these sets of crosses the three lines that displayed ovary-specific expression of *cifA*$_{w\text{Mel(TI)}}$ (L1-L3) significantly increased the viability rate of embryos when crossed with either *w*Mel and *w*Pro, indicating that the CifA variant from *w*Mel can rescue *w*Pro-induced CI (Figure 3.19a).

Primers designed to target the flanking regions of *cifA* in the *w*Mel genome were tested on DNA extracts from *w*Pro-infected mosquitoes. Sequencing of the resulting PCR product revealed that *w*Pro encoded a Type I *cifA* gene with 99% sequence similarity to that of *w*Mel. As *Wolbachia* strains can harbour multiple *cif* genes, the presence of this homologue alone did not yet explain the compatibility observed between the strains. Subsequent genome sequencing of *w*Pro revealed it contains *cif* genes from both the Type I and Type V clades. The Type V *cifB* is pseudogenised by a frameshift mutation and is therefore expected to be non-functional, while the *w*Pro Type I CifA and CifB genes share 99% amino acid similarity with the *cif* genes found in *w*Mel, which likely explains why the two strains are compatible, and why transgenic expression of *cifA*$_{w\text{Mel(TI)}}$ can rescue *w*Pro-induced CI (Figure 3.19). It is hypothesised that the difference in CI penetrance between the two strains (despite the similarity in functional *cif* gene sequences) is the result of significant differences in the relative density of *Wolbachia* in the male mosquitoes (Figure 3.20). However, it is also possible that the *cif* genes are transcribed at different levels in these strains.

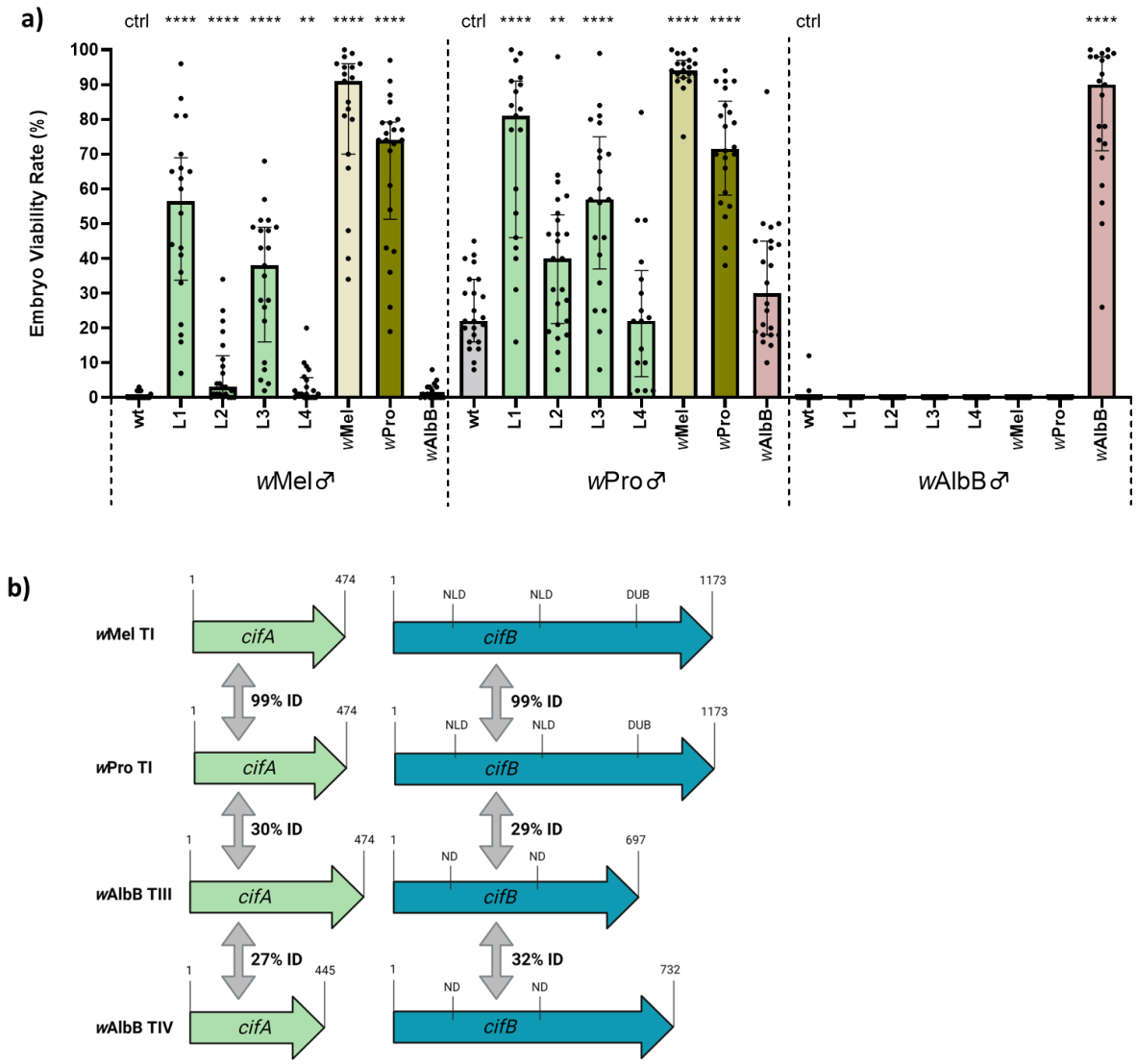


Figure 3.19: *cifA*$_{wMel(TI)}$ can rescue CI induced by the compatible *w*Mel and *w*Pro strains but not the incompatible strain *w*AlbB. (a) Graph demonstrating the rescue specificity of *cifA*$_{wMel(TI)}$. The dots represent the percentage of viable embryos laid by single females, bars represent the median and error bars denote interquartile ranges. Asterisks above bars represent significant differences relative to a control (wt female/ *Wolbachia*-infected male) cross (ctrl). Significance calculated by Mann-Whitney U-test ****=p<0.0001. **(b)** Comparison of Cifs from *w*Mel, *w*Pro, and *w*AlbB. The Type I Cifs from *w*Mel and *w*Pro share 99% identity (ID), the CifB proteins contain two sets of inactive nuclease-like domains (NLD), and a deubiquitinase (DUB) domain. *w*AlbB contains Type III and Type IV Cifs, the CifB proteins both include two sets of intact nuclease domains (ND). Length of peptide (in amino acids) indicated by numbers.

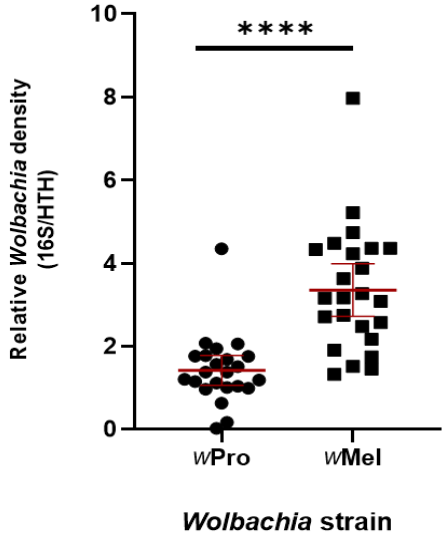


Figure 3.20: Comparison of the relative *Wolbachia* densities in adult male mosquitoes. Total *Wolbachia* densities were measured by qPCR in *w*Mel and *w*Pro carrying *Ae. aegypti* males. The *Wolbachia 16S* gene was normalised to the host *HTH* gene. Red line is the mean, error bars 95% confidence intervals. Mann-Whitney U-test used for statistical analyses ****=p<0.0001

3.2.6 Constitutive expression of *cifA*$_{w\text{Mel(TI)}}$ rescues *Wolbachia*-induced CI in *Ae. aegypti* mosquitoes.

Although expression of *cifA*$_{w\text{Mel(TI)}}$ regulated by the *exu* promoter significantly rescued CI induced by *w*Mel-carrying males, the strongest expressing line (L1) was only able to increase the embryo viability rate by approximately half (Figure 3.17b: Mdn= 53.0%, IQR = 31.8-74.3%). It was hypothesised that a stronger expression of the transgene might increase the viability rate of embryos in a CI cross. Therefore, lines expressing *cifA*$_{w\text{Mel(TI)}}$ under the control of the strong constitutive promoter *PUb* or the truncated form (*PUbt*) were tested for their ability to rescue *w*Mel-induced CI (line generation described previously). First, the five *PUb-cifA*$_{w\text{Mel(TI)}}$*-T2A-cifB*$_{w\text{Mel(TI)}}$ lines were tested for their ability to rescue CI (Figure 3.21). All lines except Line 4 had significantly higher embryo viability rates in comparison to wild-type. Line 4 had significantly lower expression of the bicistronic transcript in comparison to the other lines (based on carcass data from males- Figure 3.7b).

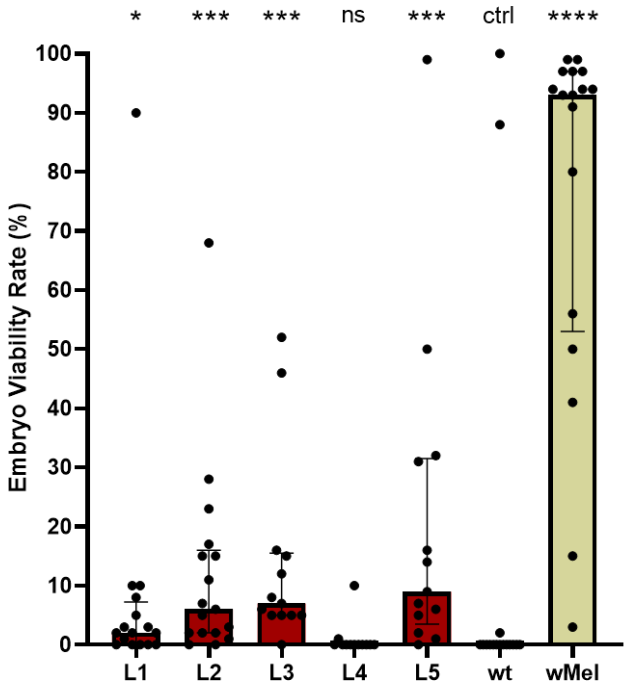


Figure 3.21: Dual expression of *cifA*$_{wMel(TI)}$ and *cifB*$_{wMel(TI)}$ under the control of the *PUb* promoter in four of the five transgenic lines generated significantly rescues *w*Mel-induced CI. Heterozygous females from five different genomic *PUb-cifA*$_{wMel(TI)}$-*T2A-cifB*$_{wMel(TI)}$ insertion lines (L1-L5) were crossed with *w*Mel-infected males. Dots represent the percentage of viable embryos laid by one female, bars represent the median and error bars denote interquartile ranges. Asterisks above bars represent significant differences relative to a control (wt female/ *Wolbachia*-infected male) cross (ctrl). Significance calculated by Mann-Whitney U-test ****=p<0.0001.

As the majority of *PUb-cifA*$_{wMel(TI)}$-*T2A-cifB*$_{wMel(TI)}$ lines rescued *w*Mel-induced CI in an expression level dependent manner, it would suggest that the T2A peptide sequence is operating to some degree - resulting in cleaved and functional CifA$_{wMel(TI)}$ production. However, the *PUb-cifA*$_{wMel(TI)}$-*T2A-cifB*$_{wMel(TI)}$ lines only increased the embryo viability rate minimally in comparison to *exu-cifA*$_{wMel(TI)}$ L1 - despite an expected higher *cifA*$_{wMel(TI)}$ expression level. The production of CifB$_{wMel(TI)}$ is not expected to have had an effect on the capacity to rescue, as *Wolbachia* are thought to express both *cif* genes simultaneously, and dual-expression in transgenic *Drosophila* studies resulted in comparable rescue to that of *cifA*$_{wMel(TI)}$ expressed individually[81]. As *cifB*$_{wMel(TI)}$ was placed downstream of the T2A sequence

it is expected that problems in T2A-mediated peptide cleavage would result in a higher abundance of upstream CifA$_{w\text{Mel(TI)}}$. Therefore, if problems in the translation of the bicistronic transcript resulted in a reduced rescue capacity it is more likely due to the production of non—functional chimera proteins and not an overabundance of CifB$_{w\text{Mel(TI)}}$. Western blotting would be a simple method to test whether the two transgenes were being translated into separate peptides, however initial tests revealed binding of both the anti-His and anti-Flag antibodies to unknown background targets in negative control samples (Figure A3). One of the unspecified targets being bound by the anti-Flag antibody had the same molecular weight expected of Flag-tagged *cifB*$_{w\text{Mel(TI)}}$ which would mask any potential signal.

The *cifA*$_{w\text{Mel(TI)}}$ transgene is either His-tagged or V5-tagged in the *PUb-cifA*$_{w\text{Mel(TI)}}$*-T2A-cifB*$_{w\text{Mel(TI)}}$ and *exu-cifA*$_{w\text{Mel(TI)}}$ lines respectively. It is possible that the His-tag affected the conformation of the protein and the V5-tag did not. Furthermore, the 3' untranslated region sequences differ between the different constructs which can result in different translation rates. To resolve these differences in construct design and determine whether the difference in CI rescue capability is the result of promoter selection or problems in transgene translation, the *PUb-cifA*$_{w\text{Mel(TI)}}$ lines were subsequently tested (Figure 3.22). Due to the high mortality rate associated with *PUb-cifA*$_{w\text{Mel(TI)}}$ L1 and L3, *PUbt-cifA*$_{w\text{Mel(TI)}}$ L2 and L3 were also tested (Figure 3.22). Although the *PUb/PUbt-cifA*$_{w\text{Mel(TI)}}$ lines had higher or comparable transgene expression to *exu-cifA*$_{w\text{Mel(TI)}}$ L1 in the ovaries, only one of the lines (*PUbt-cifA*$_{w\text{Mel(TI)}}$ L2) had a comparable rescue ability (Figure 3.22a,b). The ability of the *exu* regulatory region to promote transcript/protein deposition into developing oocytes is well documented[151,177,178]. It is therefore likely that a higher expression of the *cifA*$_{w\text{Mel(TI)}}$ in the ovaries is needed to compensate for the less targeted deposition of transcripts in developing oocytes. Accordingly, the *PUb*/*PUbt-cifA*$_{w\text{Mel(TI)}}$ line with the highest expression in the ovaries demonstrated the greatest rescue ability (Figure 3.22a,b). As the CI rescue capability of the *PUb-cifA*$_{w\text{Mel(TI)}}$*-T2A-cifB*$_{w\text{Mel(TI)}}$ lines (excluding L4) was comparable to the *PUb*/*PUbt-cifA*$_{w\text{Mel(TI)}}$ lines (excluding *PUbt-cifA*$_{w\text{Mel(TI)}}$ L2) it suggests that the low embryo viability rate observed for these lines was not the result of problems in peptide cleavage, or epitope tag/ 3' untranslated region sequence selection but rather the spatiotemporal expression of *cifA*$_{w\text{Mel(TI)}}$.

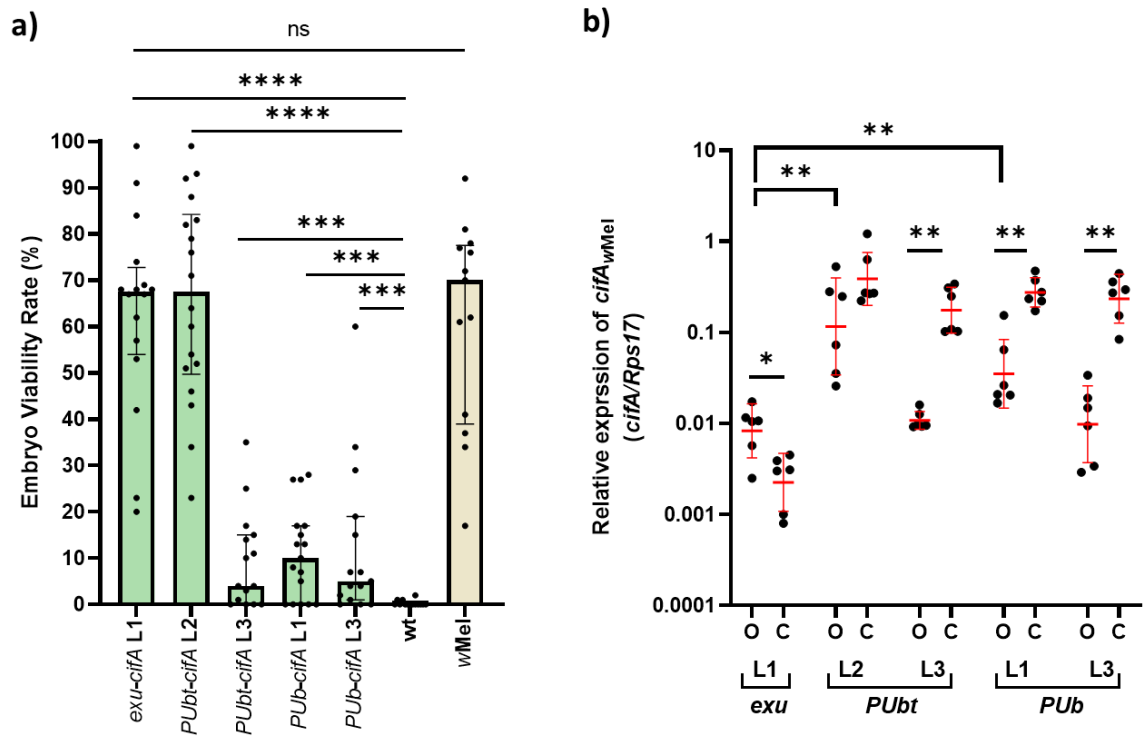


Figure 3.22: *cifA*$_{wMel(TI)}$ can significantly rescue CI when expressed in uninfected females under different promoters. a) Comparison of the rescue capability of different genomic insertion lines of transgenic mosquitoes, expressing *cifA*$_{wMel(TI)}$ under either the *exu*, *PUbt*, or *PUb* promoter, when crossed with *w*Mel males. Dots represent the percentage of viable embryos laid by one female, bars represent the median and error bars denote interquartile ranges. Significance calculated by Mann-Whitney U-test ****=p<0.0001. **b)** The expression of *cifA*$_{wMel(TI)}$ in one *exu-cifA*$_{wMel(TI)}$ line (L1) two *PUbt-cifA*$_{wMel(TI)}$ lines (L2 & L3), and two *PUb-cifA*$_{wMel(TI)}$ lines (L1 & L3) relative to housekeeping gene *rps17* in single pairs of ovaries (O) or remaining carcasses (C) 24hrs post oviposition. Red line is the geometric mean, error bars denote 95% confidence intervals, asterisks denote statistical significance.

3.2.7 Conditional *cifB*$_{wMel(TI)}$ expression results in embryonic lethality.

3.2.7.1 Generation of TRE-*cifB*$_{wMel(TI)}$ lines

Based on i) the catalytic residues encoded by *cifB* genes, ii) toxicity assays in transgenic insect cells and yeast, CifB is hypothesized to act as the primary *mod* factor/toxin. As CI canonically manifests as a delay in paternal chromosome condensation leading to a failure in chromatid segregation in early rounds of mitotic division- the CI-associated modifications (mediated by CifB) are expected to occur either during spermatogenesis or shortly after fertilisation.

Therefore, early embryonic expression of *cifA* is not expected to rescue CI induced by paternal *cifB* expression and instead, rescue in transgenic systems is the result of maternal deposition of *cifA* transcripts/gene product into the oocyte before fertilisation. As rescue is dependent on maternal possession of the transgene and not the embryo itself, this would affect the efficiency of any potential *cif*-based gene drive system (non-transgene carrying embryos/alleles benefiting from rescue through maternal deposition). However, if embryonic (non-paternal) *cifB* expression can cause lethality then a comparable embryonic *cifA* expression might be able to inhibit this effect. In this scenario, only individuals possessing the transgene would survive which would increase the relative fitness of transgenic mosquitoes. As the sole expression of *cifB*$_{w\text{Mel(TI)}}$ under the control of an embryonic promoter was expected to be lethal, a tetracycline repressible (Tet-Off) system was utilised (Figure 3.23). The Flag-tagged *cifB*$_{w\text{Mel(TI)}}$ gene was inserted downstream of a *hsp70* minimal promoter linked to a tetracycline response element (TRE) which is composed of several repeats of a tetracycline operator (*tetO*) sequence. In this instance, *cifB*$_{w\text{Mel(TI)}}$ will only be expressed when a tetracycline-repressible transactivator (tTAV) binds to the *tetO* sequences. The timing and level of *tTAV* expression in turn can be controlled by an upstream promoter: the *PUbt* promoter was selected for this study based on high expression levels in early embryos. To repress the expression of *cifB*$_{w\text{Mel(TI)}}$, mosquitoes can be reared in the presence of tetracycline (or an analogue such as doxycycline) which binds to tTAV thus preventing binding to the TRE and activation of transgene expression (Figure 3.23c).

Pre-blastoderm embryos were co-injected with the TRE-*cifB*$_{w\text{Mel(TI)}}$ donor (Figure 3.23a) and helper plasmids resulting in 5 independent genomic insertion TRE-*cifB*$_{w\text{Mel(TI)}}$ lines (see Table A1). Heterozygous males from two TRE-*cifB*$_{w\text{Mel(TI)}}$ lines (L1 and L2) were crossed with heterozygous females from the *PUbt-tTAV* line (provided by collaborators), and mosquitoes were reared in either the presence or absence of doxycycline. Although the resulting embryos looked viable, mosquitoes possessing both transgenic loci failed to hatch when reared in the absence of doxycycline (Figure 3.24). However, when mosquitoes and the resulting egg cones were hatched in the presence of doxycycline TRE-*cifB*$_{w\text{Mel(TI)}}$/*PUbt-tTAV* heterozygotes survived (Figure 3.24) - suggesting that early embryonic expression of *cifB*$_{w\text{Mel(TI)}}$ is indeed lethal. However, it is unknown whether this expression is the result of the catalytic activity of *cifB*$_{w\text{Mel(TI)}}$ or because an overexpression of any gene at this developmental stage can be lethal.

Because the toxicity of CifB can be inhibited when CifA is co-expressed (in both yeast and insect cell toxicity assays[78,80,85,86,88]), it is possible to test whether the observed CifB$_{wMel(TI)}$-induced embryonic lethality is repressed by CifA$_{wMel(TI)}$, and therefore presumably caused by the inherent toxicity of Cif*B*$_{wMel(TI)}$.

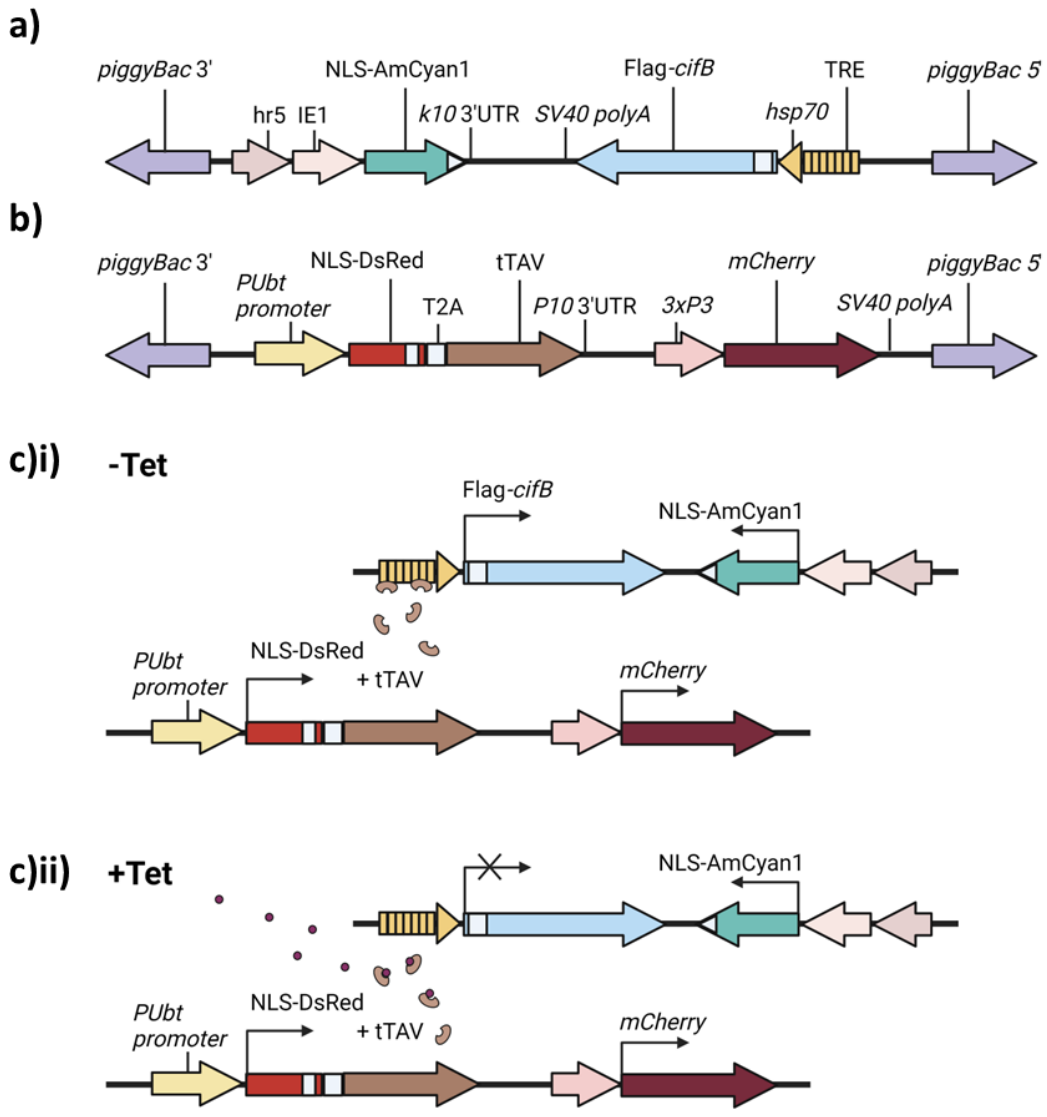


Figure 3.23: Exploiting the Tet-Off system to investigate the effect of embryonic expression of *cifB*$_{wMel(TI)}$. a) Schematic representation of the TRE-*cifB*$_{wMel(TI)}$ transposon used in this study. **b)** Schematic representation of the *PUbt-tTAV* transposon used in this study. **c)** Schematic representation of the Tet-Off system when mosquitoes are reared in the **i)** absence or **ii)** presence of tetracycline or its analogues (purple circles).

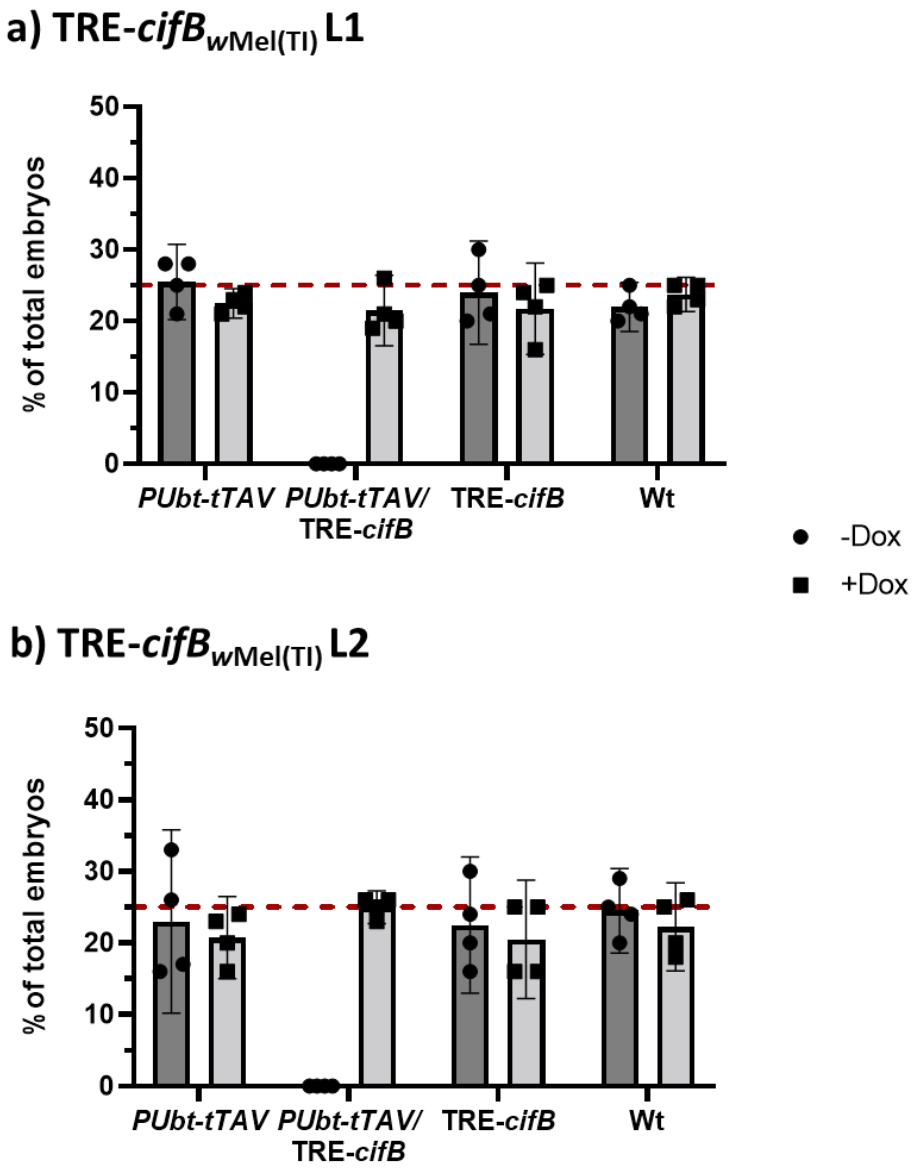


Figure 3.24: Doxycycline repressibility of embryonic lethality in two of the TRE-*cifB*$_{wMel(TI)}$ lines tested. Heterozygous females from the *PUbt-tTAV* line were crossed with heterozygous males from either TRE-*cifB*$_{wMel(TI)}$ **a)** L1 or **b)** L2 and transgene inheritance in the resulting progeny was screened via fluorescent microscopy at late larval stages. Embryonic lethality is observed in the absence of doxycycline (-Dox) as a complete absence of progeny which possess both the TRE-*cifB*$_{wMel(TI)}$ and *PUbt-tTAV* transgenes and survive to screening age. Lethality is repressed in the presence of doxycycline (+Dox) during mosquito rearing. Dots and squares represent the percentage of total embryos laid per individual female either possessing a transgenic allele or not (wt). Red dashed line represents the expected allele inheritance of 25%, bars denote the mean and error bars the S.D.

3.2.7.2 *cifA*$_{wMel(TI)}$ expression is unable to rescue *cifB*$_{wMel(TI)}$ induced embryonic lethality.

It was initially hypothesised that a *w*Mel infection in the embryo may be able to rescue the toxicity resulting from embryonic expression of *cifB*$_{wMel(TI)}$ (TRE-*cifB*$_{wMel(TI)}$/*PUbt-tTAV*). Heterozygous males from the *PUbt-tTAV* line were first mated with *w*Mel-infected females and the resulting transgenic/*w*Mel-infected female progeny were crossed to heterozygous males from two of the TRE-*cifB*$_{wMel(TI)}$ lines (L1 and L2). However, *cifA*$_{wMel(TI)}$ expression from a *w*Mel infection was unable to prevent embryonic lethality in the progeny actively expressing *cifB*$_{wMel(TI)}$ (Figure 3.25). It is possible that the endogenous expression of *cifA*$_{wMel(TI)}$ is not at a sufficiently high level to counterbalance the toxicity of *cifB*$_{wMel(TI)}$ in this transgenic system. However, as the expression of *cifB*$_{wMel(TI)}$ is lethal when the TRE is bound by tTAV, we were unable to determine the relative expression of *cifB*$_{wMel(TI)}$ in comparison to endogenous *cifA*$_{wMel(TI)}$. Furthermore, the direct comparison of transcript levels would be complicated by the physiological differences in prokaryotic endosymbiont transcription/translation/ extracellular transport and eukaryotic chromosomal expression.

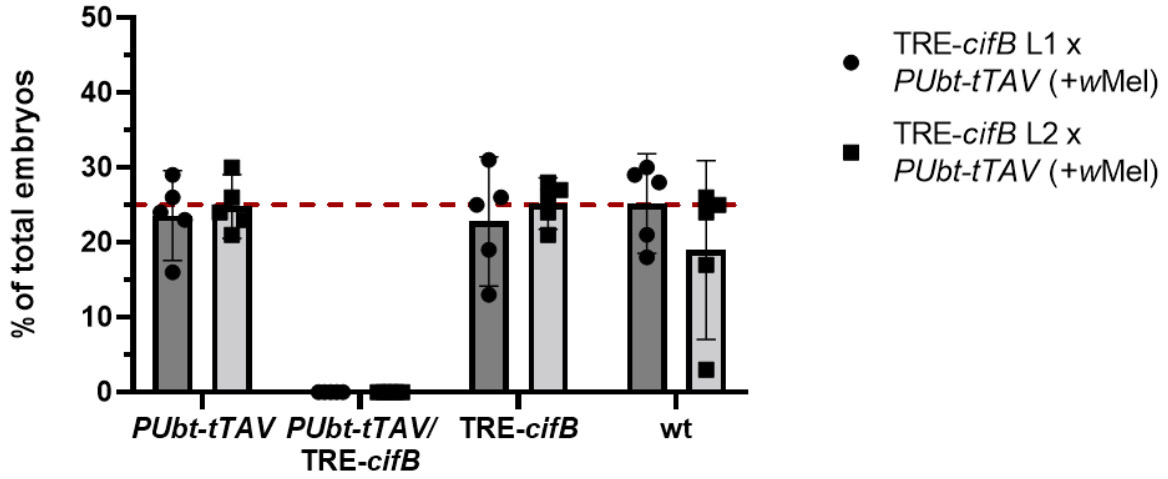


Figure 3.25: A *w*Mel infection does not rescue embryonic lethality induced by *cifB*$_{wMel(TI)}$ expression in the absence of doxycycline. wMel-infected heterozygous females from the *PUbt-tTAV* line were crossed with heterozygous males from either TRE-*cifB*$_{wMel(TI)}$ L1 or L2 and transgene inheritance in the resulting progeny was screened via fluorescent microscopy at late larval life stages. Dots and squares represent the percentage of total embryos (laid by an individual female) either possessing a transgenic allele or not (wt). Red dashed line represents the expected allele inheritance of 25%, bars denote the mean and error bars the S.D.

Because some of the *PUb-cifA*$_{w\text{Mel(TI)}}$*-T2A-cifB*$_{w\text{Mel(TI)}}$ lines had shown a capacity to rescue *Wolbachia*-mediated CI to an extent, it was hypothesised that these lines would be able to rescue lethality when the expression of *cifB*$_{w\text{Mel(TI)}}$ was embryonic and not paternal. All five *PUb-cifA*$_{w\text{Mel(TI)}}$*-T2A-cifB*$_{w\text{Mel(TI)}}$ lines were crossed into the *PUbt-tTAV* line and the resulting *PUb-cifA*$_{w\text{Mel(TI)}}$*-T2A-cifB*$_{w\text{Mel(TI)}}$*/PUbt-tTAV* female progeny were mated with heterozygous males from the TRE-*cifB*$_{w\text{Mel(TI)}}$ L1. *PUb* driven co-expression of *cifA*$_{w\text{Mel(TI)}}$ and *cifB*$_{w\text{Mel(TI)}}$ was unable to rescue the embryonic lethality resulting from activation of *cifB*$_{w\text{Mel(TI)}}$ expression (Figure 3.26). Because transcription of the bicistronic transcript encoding both *cifA*$_{w\text{Mel(TI)}}$ and *cifB*$_{w\text{Mel(TI)}}$ would result in equal amounts of the gene products (if the T2A peptide is functioning correctly), it is possible that additional strong expression of *cifB*$_{w\text{Mel(TI)}}$ in the absence of doxycycline might result in a higher abundance of CifB$_{w\text{Mel(TI)}}$.

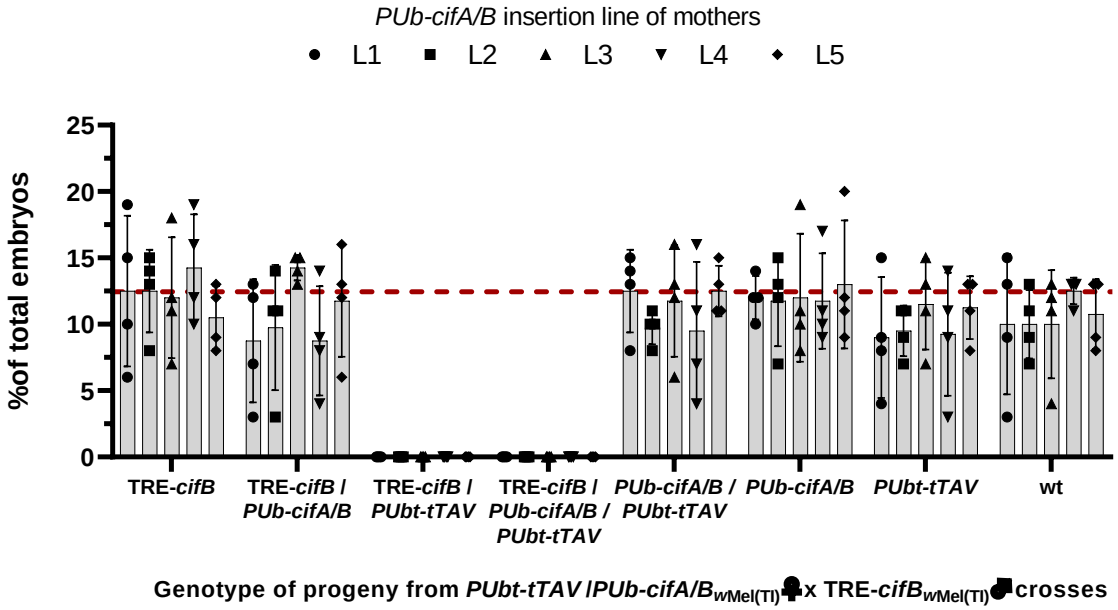


Figure 3.26: Expression of *cif*$_{w\text{Mel(TI)}}$ transgenes by all *PUb-cifA*$_{w\text{Mel(TI)}}$*-T2A-cifB*$_{w\text{Mel(TI)}}$ lines generated does not rescue embryonic lethality induced by *cifB*$_{w\text{Mel(TI)}}$ expression in the absence of doxycycline. Females heterozygous for both *PUbt-tTAV* and *PUb-cifA*$_{w\text{Mel(TI)}}$*-T2A-cifB*$_{w\text{Mel(TI)}}$ genomic insertions were crossed with heterozygous males from TRE-*cifB*$_{w\text{Mel(TI)}}$ L1 and transgene inheritance in the resulting progeny was screened via fluorescent microscopy at late larval life stages. Dots, squares, triangles, inverted triangles, and diamonds represent the percentage of total embryos (laid by an individual female) either possessing a transgenic allele or not (wt). Red dashed line represents the expected allele inheritance of 12.5%, bars denote the mean and error bars the S.D.

Although the differences in expression between different TRE-*cifB*$_{w\text{Mel(TI)}}$ lines cannot be determined, different lines show variation in the patterning and intensity of AmCyan1 fluorescence which might reflect insertions in chromatin regions of differing transcriptional activity. L3 for example had a much lower intensity of fluorescence which might suggest the transposon inserted itself into a genomic region not readily accessible to the transcription machinery and therefore might express *cifB*$_{w\text{Mel(TI)}}$ at lower levels than L1 and L2 when activated. Therefore, TRE-*cifB*$_{w\text{Mel(TI)}}$ L1 and L3 were used in future rescue experiments to investigate whether potential differences in *cifB*$_{w\text{Mel(TI)}}$ expression result in differences in rescue capacity. As the expression of *cifA*$_{w\text{Mel(TI)}}$ under the control of the *PUb* promoter is expected to be greater than the expression of *cifB*$_{w\text{Mel(TI)}}$ in the Tet-Off system (Dr Tim Harvey-Samuel pers. comm.), it was hypothesised that this difference in the relative expression of the *cif* genes would be able to inhibit embryonic lethality. Although, the expression of *cifA*$_{w\text{Mel(TI)}}$ in these lines was found to also be toxic (See section 3.2.4.1) it was hypothesised that because no transgene-dependent lethality was observed in the *PUb-cifA*$_{w\text{Mel(TI)}}$*-T2A-cifB*$_{w\text{Mel(TI)}}$ lines, that expression of both *cif* genes might mutually repress each other's toxicity, for example if they are not toxic when bound with each other. However, *cifA*$_{w\text{Mel(TI)}}$ expression under the control of the *PUb* promoter was unable to rescue embryonic lethality (Figure 3.27).

It was hypothesised that *cifA*$_{w\text{Mel(TI)}}$-induced lethality which is observed at early larval development stages might mask any potential rescue of embryonic lethality induced by *cifB*$_{w\text{Mel(TI)}}$. Therefore, the two *PUbt-cifA*$_{w\text{Mel(TI)}}$ lines (L2 and L3) which don't display any negative fitness effects of transgene inheritance yet have comparable levels of *cifA*$_{w\text{Mel(TI)}}$ expression were tested for their ability to rescue embryonic lethality induced by *tTAV* activated *cifB*$_{w\text{Mel(TI)}}$ expression. Expression of *cifA*$_{w\text{Mel(TI)}}$ by *PUbt-cifA*$_{w\text{Mel(TI)}}$ L1 and 2 did not rescue embryonic lethality (Figure 3.28). As the expression of *cifA*$_{w\text{Mel(TI)}}$ under the *PUbt* promoter is expected to be higher than the expression of *cifB*$_{w\text{Mel(TI)}}$ regulated by the Tet-Off system (Dr Tim Harvey-Samuel pers. comm.) it is puzzling why the toxicity of *cifB*$_{w\text{Mel(TI)}}$ is not inhibited as is seen in both transgenic yeast and insect cell studies. According to one potential CI induction mechanism posited by the TA model, CifA is rapidly degraded in fertilised embryos/zygotes and unless a steady expression of *cifA* is maintained by a *Wolbachia* infection then residual CifB deposited by the sperm will result in lethality[90]. If this theory is correct, then the transgenic CifA might degrade more quickly relative to transgenic CifB during

embryogenesis, resulting in a lower total abundance of the former and therefore a lethality phenotype. Alternatively, if the toxicity of *cifB*$_{w\text{Mel(TI)}}$ does not result from its inherent catalytic activity (perhaps due to inappropriate protein folding) but in fact is an artefact of overexpression, then the inability of *cifA*$_{w\text{Mel(TI)}}$ to rescue embryonic lethality might be explained. To test whether a similar embryonic lethal phenotype is observed when an alternative gene is expressed in the Tet-Off system a TRE-*cifA*$_{w\text{Mel(TI)}}$ line was generated (see Table A1).

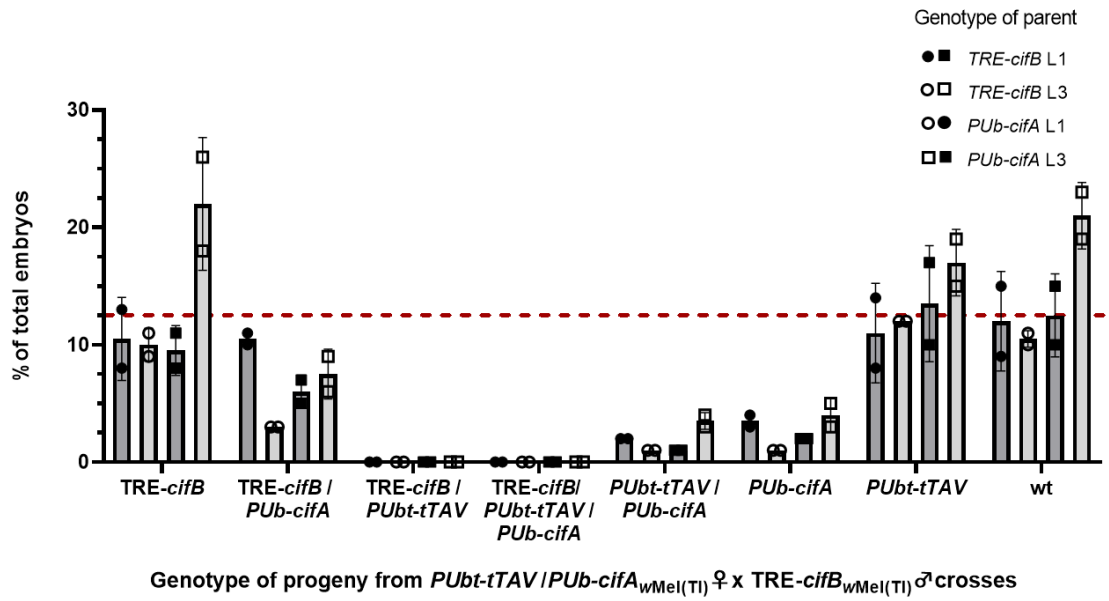


Figure 3.27: Expression of *cifA*$_{w\text{Mel(TI)}}$ by *PUb-cifA*$_{w\text{Mel(TI)}}$ lines does not rescue embryonic lethality induced by *cifB*$_{w\text{Mel(TI)}}$ expression in the absence of doxycycline. Heterozygous *PUbt-tTAV /PUb-cifA*$_{w\text{Mel(TI)}}$ mosquitoes were crossed with heterozygous mosquitoes from either TRE-*cifB*$_{w\text{Mel(TI)}}$ L1 (filled symbol) or L3 (open symbol) and transgene inheritance in the resulting progeny was screened via fluorescent microscopy at late larval life stages. Dots (*PUb-cifA*$_{w\text{Mel(TI)}}$ L1), and squares (*PUb-cifA*$_{w\text{Mel(TI)}}$ L3) represent the percentage of total embryos (laid by a pool of females) either possessing a transgenic allele or not (wt). Red dashed line represents the expected allele inheritance of 12.5%, bars denote the mean and error bars the S.D.

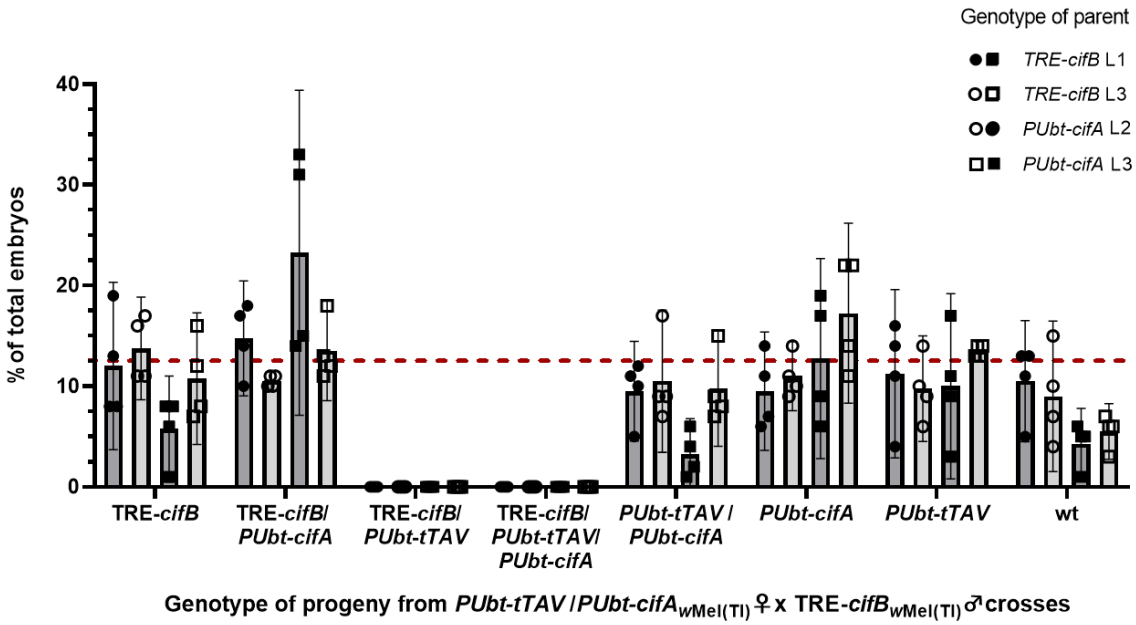


Figure 3.28: Expression of *cifA*$_{wMel(TI)}$ by *PUbt-cifA*$_{wMel(TI)}$ lines does not rescue embryonic lethality induced by *cifB*$_{wMel(TI)}$ expression in the absence of doxycycline. Females heterozygous for both *PUbt-tTAV* and *PUbt-cifA*$_{wMel(TI)}$ genomic insertions were crossed with heterozygous males from either TRE-*cifB*$_{wMel(TI)}$ L1(filled symbol) or L3 (open symbol) and transgene inheritance in the resulting progeny was screened via fluorescent microscopy at late larval life stages. Dots (*PUbt-cifA*$_{wMel(TI)}$ L2), and squares (*PUbt-cifA*$_{wMel(TI)}$ L3) represent the percentage of total embryos (laid by an individual female) either possessing a transgenic allele or not (wt). Red dashed line represents the expected allele inheritance of 12.5%, bars denote the mean and error bars the S.D.

To test whether *tTAV*-induced embryonic expression of *cifA*$_{wMel(TI)}$ (Figure 3.29) results in lethality, heterozygotes from the TRE-*cifA*$_{wMel(TI)}$ line were crossed with *PUbt-tTAV* heterozygotes and the transgene inheritance was screened in the progeny (Figure 3.30). In comparison to the three TRE-*cifB*$_{wMel(TI)}$ lines tested, the inheritance of both *PUbt-tTAV* and TRE-*cifA*$_{wMel(TI)}$ insertions did not always result in death. However, embryonic expression of *cifA*$_{wMel(TI)}$ in the Tet-Off system did result in a high mortality rate similar to what is seen when *cifA*$_{wMel(TI)}$ is expressed under the control of the *PUb* promoter. Although this result might suggest that *cifB* $_{wMel(TI)}$ is more toxic than *cifA*$_{wMel(TI)}$, because only one line was generated, it remains possible that other TRE-*cifA*$_{wMel(TI}$ lines generated would result in complete lethality similar to what is seen for the TRE-*cifB*$_{wMel(TI)}$ lines.

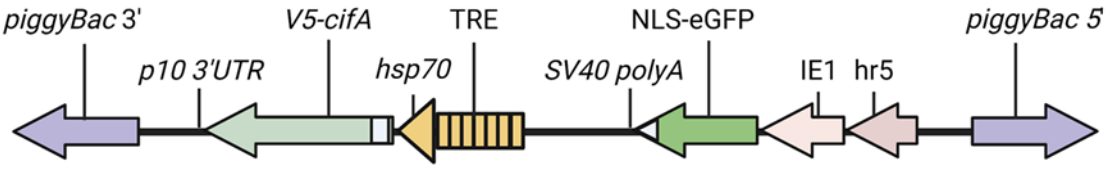


Figure 3.29: Schematic representation of the TRE-*cifA*$_{wMel(TI)}$ transposon used in this study.

To test whether the simultaneous activation of *cifA*$_{wMel(TI)}$ and *cifB*$_{wMel(TI)}$ expression would inhibit the lethality observed when the transgenes are expressed individually, the TRE-*cifA*$_{wMel(TI)}$ line was crossed into either TRE-*cifB*$_{wMel(TI)}$ L1 or L3 and the resulting TRE-*cifA*$_{wMel(TI)}$/ TRE-*cifB*$_{wMel(TI)}$ heterozygous females were mated with heterozygous *PUbt-tTAV* males. Embryonic expression of *cifA*$_{wMel(TI)}$ and *cifB*$_{wMel(TI)}$ induced by tTAV binding to the TRE resulted in complete embryonic lethality and therefore the two Cif peptides did not appear to repress the toxicity of the other (Figure 3.31).

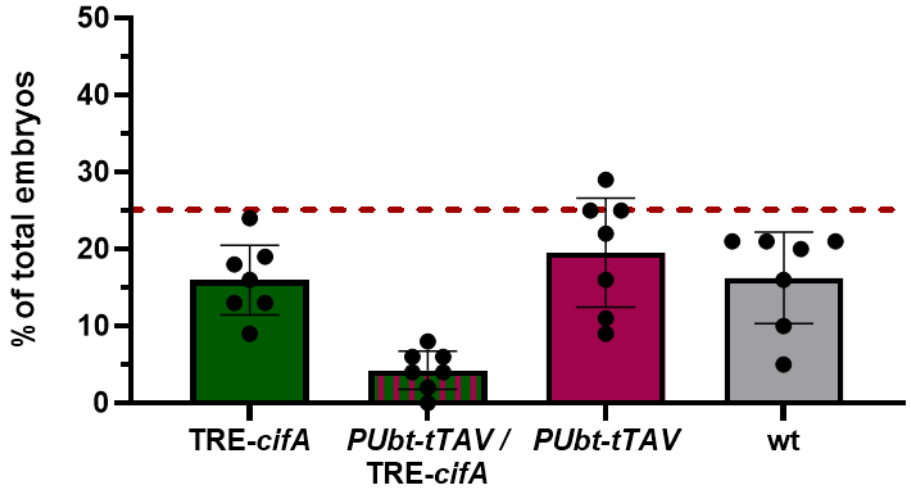


Figure 3.30: Embryonic expression of *cifA*$_{wMel(TI)}$ results in a high mortality rate in the absence of doxycycline. Heterozygous females from the *PUbt-tTAV* line were crossed with heterozygous males from the TRE-*cifA*$_{wMel(TI)}$ line and transgene inheritance in the resulting progeny was screened via fluorescent microscopy at late larval life stages. Dots represent the percentage of total embryos (laid by an individual female) either possessing a transgenic allele or not (wt). Red dashed line represents the expected allele inheritance of 25%, bars denote the mean and error bars the S.D.

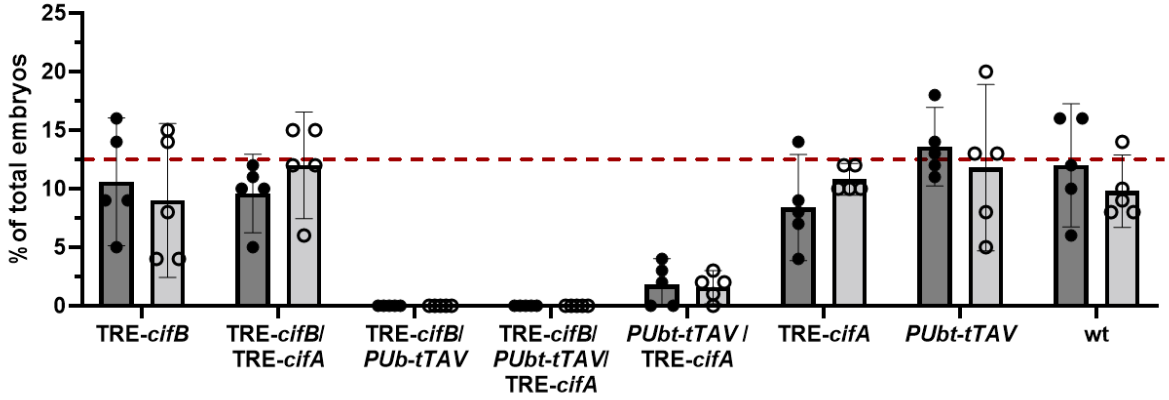


Figure 3.31: Embryonic expression of both *cifA*$_{wMel(TI)}$ and *cifB*$_{wMel(TI)}$ results in lethality in the absence of doxycycline. Heterozygous TRE-*cifA*$_{wMel(TI)}$ / TRE-*cifB*$_{wMel(TI)}$ L1 (filled symbol) or L3 (open symbol) mosquitoes were crossed with heterozygous mosquitoes from the *PUbt-tTAV* line and transgene inheritance in the resulting progeny was screened via fluorescent microscopy at late larval life stages. Dots represent the percentage of total embryos (laid by a single female) either possessing a transgenic allele or not (wt). Red dashed line represents the expected allele inheritance of 12.5%, bars denote the mean and error bars the S.D.

3.3 Summary and conclusions

Transgenic lines of *Ae. aegypti* mosquitoes expressing both *cifA*$_{wMel(TI)}$ and *cifB*$_{wMel(TI)}$ in the testes under the control of a single promoter failed to result in a male sterility phenotype despite several of the *β2t/ β2t*-cifA*$_{wMel(TI)}$*-T2A-cifB*$_{wMel(TI)}$ lines displaying testes-specific expression of the *cif* transgenes. One initial hypothesis was that a higher expression of the transgenes was necessary for CI induction, however none of the five different genomic *PUb-cifA*$_{wMel(TI)}$*-T2A-cifB*$_{wMel(TI)}$ insertion lines (which had higher expression levels than the testes-specific promoter lines) displayed a significant sterility phenotype. Although transcription of the bicistronic sequence was detected by RT-qPCR, problems in its translation might not equate to the expected amounts of gene product. For example, if the ability of the T2A peptide to promote ribosomal skipping was impaired this could result in non-equimolar quantities of the Cif peptides and/or non-functional chimeric proteins. As the penetrance of CI has been shown to be attenuated by a higher expression of *cifA* relative to *cifB*[93], it is possible that problems in peptide self-cleavage resulted in a higher abundance of CifA. Although, western blots could not be conducted, evidence for the correct Cif peptide cleavage came from the fact that the *PUb-cifA*$_{wMel(TI)}$*-T2A-cifB*$_{wMel(TI)}$ lines could rescue CI-

induced by *Wolbachia*-infected males in an expression dependent manner which suggested that some functional CifA$_{wMel(TI)}$ was being produced. If both *cif* gene products were translated properly then it is not clear why high levels of expression in these lines did not result in male sterility as is seen in other transgenic models. As was seen in the CI rescue experiments, a higher expression of the transgene does not always result in the expected phenotype- which is likely based on expression distribution between different tissue cell types. There remained the possibility that the transgenes (under the control of the *PUb* promoter) did not express in the earlier stages of spermatogenesis which might have been important for CI induction. However, immunofluorescent staining of testes from *PUbt-cifA*$_{wMel(TI)}$ L2 males, showed the truncated form of the *PUb* promoter drove *cifA*$_{wMel(TI)}$ expression in all cells contained within the testes. Consequently, a similar expression pattern would be expected in the *PUb-cifA*$_{wMel(TI)}$*-T2A-cifB*$_{wMel(TI)}$ lines. However, due to the unspecific binding of the anti-His and anti-Flag antibodies this could not be visualised. Future work will employ antibodies targeting conserved Cif epitopes which will provide data on both transgenic and endogenous Cif$_{wMel(TI)}$ protein levels and localisation.

To test whether the lack of male-sterility was the result of CifA$_{wMel(TI)}$ attenuating the toxicity of CifB$_{wMel(TI)}$, transgenic lines of mosquitoes expressing solely *cifB*$_{wMel(TI)}$ were generated. RT-qPCR revealed that the expression of the transcript was relatively low (comparable to baseline expression) in the testes of males from these lines which might explain why a sterility phenotype was not observed. However, it could also be the result of several factors, namely i) the spatiotemporal expression pattern of the transgenes was incorrect ii) *cifA*$_{wMel(TI)}$ expression is needed alongside *cifB*$_{wMel(TI)}$ in order to induce CI, or iii) the "toxicity" of CifB$_{wMel(TI)}$ was compromised, for example by inappropriate folding or an absence of essential co-factors present when expressed by *Wolbachia*.

Ubiquitous expression of *cifB*$_{wMel(TI)}$ in a tetracycline-repressible system resulted in embryonic lethality when mosquitoes were reared in the absence of tetracycline. This could imply that CifB$_{wMel(TI)}$ is toxic, however as this toxicity was unable to be rescued by *cifA*$_{wMel(TI)}$ expression it remains possible that the lethality observed is the result of overexpression and not the catalytic activity of CifB$_{wMel(TI)}$. Mutation of predicted catalytic residues in the TRE-*cifB*$_{wMel(TI)}$ lines, might resolve where the toxicity stems from. Or perhaps differences in *cifB* toxicity requires *cifB*$_{wMel(TI)}$ to be expressed earlier in spermatogenesis than *cifB*$_{wPip(TI)}$ or *cifB*$_{wAlbB(TIV)}$

[which were later found to induce male sterility when their expression was regulated by the *β2t** promoter (Chapter 4)] to have an effect. For example, the *β2t** promoter is not active in the germ cell/spermatogonia regions of the testes[173]. The *PUbt* promoter could drive *cifB*$_{w\text{Mel(TI)}}$ expression in these regions as was seen in the *PUbt-cifA*$_{w\text{Mel(TI)}}$ lines, however based on the results of the tetracycline repressible system *PUbt-cifB*$_{w\text{Mel(TI)}}$ individuals would be expected to die before reaching adulthood. Therefore, alternative *Ae. aegypti* germline promoters should be sought and tested.

To determine whether *cifA*$_{w\text{Mel(TI)}}$ expression was necessary for CI induction, transgenic lines that expressed *cifA*$_{w\text{Mel(TI)}}$ under the control of testes-specific *β2t** promoter were generated. Expression of *cifA*$_{w\text{Mel(TI)}}$ alone had no effect on the fertility of transgenic males and after *β2t*-cifA*$_{w\text{Mel(TI)}}$ lines were crossed with *β2t*-cifB*$_{w\text{Mel(TI)}}$ lines the resultant male progeny possessing both transgenic loci remained fertile. It is possible that the relative expression of *cifA*$_{w\text{Mel(TI)}}$ and *cifB*$_{w\text{Mel(TI)}}$ from different genomic loci did not result in the correct balance necessary for the induction of CI. A higher abundance of CifA$_{w\text{Mel(TI)}}$ might attenuate the toxicity of CifB$_{w\text{Mel(TI)}}$, whilst insufficient *cifA*$_{w\text{Mel(TI)}}$ might result in the selective killing of sperm precursors with a higher CifB$_{w\text{Mel(TI)}}$ abundance. However, as no sperm defects are observed when *cifB*$_{w\text{Mel(TI)}}$ is expressed independently, the lack of CI phenotype is more likely the result of a deficiency in the toxicity of transgenic CifB$_{w\text{Mel(TI)}}$.

Although recapitulation of CI induction through the expression of the *w*Mel *cif* genes was not achieved, recapitulation of the CI rescue phenotype was. This study has shown that expression of *cifA*$_{w\text{Mel(TI)}}$ in transgenic mosquito ovaries is sufficient to significantly rescue embryonic lethality in crosses with males infected with compatible strains (*w*Mel and *w*Pro) but not the canonically incompatible strain *w*AlbB. The compatibility between *w*Pro and *w*Mel is expected to be the result of both strains possessing *cif* genes with high sequence similarity. As the wPro Type IV *cifB* gene is pseudogenised, and the Type I *cifB* genes share 99% protein sequence similarity, the reason for the incomplete CI penetrance phenotype observed in incompatible crosses is likely due to low densities of *w*Pro in testes. Rescue was found to be dependent on the deposition of CifA in developing oocytes which was affected by both the level of transgene expression and promoter selection.

A concern surrounding the use of *Wolbachia*-mediated vector replacement strategies is that viral evolution could lead to evasion of the bacterium's blocking mechanisms. In that case it

could be beneficial to develop a method for removing *Wolbachia* from an infected population, to facilitate subsequent replacement with a different *Wolbachia* strain. Release of uninfected mosquitoes at high frequencies would lead to a reduction in the *Wolbachia* frequency within a population, however, as wild-type *Ae. aegypti* are likewise competent vectors this does not provide an adequate replacement strategy. In 2004, Sinkins and Godfray modelled the use of a CI rescue factor to drive a transgene into a *Wolbachia*-infected population[179]. In this self-limiting gene drive, the spread of the rescue construct would displace *Wolbachia* from the population before itself being displaced once the bacterium (and therefore its selective advantage) was lost. This drive can only be achieved if the bacterium is not inherited by all of the progeny of an infected female, and these imperfect maternal transmission conditions have been observed when *w*Mel-carrying mosquitoes are exposed to high temperatures during development[113,115,124]. This strain is highly sensitive to the conditions of cyclical heat stress often observed in tropical climates, and is far more heat sensitive compared to some alternative strains *e.g., w*AlbB[113].

Now that the factor responsible for rescuing *w*Mel-induced CI has been discovered (*cifA*$_{w\text{Mel(TI)}}$) and this phenotype has been recapitulated in transgenic *Ae. aegypti* mosquitoes, the legitimacy of this model can be tested in future studies. Based on the surrounding literature and comparisons between lines generated in this study, it was hypothesised that increasing the level of *cifA*$_{w\text{Mel(TI)}}$ expression in ovaries would increase the CI rescue capability of transgenic females[81,93,172]. However, several lines expressing *cifA* under the control of the *PUb* promoter or its truncated form resulted in high mortality rates. Additionally, lethality is not observed in TRE- *cifA*$_{w\text{Mel(TI)}}$ unless the expression of *cifA*$_{w\text{Mel(TI)}}$ is activated by expression of *tTAV* (controlled by the *PUbt* promoter). This might indicate that the expression of *cifA*$_{w\text{Mel(TI)}}$ is toxic at high levels, however it is important to note that *PUbt-cifA*$_{w\text{Mel(TI)}}$ L2 had the highest expression of all *cifA*$_{w\text{Mel(TI)}}$ lines but did not exhibit the same lethality phenotype. If *cifA*$_{w\text{Mel(TI)}}$ expression is toxic this might impact the ability for lines expressing the transgene to drive through a *Wolbachia*-infected population.

Chapter 4:

Investigating CI-mediation via transgenic expression of *cif* genes from *w*AlbB and *w*Pip in *Ae. aegypti* mosquitoes.

4. Investigating CI-mediation via transgenic expression of *cif* genes from *w*AlbB and *w*Pip in *Ae. aegypti* mosquitoes.

4.1 Introduction

Although naturally co-infected with two strains of *Wolbachia* (*w*AlbA and *w*AlbB), *Ae. albopictus* mosquitoes remain competent vectors of several arboviruses[180–184]. This vector competence is attributable to the localisation of the *Wolbachia* infections, which are largely restricted to the reproductive tissues. Co-evolutionary pressures on native infections are expected to restrict *Wolbachia* densities in tissues not involved in vertical transmission and thus favour localisation of *Wolbachia* to the germline[129]. Transinfection of *w*AlbB into *Ae. aegypti* results in a more diffuse tissue distribution and renders the mosquitoes refractory to many important arboviruses[9,109,185,186]. Unlike native *w*Mel infections in *D. melanogaster* the CI induced by *w*AlbB is both strong and stable in both its native host and transinfected *Ae. aegypti*[110]. The combination of both strong pathogen inhibition and CI induction, in addition to greater heat stability compared to the alternative *w*Mel strain, has led to the selection of *w*AlbB for both population replacement and suppression strategies involving transinfected *Ae. aegypti.* Sequencing of the *w*AlbB genome revealed that this strain encodes two sets of cognate *cif* genes of different types (Type III and IV)[187]. Both sets appear to be intact and are expected to be functional, however, to date there have been no studies into their functionality.

Like *Ae.* albopictus, members of the mosquito species complex *C. pipiens* also remain notable disease vectors despite carrying a native *Wolbachia* infection (*w*Pip)[188–190]. However, transfer of *w*Pip to *Ae. aegypti* does not result in a pathogen inhibition phenotype despite reaching high densities[111]. *w*Pip is highly diverse and can be divided into 5 phylogenetically distinct groups (*w*Pip-I to wPip-V)[191]. Before the discovery of the *cif* genes, it was found that crosses between mosquito lines infected with different variants of *w*Pip resulted in a complex pattern of incompatibility as well as variation in the strength of CI induction[192]. The *w*Pip reference genome *w*Pip(Pel) indicated that *w*Pip possessed one set of Type I and Type IV *cif* genes, however other studies have shown that *w*Pip variants might encode several copies of the Type I *cif* operon[193,194]. Additionally, whilst the sequences of the Type IV set of *cif* genes do not seem to differ both between and within *w*Pip groups, the Type I *cif* genes display significant polymorphism[193]. Therefore, the compatibility matrix observed between *C. pipiens*

lines likely results from the diversity of Type I *cif* copies. In support of this theory, structural analysis of the Type I *cif* genes from divergent *w*Pip strains revealed that polymorphisms in the binding interface between CifA and CifB resulted in an inability to bind[85]. As rescue is dependent on CifA:CifB binding this inability to bind between *w*Pip *cif* homologs would result in incompatibility[86].

Researchers have been unable to generate *D. melanogaster* lines which transgenically express $cifB_{w\text{Pip(TI)}}$ alone, and consequently both $cif_{w\text{Pip(TI)}}$ genes are needed to generate viable transgenic males capable of inducing CI. However, the induction of CI with only $cifB_{w\text{Pip(TI)}}$ expression has been achieved in transgenic *An. gambiae* mosquitoes and thus the two-by-one-model of CI might not be conserved between different host species. Therefore, the Type I $cif_{w\text{Pip(TI)}}$ genes were selected to form a comparison with work conducted in either *Drosophila* or *Anopheles* in a new transgenic model and as a direct comparison to the study of $cif_{w\text{Mel(TI)}}$ genes in this organism (Chapter 3). Like Type I *cif* genes, those of Type IV have been studied in *D. melanogaster* and adhere to the two-by-one model, however it remains to be seen whether this is true in *Ae. aegypti*. Although, the *w*Pip Type IV genes would form a better comparison to the studies in *D. melanogaster,* the *w*AlbB Type IV *cif* genes were selected for this study based on their relevance to *w*AlbB-based *Ae. aegypti* population control methods.

An initial aim of this chapter was to generate transgenic *Ae. aegypti* mosquito lines which express either $cifB_{w\text{AlbB(TIV)}}$ or $cifB_{w\text{Pip(TI)}}$ individually in the testes of adult mosquitoes. It was hypothesised that expression of these *cif* genes would result in conditional male sterility and based on differences in their expected catalytic activity it was predicted that there might be differences in their dependency of cognate *cifA* expression during spermatogenesis. If indeed *cifA*/B co-expression was needed to recapitulate canonical CI induction, a secondary aim was to generate transgenic lines that expressed the corresponding *cifA* in the testes and characterise whether co-expression enhanced or inhibited the expected toxicity of *cifB* expression. It was hoped that this work might i) elucidate the role CifA plays in CI induction**,** ii) indicate which model of CI-mediation is most parsimonious, and iii) determine whether this model is conserved between *cif* genes of differing Types. To replicate CI rescue in a transgenic system, *Ae. aegypti* lines which express either $cifA_{w\text{AlbB(TIV)}}$ or $cifA_{w\text{Pip(TI)}}$ in female reproductive tissues would be generated and their effect on fertility when crossed with male mosquitoes expressing the corresponding *cif* genes would be investigated. It was hypothesised that if both

rescue and induction phenotypes could be generated through transgenesis then it would provide proof-of-principle for a *cif*-based gene drive system in this vector species. Furthermore, it was thought that the information gathered in this study would allow for future optimisation of this system.

4.2 Results and Discussion

4.2.1 Testes-specific expression of *cifB*$_{wAlbB(TIV)}$ induces complete sterility in *Ae. aegypti* mosquitoes.

4.2.1.1 Generation of *β2t*-cifB*$_{wAlbB(TIV)}$ lines

As the *β2t** promoter was found to promote testes-specific expression of *cifA/cifB*$_{wMel(TI)}$, this sequence was selected to drive the expression of *cifB*$_{wAlbB(TIV)}$. The synthesised *cifB*$_{wAlbB(TIV)}$ gene was codon optimised for expression in *Ae. aegypti* and cloned into the *β2t**- *cifB*$_{wMel(TI)}$ donor plasmid. After embryo microinjection of the *β2t*-cifB*$_{wAlbB(TIV)}$ donor transposon (Figure 4.1) a high mortality rate (49%) was observed in transient G_{0s} at later developmental stages. Of the 25 transient G_0 males tested for germline insertion, 15 were sterile (58%) and those that produced viable young did not have a germline insertion. However, three independent genomic insertion lines were generated, and maintained through transgenic females (see Table A1).

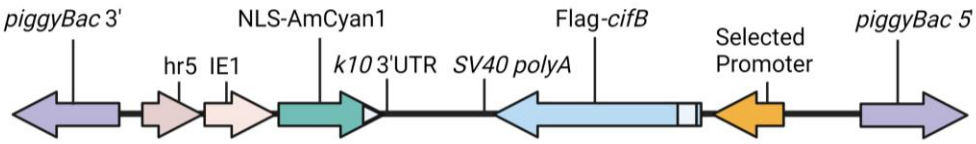


Figure 4.1: Schematic representation of the transposon used to investigate the effect of either *cifB*$_{wAlbB(TIV)}$ or *cifB*$_{wPip(TI)}$ expression on male fertility.

Heterozygous males from each of the three lines were found to be completely sterile when crossed with either wild-type or *w*AlbB-infected females (Figure 4.2a) and expression of *cifB*$_{wAlbB(TIV)}$ was found to be relatively high and testes-specific (Figure 4.2b). Mating behaviour was observed between transgenic males and the females, however dissection of the spermathecae revealed that none were inseminated.

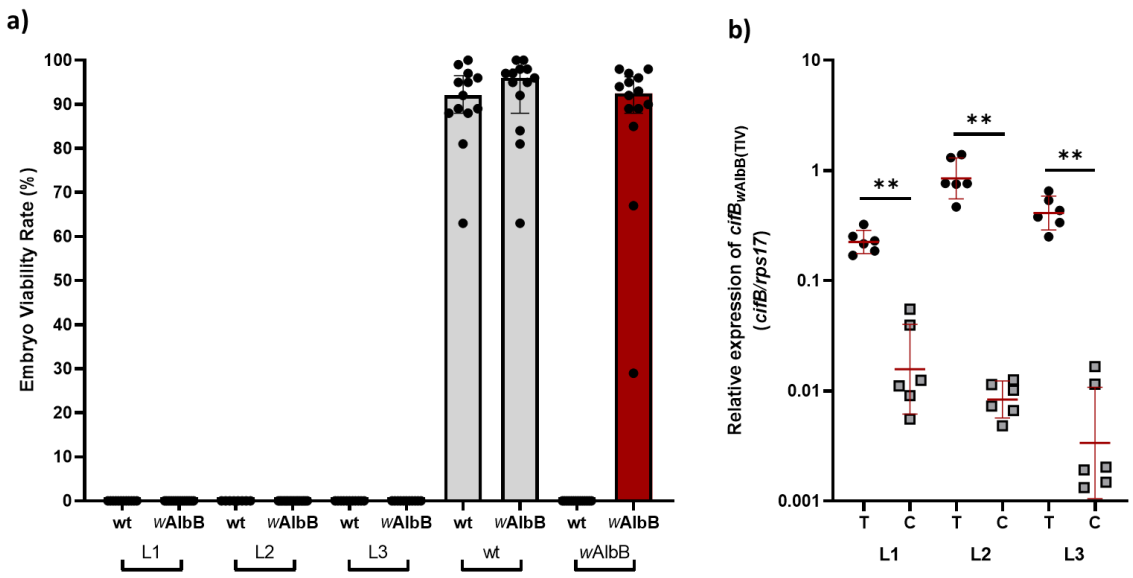


Figure 4.2: Testes-specific expression of *cifB*$_{wAlbB(TIV)}$ induces non-rescuable sterility. Heterozygous males from three different genomic *β2t*-cifB*$_{wAlbB(TIV)}$ insertion lines (L1-L3) significantly reduces the viability rate of the progeny when crossed with either wild-type (wt) or *w*AlbB-infected females. Dots represent the percentage of viable embryos laid by single females, bars represent the median and error bars denote interquartile ranges. **b)** The expression of *cifB*$_{wAlbB(TIV)}$ relative to housekeeping gene *rps17* in pooled adult testes (T) and the remaining carcasses (C) from the three *β2t*-cifB*$_{wAlbB(TIV)}$ lines . Lines denote geometric mean, error bars denote 95% CI. Significance calculated by Mann-Whitney U-test. **=p<0.01

The seminal vesicles and testes of transgenic males were inspected by microscopy which revealed that they lacked typical signals of mature sperm development, with imaging showing that the spermatocytes had failed to elongate (Figure 4.3). This 'spermless male' phenotype is similar to that reported in some previously published studies in *Ae. aegypti* which knocked-out the *β2t* gene, which encodes a protein subunit integral to sperm development[143,144]. Because all three independent insertion lines displayed the same sterility phenotype, disruption of spermatogenesis based on integration of the transposon in genes involved in this process seemed unlikely.

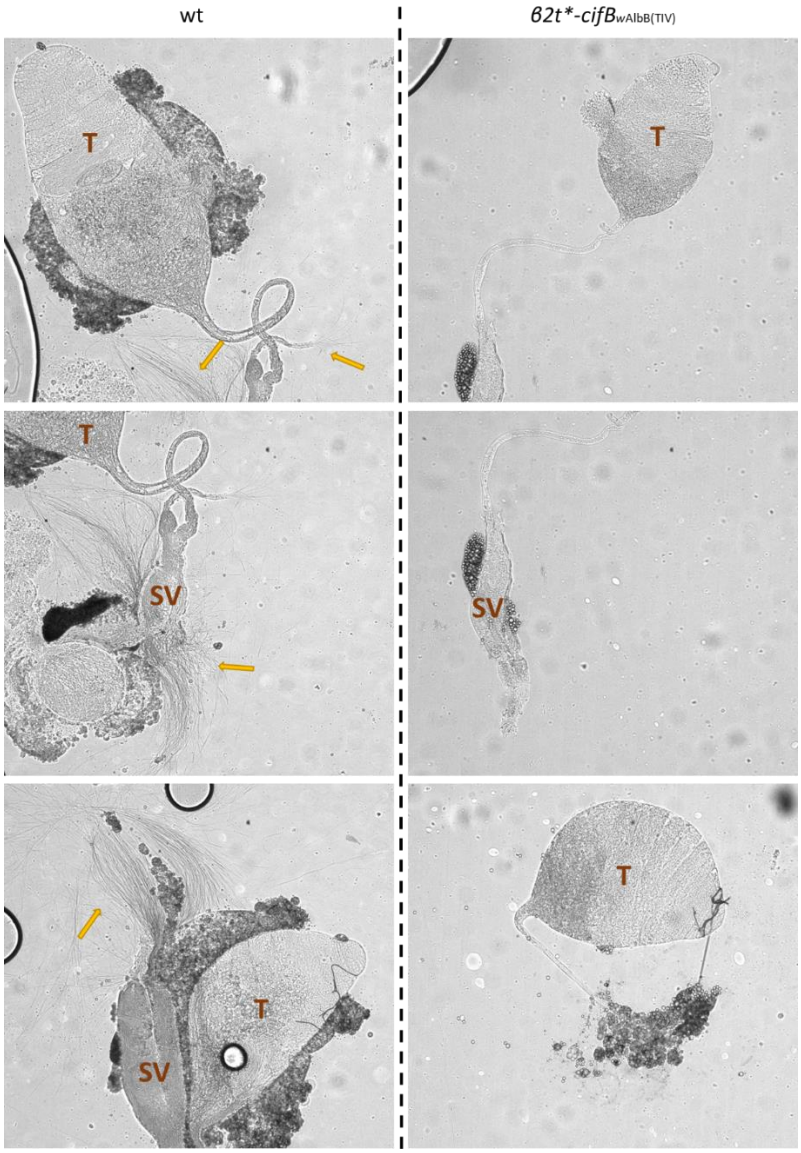


Figure 4.3: Paternal expression of *cifB*$_{wAlbB(TIV)}$ inhibits the production of mature sperm. Brightfield imaging of testes (T) and seminal vesicles (SV) from wild-type (wt) and *β2t*-cifB*$_{wAlbB(TIV)}$ male mosquitoes. An abundance of mature sperm (yellow arrows) was released from wt tissues however tissues dissected from *β2t*-cifB*$_{wAlbB(TIV)}$ male mosquitoes were devoid of mature sperm.

As *cifB*$_{wAlbB(TIV)}$ is expected to act as a nuclease, it was hypothesised that the sterility phenotype might be the result of this catalytic activity. TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assays of testes squashes from transgenic males expressing *cifB*$_{wAlbB(TIV)}$ revealed a considerable amount of DNA breaks in comparison to wild-type testes (Figure 4.4) which indicated that *cifB*$_{wAlbB(TIV)}$ is indeed a functional nuclease and

that the "spermless" sterility phenotype observed for all three *β2t*-cifB*$_{wAlbB(TIV)}$ lines likely results from DNA damage terminating spermatogenesis.

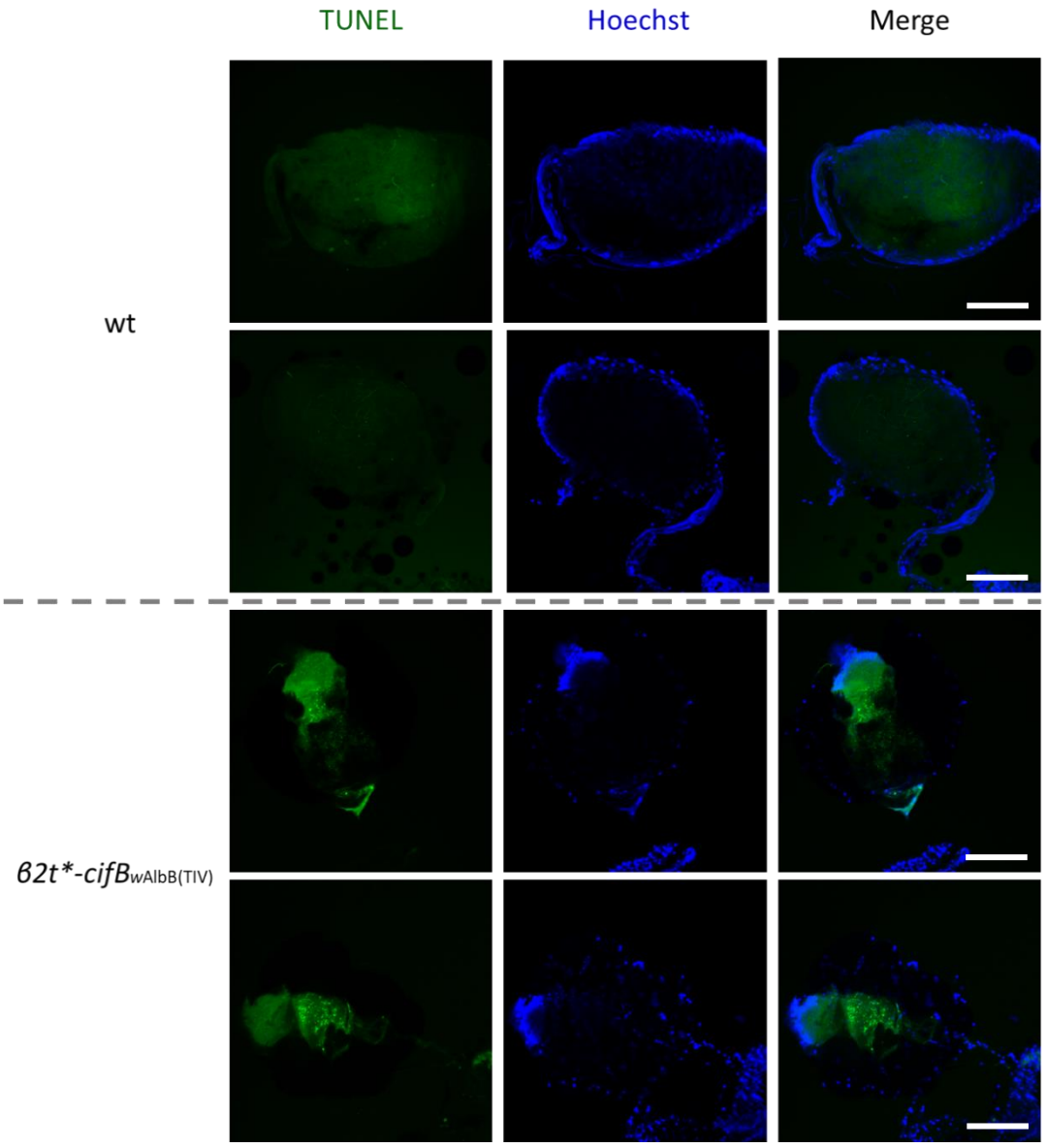


Figure 4.4: Testes-specific *cifB*$_{wAlbB(TIV)}$ expression induces DNA breaks. Representative images of TUNEL staining of squashed testes from age-matched wild-type (wt) males or heterozygous males from *β2t*-cifB*$_{wAlbB(TIV)}$ L1. DNA breaks marked by TUNEL staining (green foci) are abundant in *cifB*$_{wAlbB(TIV)}$ expressing but not wt male testes. In total 9 wt and 8 *β2t*-cifB*$_{wAlbB(TIV)}$ L1 testes were imaged, Scale bars indicate 100 µm.

4.2.2 Testes-specific expression of both *cifA*$_{wAlbB(TIV)}$ and *cifB*$_{wAlbB(TIV)}$ induces rescuable CI in *Ae. aegypti* mosquitoes.

4.2.2.1 Generation of *β2t*-cifA*$_{wAlbB(TIV)}$ lines

As binding between CifA and CifB suppresses toxicity in temperature sensitive yeast and insect cell assays it was hypothesised that testes-specific expression of *cifA*$_{wAlbB(TIV)}$ might inhibit CifB$_{wAlbB(TIV)}$ nuclease activity/toxicity and permit spermatogenesis to proceed[78,80,85,86,88,92]. Therefore, lines expressing *cifA*$_{wAlbB(TIV)}$ under the control of the *β2t** promoter (Figure 4.5) were generated through embryo microinjection (see Table A1). Testes-specific expression was confirmed for two of the three independent genomic *β2t*-cifA*$_{wAlbB(TIV)}$ insertion lines (L1 and L2), however, expression of the transgene in the third was not detected by RT-qPCR (Figure 4.6).

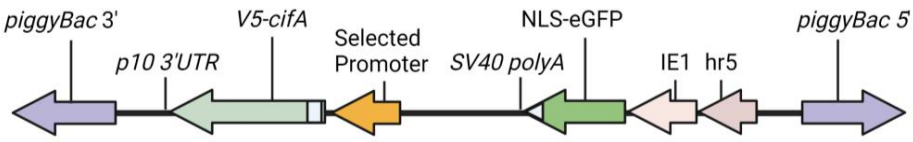


Figure 4.5: Schematic representation of the transposon used to investigate the effect of *cifA*$_{wAlbB(TIV)}$ expression on male fertility.

Heterozygous males from each of the three lines were crossed with either wild-type or *w*AlbB-infected females to test for any fertility effect of testes-specific *cifA*$_{wAlbB(TIV)}$ expression. The median embryo viability rate of these crosses did not decrease in comparison to the wild-type control cross, suggesting that expression of *cifA*$_{wAlbB(TIV)}$ did not affect male fertility (Figure 4.7a). To test whether *cifA*$_{wAlbB(TIV)}$ expression might attenuate the toxicity of *cifB*$_{wAlbB(TIV)}$ expression, heterozygous males from all three *β2t*-cifA*$_{wAlbB(TIV)}$ lines were crossed with heterozygous females from each of the three *β2t*-cifB*$_{wAlbB(TIV)}$ lines to generate males heterozygous for both *β2t*-cifA*$_{wAlbB(TIV)}$ and *β2t*-cifB*$_{wAlbB(TIV)}$ genomic insertions. These males were crossed with either wild-type or *w*AlbB-infected females and after oviposition, spermathecae were dissected from the females and the number of inseminated females was calculated (Figure 4.7a). The rescue of spermatogenesis was found to be dependent on both *cifA*$_{wAlbB(TIV)}$ and *cifB*$_{wAlbB(TIV)}$ expression levels which varied due to the positional effect of genomic insertion sites (Figure 4.7b). In some combinations such as *β2t*-cifB*$_{wAlbB(TIV)}$ L1 and

either *β2t*-cifA*$_{wAlbB(TIV)}$ L1 or L2, sperm was found in all mated female spermathecae. Despite a rescue of sperm production, all crosses between males heterozygous for *cifA*$_{wAlbB(TIV)}$ and *cifB*$_{wAlbB(TIV)}$ and wild-type females resulted in inviable offspring. However, if the females were infected with *w*AlbB there was a significant increase in the viability rate of the offspring which indicated that the conditional sterility observed for *β2t*-cifA*$_{wAlbB(TIV)}$; *cifB*$_{wAlbB(TIV)}$ males reflected CI induction (Figure 4.7a).

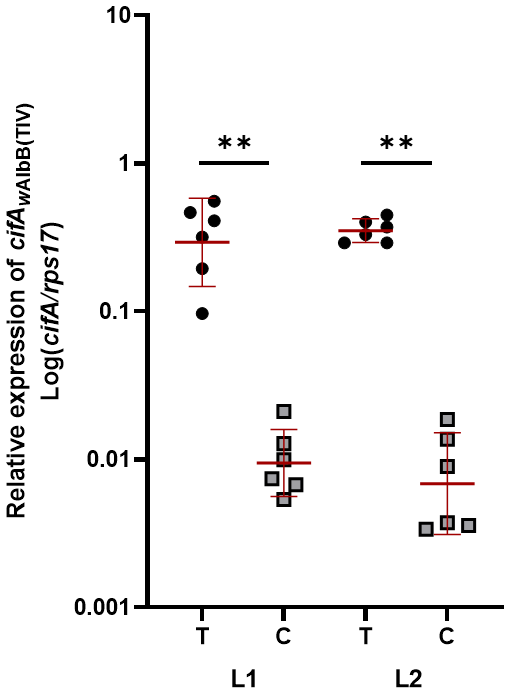


Figure 4.6: The expression of *cifA*$_{wAlbB(TIV)}$ relative to housekeeping gene *rps17* in pooled adult testes (T) and the remaining carcasses (C) from two of the three *β2t*-cifA*$_{wAlbB(TIV)}$ lines. The third line had near undetectable expression of *cifA*$_{wAlbB(TIV)}$ in both the testes and carcasses. Lines denote geometric mean, error bars denote 95% CI. Significance calculated by Mann-Whitney U-test. **=p<0.01

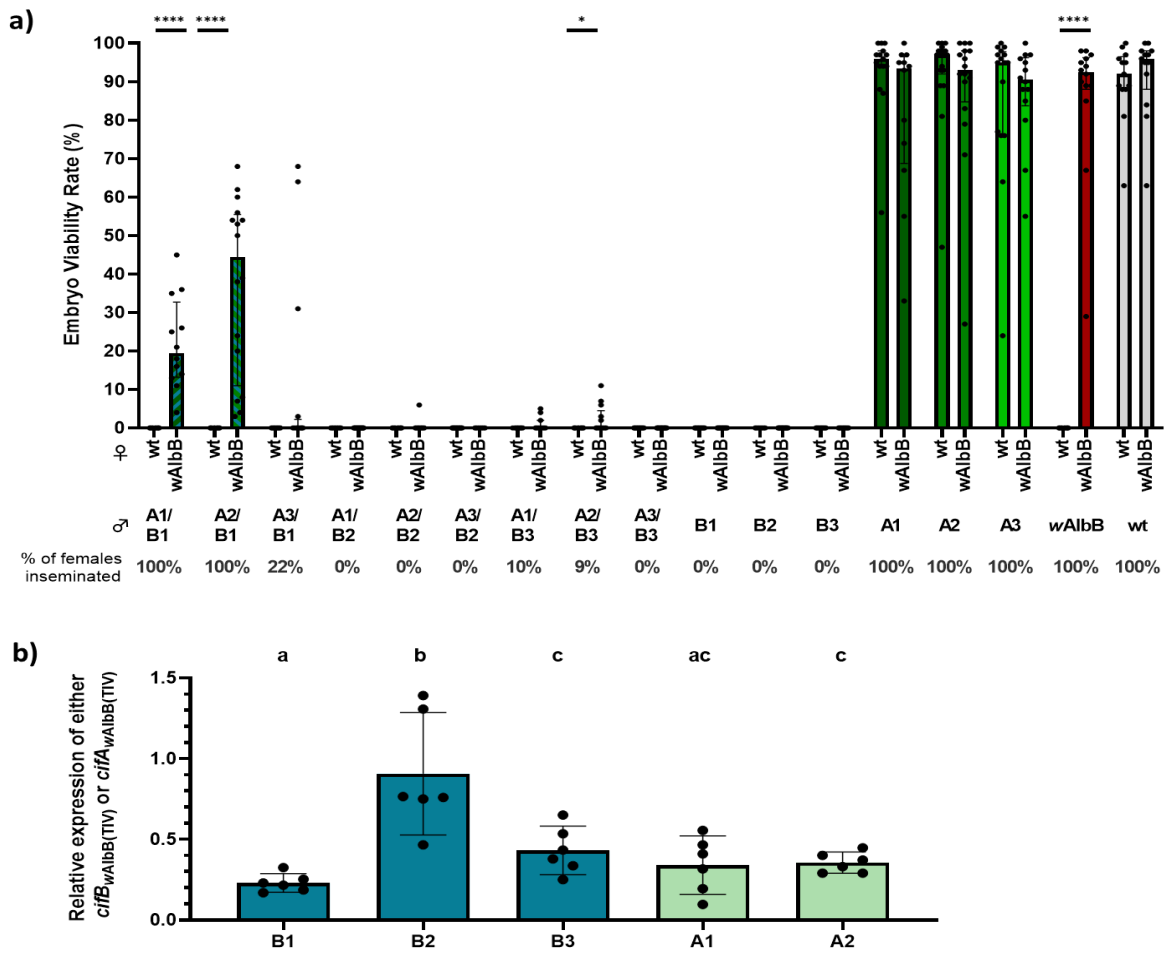


Figure 4.7: Testes-specific expression of *cifA*$_{wAlbB(TIV)}$ can rescue impairment of spermatogenesis caused by *cifB*$_{wAlbB(TIV)}$ expression and expression of both *cif* transgenes can induce rescuable CI. a) The effect of sole expression or co-expression of *cifA*$_{wAlbB(TIV)}$ and *cifB*$_{wAlbB(TIV)}$ (under the control of the *β2t** promoter) on male fertility. Expression of *cifB*$_{wAlbB(TIV)}$ by three different genomic insertion lines (B1-3) induced complete/non-rescuable sterility. Expression of *cifA*$_{wAlbB(TIV)}$ (by three different genomic insertion lines (A1-3) did not affect male fertility. However, expression of both *cifA*$_{wAlbB(TIV)}$ and *cifB*$_{wAlbB(TIV)}$ induced sterility when crossed with wild-type (wt) females which was rescuable when females were infected with *w*AlbB. Differences in insertion site affected the ability of *cifA*$_{wAlbB(TIV)}$ to rescue spermatogenesis as indicated by the percentage of females inseminated, as well as the rescue capacity of *w*AlbB-infected females. Dots represent the percentage of viable embryos laid by one female, bars represent the median and error bars denote interquartile ranges. Significance calculated by Mann-Whitney U-test. ****=p<0.0001 **b)** The mean relative expression of either *cifA*$_{wAlbB(TIV)}$ or *cifB*$_{wAlbB(TIV)}$ relative to housekeeping gene *rps17* in pooled adult testes. Letters indicate significant differences calculated by Mann-Whitney U-tests.

The ability of *w*AlbB to rescue CI induced by *ß2t*-cifA*$_{wAlbB(TIV)}$; *cifB*$_{wAlbB(TIV)}$ males was also dependent on the combination of genomic insertion sites involved in the cross. TUNEL assays of squashed testes from *ß2t*-cifA*$_{wAlbB(TIV)}$ L2; *cifB*$_{wAlbB(TIV)}$ L1 males revealed that although elongated sperm was being produced, there were still a considerable amount of DNA breaks in comparison to wild-type testes (Figure 4.8). This might suggest that spermatid DNA damage is the root cause of CI induction involving Type IV *cif* genes and that the role of CifA is to either restrict the extent of DNA damage during spermatogenesis or to direct the nuclease activity of CifB to specific target sites. If this is true in the natural context of *Wolbachia* infection, then the modifications associated with CI induction would occur during spermatogenesis which would provide supporting evidence for the HM model of CI mediation.

However, TUNEL assays performed on *w*AlbB-infected testes revealed that although this strain encodes two Types of *cifB* genes with expected nuclease activity a high abundance of DNA breaks similar to that seen in transgenic *cifB*$_{wAlbB(TIV)}$ lines was not observed (Figure 4.8). As we have shown CifB$_{wAlbB(TIV)}$ is capable of cleaving spermatid DNA *in situ*, the lack of DNase activity in *w*AlbB-infected testes suggests that the modifications associated with CI perhaps do not occur during spermatogenesis. Therefore, the abundance of DNA breaks observed in *ß2t*-cifA*$_{wAlbB(TIV)}$; *cifB*$_{wAlbB(TIV)}$ male testes might reflect an imbalance of expression between the two *cif* genes. If the modifications (nuclease activity) are expected to occur after fertilisation and not during spermatogenesis, then DNA damage induced by *cifB*$_{wAlbB(TIV)}$ expression in some but not all spermatozoa produced might explain why full rescue by *w*AlbB-infected females was not observed. To further investigate this possibility, lines encoding both *cifA*$_{wAlbB(TIV)}$ and *cifB*$_{wAlbB(TIV)}$ linked with a T2A peptide sequence were generated in order to more likely achieve equimolar ratios.

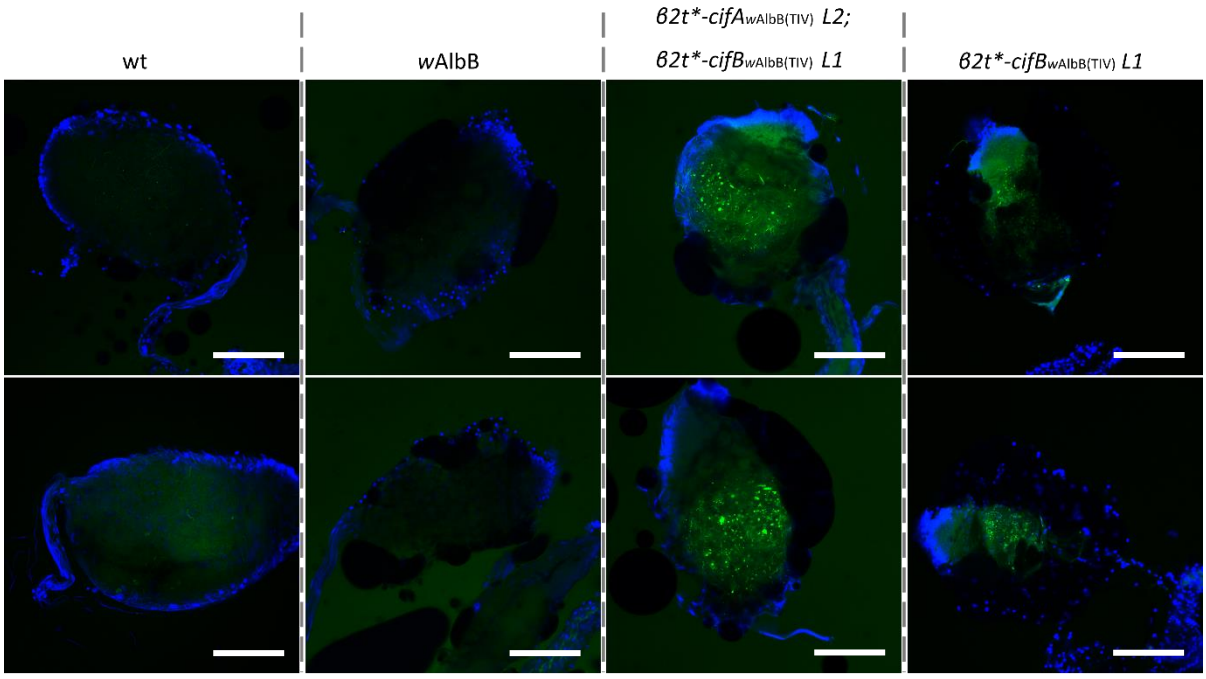


Figure 4.8: Frequent DNA breaks observed in testes which expressed either *cifB*$_{wAlbB(TIV)}$ alone or with *cifA*$_{wAlbB(TIV)}$ is not observed in *w*AlbB infected males. Representative images of TUNEL staining on testes squashes from age-matched wild-type (wt) and *w*AlbB-infected male mosquitoes, as well as those heterozygous for either *cifB*$_{wAlbB(TIV)}$ (*Ø2t*-cifB*$_{wAlbB(TIV)}$ L1) alone or both *cifA*$_{wAlbB(TIV)}$ and *cifB*$_{wAlbB(TIV)}$ (*Ø2t*-cifA*$_{wAlbB(TIV)}$ L2; *Ø2t*-cifB*$_{wAlbB(TIV)}$ L1). DNA breaks are marked by TUNEL staining (green foci), DNA is marked with Hoechst stain (blue). In total, 9 wt, 10 *w*AlbB, 3 *Ø2t*-cifA*$_{wAlbB(TIV)}$ L2; *Ø2t*-cifB*$_{wAlbB(TIV)}$ L1, and 8 *Ø2t*-cifB*$_{wAlbB(TIV)}$ L1 testes were imaged, scale bars indicate 100 µm.

4.2.2.2 Generation of *β2t*-cifA*$_{wAlbB(TIV)}$-T2A-*cifB*$_{wAlbB(TIV)}$ lines

In total, 4 independent *β2t*-cifA*$_{wAlbB(TIV)}$-T2A-*cifB*$_{wAlbB(TIV)}$ (Figure 4.9) insertion lines were generated (see Table A1).

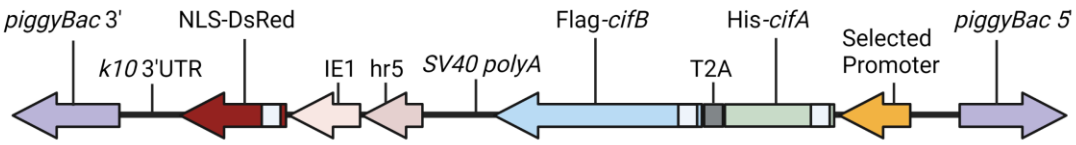


Figure 4.9: Schematic representation of the transposon used to investigate the effect of *cifA*$_{wAlbB(TIV)}$ and *cifB*$_{wAlbB(TIV)}$ co-expression on male fertility.

Heterozygous males from each of the four lines were rendered completely sterile in crosses with wild-type females, however females carrying *w*AlbB were able to significantly increase the median embryo viability rate (Figure 4.10). Therefore, the conditional sterility induced by dual expression of *cifA*$_{wAlbB(TIV)}$ and *cifB*$_{wAlbB(TIV)}$ was indicative of CI. Dissection of the spermathecae from females involved in the crosses revealed that they were all inseminated which further supported the role of CifA$_{wAlbB(TIV)}$ in attenuating the toxicity of CifB$_{wAlbB(TIV)}$ during spermatogenesis (Figure 4.10). Although, *w*AlbB-infected females were able to significantly increase the embryo viability rate, the level of rescue was significantly lower when crossed to transgenic males than males likewise infected with *w*AlbB. It was possible that endogenous *cifA*$_{wAlbB(TIV)}$ expression by the bacterium was not enough to fully rescue CI induced by transgenic males and perhaps transgenic expression of *cifA*$_{wAlbB(TIV)}$ might have been better suited. Therefore, transgenic lines expressing *cifA*$_{wAlbB(TIV)}$ under the *exu* promoter were generated.

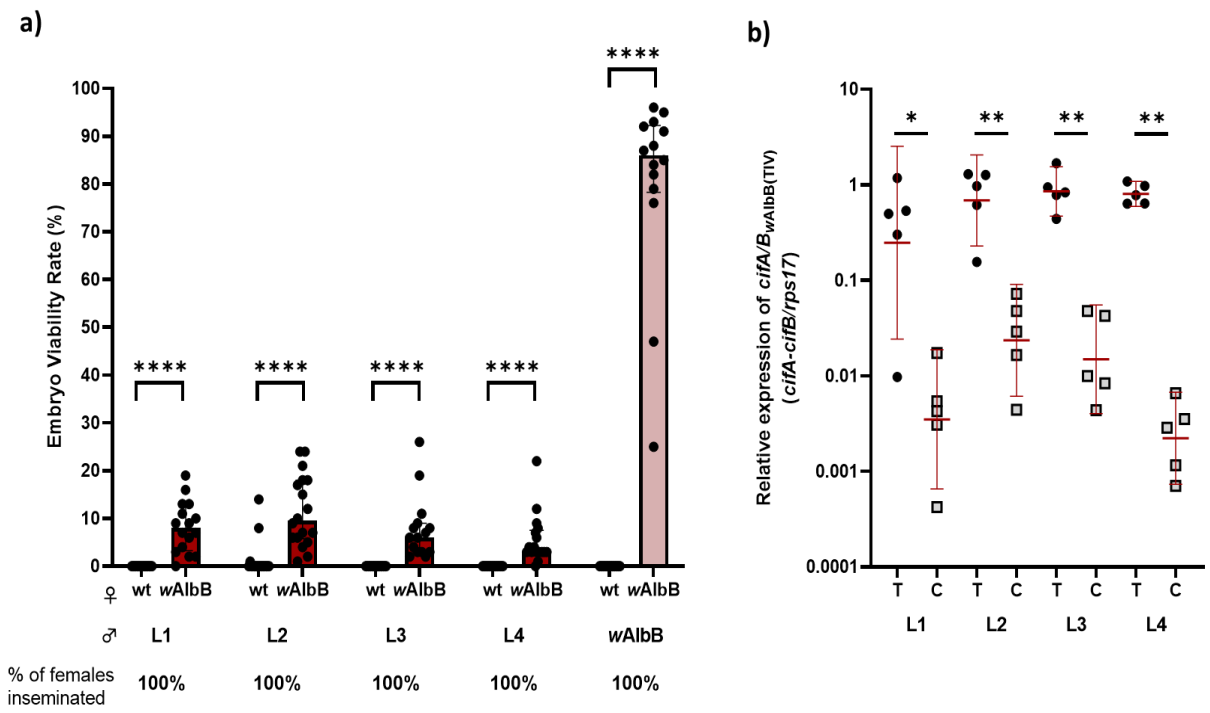


Figure 4.10: Testes-specific dual-expression of *cifA*$_{wAlbB(TIV)}$ and *cifB*$_{wAlbB(TIV)}$ from a single locus results in rescuable CI. Heterozygous males from each of the four different genomic insertion *β2t*-cifA*$_{wAlbB(TIV)}$-T2A- *cifB*$_{wAlbB(TIV)}$ lines (L1-L4) significantly reduced the viability rate of the progeny when crossed with wild-type (wt) females. The median embryo viability rate was significantly increased if the females were infected with the *w*AlbB strain of *Wolbachia*. *cifA;B*$_{wAlbB(TIV)}$ co-expression did not inhibit the production of sperm as was indicated by the percentage of females inseminated in each cross. Dots represent the percentage of viable embryos laid by one female, bars represent the median and error bars denote interquartile ranges. **b)** The expression of *cifA/B*$_{wMel(TI)}$ relative to housekeeping gene *rps17* in pooled adult testes (T) and remaining carcass (C). Lines denote geometric mean, error bars denote geometric SD. Significance calculated by Mann-Whitney U-test.

4.2.2.3 Generation of *exu-cifA*$_{wAlbB(TIV)}$ lines

Three independent genomic insertion lines were generated (see Table A1) through embryo microinjection of the *exu-cifA*$_{wAlbB(TIV)}$ donor plasmid (Figure 4.11).

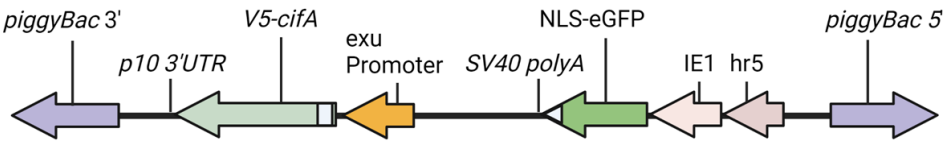


Figure 4.11: Schematic representation of the transposon used to investigate the effect of either *cifA*$_{wAlbB(TIV)}$ or *cifA*$_{wPip(TI)}$ expression on the ability to rescue CI.

Maternal expression was not found to affect the fertility of heterozygous females when mated with wild-type males (Figure 4.12a). As *w*AlbB encodes two sets of *cif* genes (Type III and IV) that are predicted to be bidirectionally incompatible, it was not expected that transgenic expression of *cifA*$_{wAlbB(TIV)}$ would rescue CI induced by the bacterium (in the absence of *cifA*$_{wAlbB(TIII)}$). Therefore, to test the ability for maternal expression of *cifA*$_{wAlbB(TIV)}$ to rescue CI, heterozygous females from each *exu-cifA*$_{wAlbB(TIV)}$ line were crossed to heterozygous males from *β2t*-cifA*$_{wAlbB(TIV)}$-T2A-*cifB*$_{wAlbB(TIV)}$ L1. Only one of the lines (L3) was able to significantly increase the median embryo viability rate from 0% (Figure 4.12: Mdn = 4.5% IQR- 2.0-5.75%). Interestingly, the level of rescue provided by maternal *cifA*$_{wAlbB(TIV)}$ expression in *exu-cifA*$_{wAlbB(TIV)}$ L3 was only slightly lower than that provided by a *w*AlbB infection (Figure 4.12: Mdn =8.0%, IQR=3.3-12.5%). This line was found to have a high ovary specific expression of *cifA*$_{wAlbB(TIV)}$ however, the expression in the ovaries was comparable to *exu-cifA*$_{wAlbB(TIV)}$ L1 which did not significantly increase the median viability rate of embryos (Figure 4.12). It is possible that the *exu-cifA*$_{wAlbB(TIV)}$ L3 has a higher capacity to deposit *cifA*$_{wAlbB(TIV)}$ in developing oocytes comparative to L1.

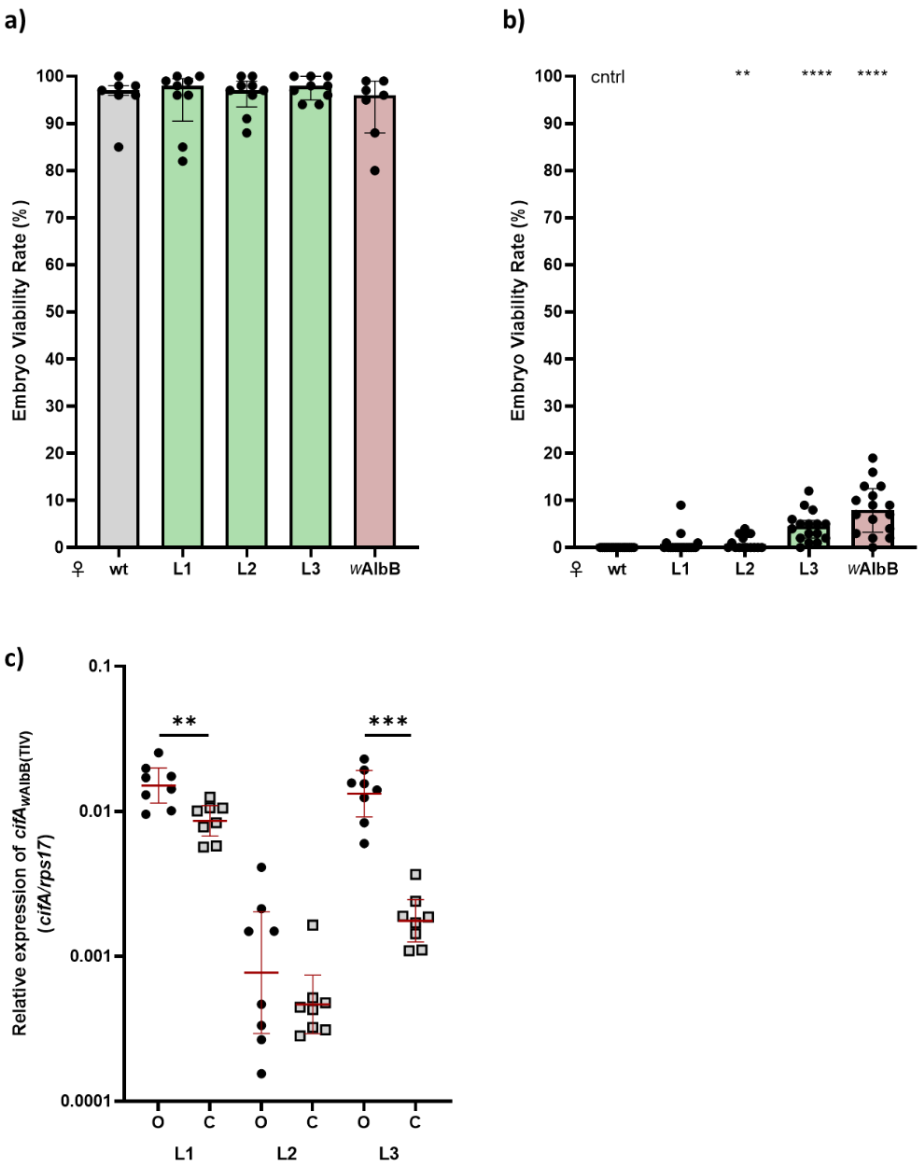


Figure 4.12: The effect of maternal *cifA*$_{wAlbB(TIV)}$ expression on female fertility in crosses between either wild-type males or males expressing both *cif*$_{wAlbB(TIV)}$ genes in the testes. a) Transgenic expression of *cifA*$_{wAlbB(TIV)}$ under the control of the *exu* promoter did not affect fertility in crosses with wild-type (wt) males. **b)** Out of the three *exu-cifA*$_{wAlbB(TIV)}$ lines, one significantly increased median embryo viability when crossed with *β2t*-cifA*$_{wAlbB(TIV)}$-T2A-*cifB*$_{wAlbB(TIV)}$ males. In both **a)** and **b)** dots represent the percentage of viable embryos laid by one female, bars denote the median and error bars the interquartile ranges. **c)** The expression of *cifA*$_{wAlbB(TIV)}$ in three *exu-cifA*$_{wAlbB(TIV)}$ lines (L1-3) relative to housekeeping gene *rps17* in single pairs of ovaries (O) or remaining carcasses (C) 24hrs post oviposition. Red line is the geometric mean, error bars denote 95% confidence intervals, asterisks denote statistical significance.

As the level of rescue was found to be dependent on the balance of CifA$_{wAlbB(TIV)}$ and CifB$_{wAlbB(TIV)}$ from experiments involving their expression from different genomic insertion sites, it remained possible that the correct balance of the two transgenes in the testes had not been met despite being expressed at near equimolar quantities using the T2A sequence. Although the *cif* genes are thought to be transcribed as an operon, they can display significant transcriptional differences[83,195]. For several strains (including *w*Mel) the level of *cifA* transcripts are higher than those of *cifB*[83,195]. Therefore, it was hypothesised that a higher relative expression of *cifA*$_{wAlbB(TIV)}$ compared to *cifB*$_{wAlbB(TIV)}$ might be needed to prevent excessive DNA damage in the testes and permit efficient rescue in the embryos. Accordingly, to provide an additional genomic copy of *cifA*$_{wAlbB(TIV)}$, males from *β2t*-cifA*$_{wAlbB(TIV)}$ L2 were crossed with females from *β2t*-cifA*$_{wAlbB(TIV)}$-T2A-*cifB*$_{wAlbB(TIV)}$ L1 and the resulting double heterozygous males were crossed with either wild-type, *w*AlbB-infected, or *exu-cifA*$_{wAlbB(TIV)}$ L3 females. Additional expression of *cifA*$_{wAlbB(TIV)}$ was not found to attenuate CI induction but was found to considerably increase the viability rate of embryos in crosses with *w*AlbB-infected females and uninfected females expressing *cifA*$_{wAlbB(TIV)}$ (Figure 4.13). Therefore, increasing the relative paternal dosage of CifA$_{wAlbB(TIV)}$ can improve the capacity for embryonic CifA$_{wAlbB(TIV)}$ to rescue incompatibility.

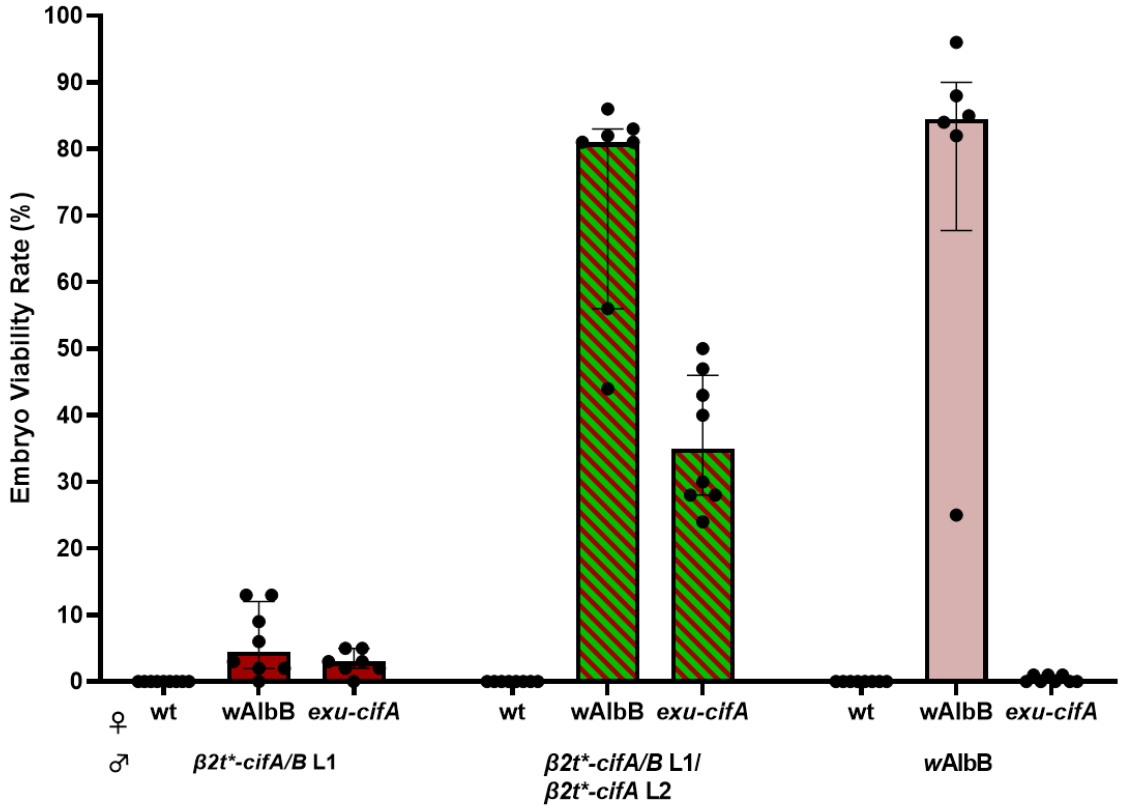


Figure 4.13: Additional *cifA*$_{wAlbB(TIV)}$ expression does not attenuate CI penetrance but increases the rescue capability of *w*AlbB-infected or transgenic *cifA*$_{wAlbB(TIV)}$ expressing females. Either males (♂) heterozygous for *β2t*-cifA*$_{wAlbB(TIV)}$-T2A- *cifB*$_{wAlbB(TIV)}$ L1 or both *β2t*-cifA*$_{wAlbB(TIV)}$ L2 and *β2t*-cifA*$_{wAlbB(TIV)}$-T2A-*cifB*$_{wAlbB(TIV)}$ L1 insertions were mated with wild-type (wt), *w*AlbB-infected, or heterozygous *exu-cifA*$_{wAlbB(TIV)}$ L3 females (♀). *w*AlbB-infected males were used for comparison. Dots represent the percentage of viable embryos laid by one female, bars represent the median and error bars denote interquartile ranges.

4.2.2.4 Generation of *topi-cifA*$_{wAlbB(TIV)}$-T2A-*cifB*$_{wAlbB(TIV)}$ lines

From the previous results it was theorised that a higher paternal *cifA*$_{wAlbB(TIV)}$ expression comparative to *cifB*$_{wAlbB(TIV)}$ was needed to attenuate the toxicity of CifB$_{wAlbB(TIV)}$ during spermatogenesis. This is potentially due to CifA having an expected higher turnover rate than CifB and therefore more CifA is needed to prevent unbound CifB modifications to the chromatin. It was hypothesised that an overall lower expression of *cifB*$_{wAlbB(TIV)}$ might limit the damage caused by the resulting peptides during spermatogenesis and thus allow for a strong rescue phenotype. As the *topi* promoter was found to promote lower levels of transcription than the *β2t** promoter (see Section 3.2.1.5: Figure 3.6) this regulatory sequence was selected to drive the *cif*$_{wAlbB(TIV)}$ genes.

In each of the four independent genomic *topi-cifA*$_{wAlbB(TIV)}$-T2A-*cifB*$_{wAlbB(TIV)}$ insertion lines generated (see Table A1) the expression of *cifA*$_{wAlbB(TIV)}$/*cifB*$_{wAlbB(TIV)}$ was found to be significantly higher in the testes compared to remaining carcasses (Figure 4.14b) - yet significantly lower than when transcription was activated by the *β2t** promoter (14-fold decrease: Figure 4.14c). To test the capacity of *topi-cifA*$_{wAlbB(TIV)}$-T2A-*cifB*$_{wAlbB(TIV)}$ males to induce CI, heterozygous males from each of the four independent insertion lines were crossed with either wild-type or *w*AlbB-infected females (Figure 4.14a). When females were uninfected all the resulting embryos were inviable despite all the females being inseminated. When females were infected with *w*AlbB, the median embryo viability rate was increased substantially to a level almost comparable with the *w*AlbB♂ x *w*AlbB♀ control crosses. Furthermore, maternal expression of *cifA*$_{wAlbB(TIV)}$ (*exu-cifA*$_{wAlbB(TIV)}$ L3) was found to significantly increase the median embryo viability rate (Figure 4.14a) to a level comparable to what was observed when a much higher *cifA*$_{wAlbB(TIV)}$ expression relative to *cifB*$_{wAlbB(TIV)}$ in the testes was achieved (Figure 4.13). Therefore modulating the Cif dosage in the testes has an effect on the ability of CifA in embryos to rescue, and likely involves limiting CifB-induced damage before CifB is loaded into sperm nuclei. Lowering the overall paternal dosage of CifB (with the *topi* promoter) also likely improves rescue capacity by lowering the amount of CifB deposited into embryos via sperm.

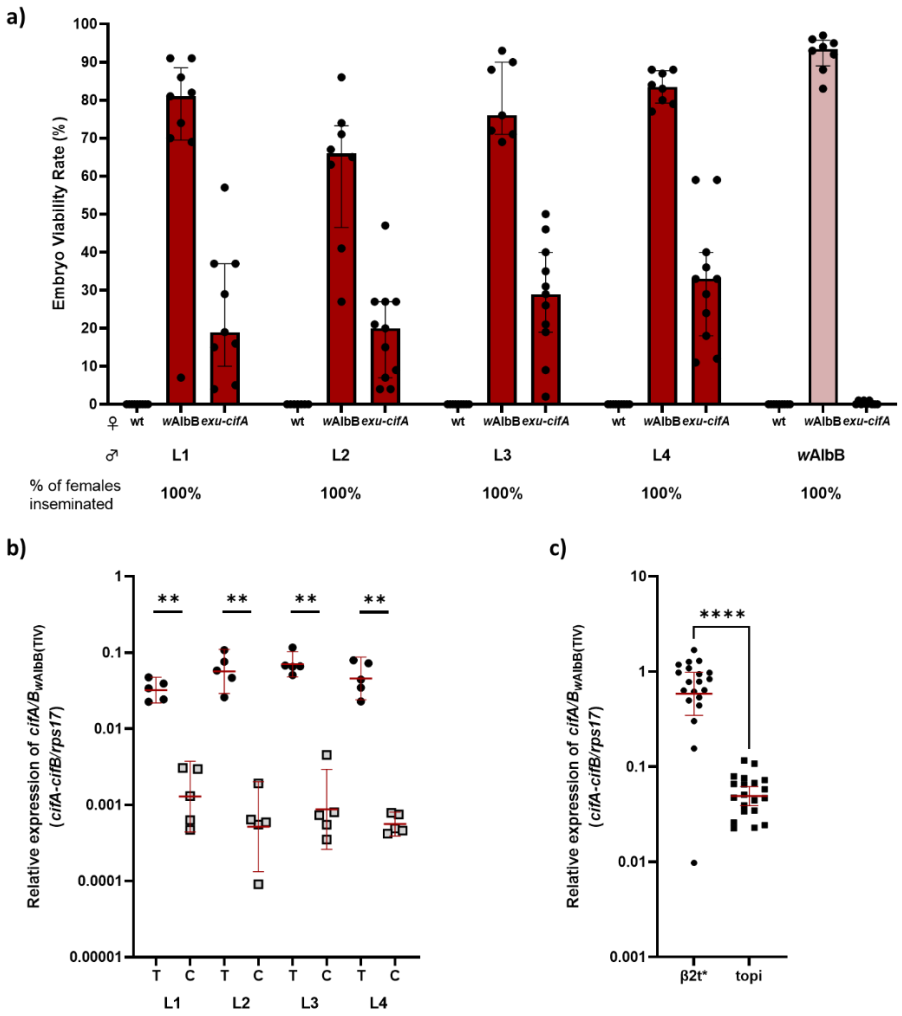


Figure 4.14: Testes-specific dual-expression of *cifA*$_{wAlbB(TIV)}$ and *cifB*$_{wAlbB(TIV)}$ from a single locus under the regulation of the *topi* promoter results in rescuable CI. a) Heterozygous males from each of the four different genomic insertion *topi-cifA*$_{wAlbB(TIV)}$-T2A-*cifB*$_{wAlbB(TIV)}$ lines (L1-L4) significantly reduced the viability rate of the progeny when crossed with wild-type (wt) females. The median embryo viability rate was significantly increased if the females were infected with *w*AlbB or if females expressed *cifA*$_{wAlbB(TIV)}$ (*exu-cifA*). *cifA/B*$_{wAlbB(TIV)}$ co-expression did not inhibit the production of sperm as was indicated by the percentage of females inseminated in each cross. Dots represent the percentage of viable embryos laid by one female, bars represent the median and error bars denote interquartile ranges. **b)** The expression of *cifA/B*$_{wAlbB(TIV)}$ relative to housekeeping gene *rps17* in pooled adult testes (T) and remaining carcass (C). Lines denote geometric mean, error bars denote geometric SD. Significance calculated by Mann-Whitney U-test. **c)** Comparison of the levels of *cifA/B*$_{wAlbB(TIV)}$ expression in the testes regulated by either the *β2t** or *topi* promoters. Significance calculated by Mann-Whitney U-test.

4.2.3 Testes-specific expression of *cifB*$_{w\text{Pip(TI)}}$ induces sterility in *Ae. aegypti* mosquitoes.

4.2.3.1 Generation of *β2t*-cifB*$_{w\text{Pip(TI)}}$ lines

As previously shown, the co-expression of *cifA*$_{w\text{AlbB(TIV)}}$ and *cifB*$_{w\text{AlbB(TIV)}}$ was needed to induce rescuable CI in transgenic *Ae. aegypti*. However, the expression of only *cifB*$_{w\text{Pip(TI)}}$ was found to elicit the same phenotype in *An. gambiae*[93], and therefore paternal *cifA*$_{w\text{Pip(TI)}}$ expression might likewise be unnecessary in *Ae. aegypti*. To test this hypothesis, lines expressing *cifB*$_{w\text{Pip(TI)}}$ under the *β2t** promoter were generated (see Table A1). Paternal expression of *cifB*$_{w\text{Pip(TI)}}$ resulted in a significant reduction in embryo viability when heterozygous males from each of the two independent lines (see Table A1) were crossed with wild-type females (Figure 4.15a). Although a minority of the eggs appeared viable (Figure A2), they did not hatch which suggests a later stage of developmental arrest (Figure 4.15b). This apparent delay in embryonic developmental arrest was also observed in the transgenic *An. gambiae* study as well as in crosses between infected *C. pipiens* mosquitoes, which suggests that this is characteristic of *w*Pip *cif* genes[73,93,192,193]. RT-qPCR confirmed that expression of the transgene in the testes was higher when compared to the remaining carcasses which indicates active expression in the testes (Figure 4.15c). Interestingly, the level of *cifB*$_{w\text{Pip(TI)}}$ expression was very low in both independent insertion lines, and comparable to levels observed in the *β2t**-*cifB*$_{w\text{Mel(TI)}}$ lines (Figure 4.15d). Therefore, the lack of sterility phenotype observed when *cifB*$_{w\text{Mel(TI)}}$ was expressed on its own can no longer be explained by low expression levels in the testes.

As the *β2t** promoter has been shown to drive high levels of transcription of *cifA*$_{w\text{Mel(TI)}}$/*cifB*$_{w\text{Mel(TI)}}$, *cifA*$_{w\text{Mel(TI)}}$, *cifA*$_{w\text{AlbB(TIV)}}$/*cifB*$_{w\text{AlbB(TIV)}}$, *cifB*$_{w\text{AlbB(TIV)}}$ and *cifA*$_{w\text{AlbB(TIV)}}$ in the testes it is unknown why low expression levels of either *cifB*$_{w\text{Mel(TI)}}$ and *cifB*$_{w\text{Pip(TI)}}$ are recorded when the genes are expressed alone. One explanation is that both these *cif* genes share a functional DUB domain. When the DUB domain of *cifB*$_{w\text{Pip(TI)}}$ was inactivated through mutation, the toxicity of the peptide was maintained however a decrease of CifB$_{w\text{Pip(TI)}}$ transfer to oocytes was recorded[88]. Therefore, it is possible that this domain is involved in preventing the degradation of CifB and not involved in the modifications associated with CI. If the DUB domain does indeed increase the protein stability, an accumulation of CifB would result in cell lethality unless bound by its cognate CifA. Therefore, it is possible only lines expressing either *cifB*$_{w\text{Mel(TI)}}$ and *cifB*$_{w\text{Pip(TI)}}$ at very low levels were viable and therefore

maintained during experimentation and could explain why lines that expressed both *cifA*$_{wMel(TI)}$ and *cifB*$_{wMel(TI)}$ at levels expected of the selected promoter were obtained, but not when *cifB*$_{wMel(TI)}$ was expressed individually.

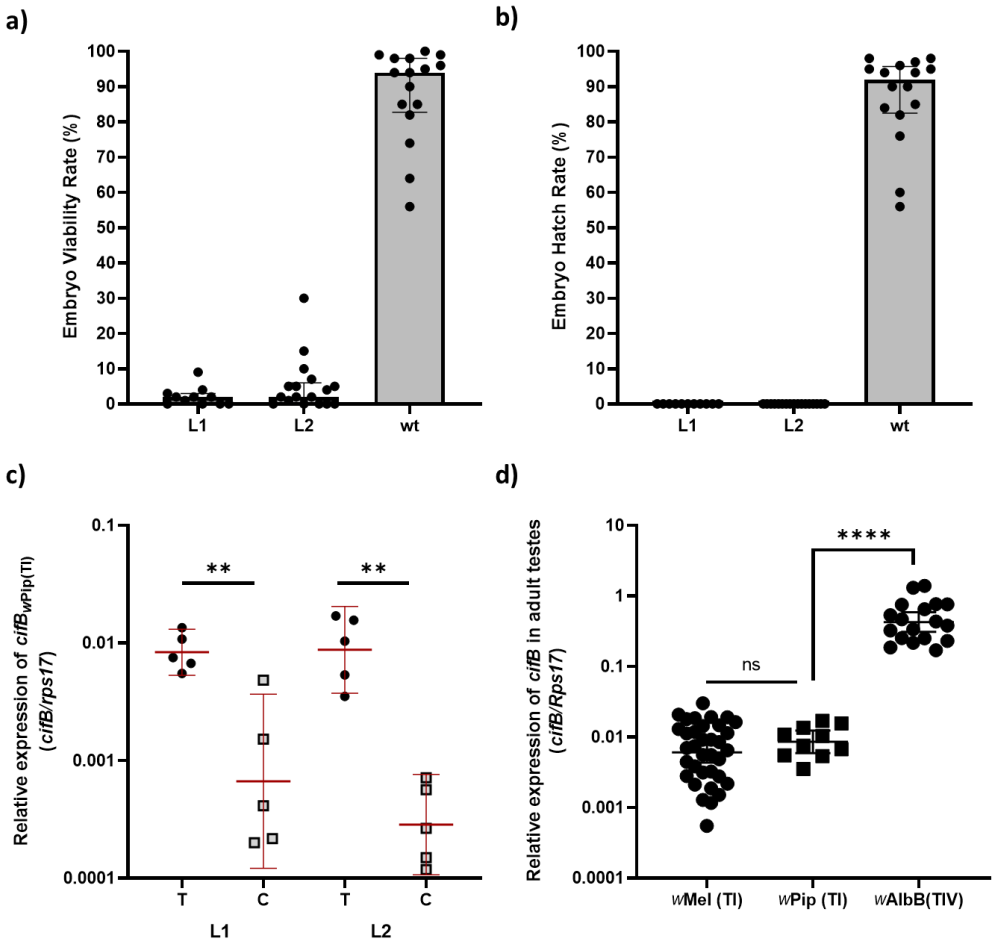


Figure 4.15: Testes-specific expression of *cifB*$_{wPip(TI)}$ resulted in male sterility. Crosses between heterozygous *β2t*-cifB*$_{wPip(TI)}$ males from two independent insertion lines (L1-2) and wild-type (wt) females resulted in **a)** a near-complete reduction in the embryo viability rate, and **b)** a complete reduction in the embryo hatch rate. Dots represent either the embryo viability or hatch rates recorded for the progeny of a single female, bars represent the median and error bars denote interquartile ranges. **c)** The expression of *cifB*$_{wPip(TI)}$ relative to housekeeping gene *rps17* in pooled adult testes (T) and remaining carcass (C). Lines denote geometric mean, error bars denote geometric SD. Significance calculated by Mann-Whitney U-test. **d)** Comparison of the levels of *cifB*$_{wMel(TI)}$, *cifB*$_{wPip(TI)}$, and *cifB*$_{wAlbB(TIV)}$ expression in the testes regulated by the *β2t** promoter. Significance calculated by Mann-Whitney U-test.

Unlike what was observed upon testes-specific *cifB*$_{w\text{AlbB(TIV)}}$ expression, spermatogenesis was not impeded and *β2t*-cifB*$_{w\text{Pip(TI)}}$ males produced mature sperm which could be seen in the dissected spermathecae from mated females. Based on prior *in vitro* studies and the fact that *cifB*$_{w\text{Pip(TI)}}$ does not encode the key catalytic residues in its PD-(D/E)XK-like nuclease domains, CifB$_{w\text{Pip(TI)}}$ is not expected to exhibit DNase activity. However, CifB$_{w\text{Mel(TI)}}$ which was also predicted to not display nuclease activity was found to cleave both single-stranded and double-stranded DNA *in vitro* and in *D. melanogaster* testes. To test whether CifB$_{w\text{Pip(TI)}}$ exhibits nuclease activity when expressed in *Ae. aegypti* tissues TUNEL assays were performed on testes dissected from heterozygous *β2t*-cifB*$_{w\text{Pip(TI)}}$ L1 males (Figure 4.16). As predicted, *cifB*$_{w\text{Pip(TI)}}$ expression did not result in an abundance of DNA breaks - as was seen when the predicted nuclease *cifB*$_{w\text{AlbB(TIV)}}$ was expressed.

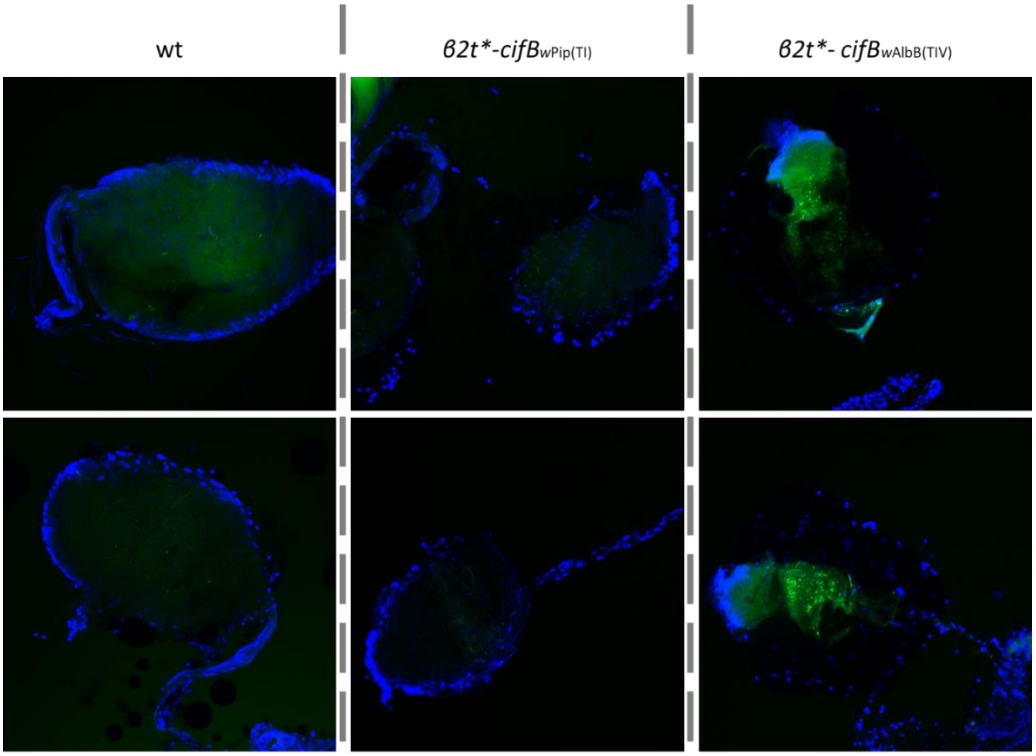


Figure 4.16: A comparison of the nuclease capability of CifB$_{w\text{Pip(TI)}}$ and CifB$_{w\text{AlbB(TIV)}}$ *in situ*. TUNEL staining on testes squashes from age-matched wild-type (wt), as well as those heterozygous for either *cifB*$_{w\text{Pip(TI)}}$ (*β2t*-cifB*$_{w\text{Pip(TIV)}}$ L1) or *cifB*$_{w\text{AlbB(TIV)}}$ (*β2t*-cifB*$_{w\text{AlbB(TIV)}}$ L1). DNA breaks are marked by TUNEL staining (green foci), DNA is marked with Hoechst stain (blue). Compared to the negative controls (wt), DNA breaks are observed in testes which express *cifB*$_{w\text{AlbB(TIV)}}$ but not *cifB*$_{w\text{Pip(TI)}}$ highlighting differences in the enzymatic activity of these Cif peptides.

4.2.4 Maternal expression of *cifA*$_{w\text{Pip(TI)}}$ delays developmental arrest induced by paternal *cifB*$_{w\text{Pip(TI)}}$ expression.

4.2.4.1 Generation of *exu-cifA*$_{w\text{Pip(TI)}}$ lines.

Unfortunately, a transinfected *w*Pip(Pel) *Ae. aegypti* line was unavailable for crossing experiments. Therefore, to determine whether *cifB*$_{w\text{Pip(TI)}}$ induced sterility was rescuable and therefore represented canonical CI induction, *exu-cifA*$_{w\text{Pip(TI)}}$ lines (figure 4.11) were generated. To test whether maternal *cifA*$_{w\text{Pip(TI)}}$ expression could rescue *β2t*-cifB*$_{w\text{Pip(TI)}}$ male infertility, *exu-cifA*$_{w\text{Pip(TI)}}$ heterozygous females were crossed with heterozygous *β2t*-cifB*$_{w\text{Pip(TI)}}$ males. One of the six *exu-cifA*$_{w\text{Pip(TI)}}$ independent insertions (see Table A1) was male linked, and therefore this line (L2) was not used in crosses. Maternal expression of *cifA*$_{w\text{Pip(TI)}}$ was found to significantly increase the proportion of developed embryos which was suggestive of CI rescue, however, these crosses did not result in a significant increase in the embryo hatch rate (Figure 4.17). Crosses between *C. pipiens* mosquitoes carrying incompatible variants of the *w*Pip strain can often result in a later developmental arrest.

Cytological analysis of the embryos revealed that in the absence of an inherited *Wolbachia* infection, delays in paternal chromatin condensation and separation led to chromatin bridging, aneuploid nuclei and an early arrest of embryogenesis[73]. However, if the embryos inherited a *Wolbachia* strain incompatible with that carried by the father, then the paternal chromatin was completely excluded in the first zygotic division, resulting in developed haploid embryos that were unable to hatch[73]. This would suggest that the expression of incompatible *cif* genes by *Wolbachia* in the embryos might have an additive effect on the asynchronicity of the pronuclei. It seems unlikely that maternal expression of the cognate *cifA* would increase the severity of CI defects, and thus haploid development. A more parsimonious explanation is that the maternal *cifA*$_{w\text{Pip(TI)}}$ expression achieved by the *exu-cifA*$_{w\text{Pip(TI)}}$ lines was able to delay the developmental arrest time of embryos but not prevent embryonic lethality. It should be noted that hatching was only recorded for progeny of transgenic mothers and never wild-type mothers which would suggest that in a minority of cases maternal *cifA*$_{w\text{Pip(TI)}}$ expression is able to prevent embryonic lethality. Theoretically, the expression regulated by the *exu* promoter might not be strong enough to rescue the sterility induced by *β2t*-cifB*$_{w\text{Pip(TI)}}$ male.

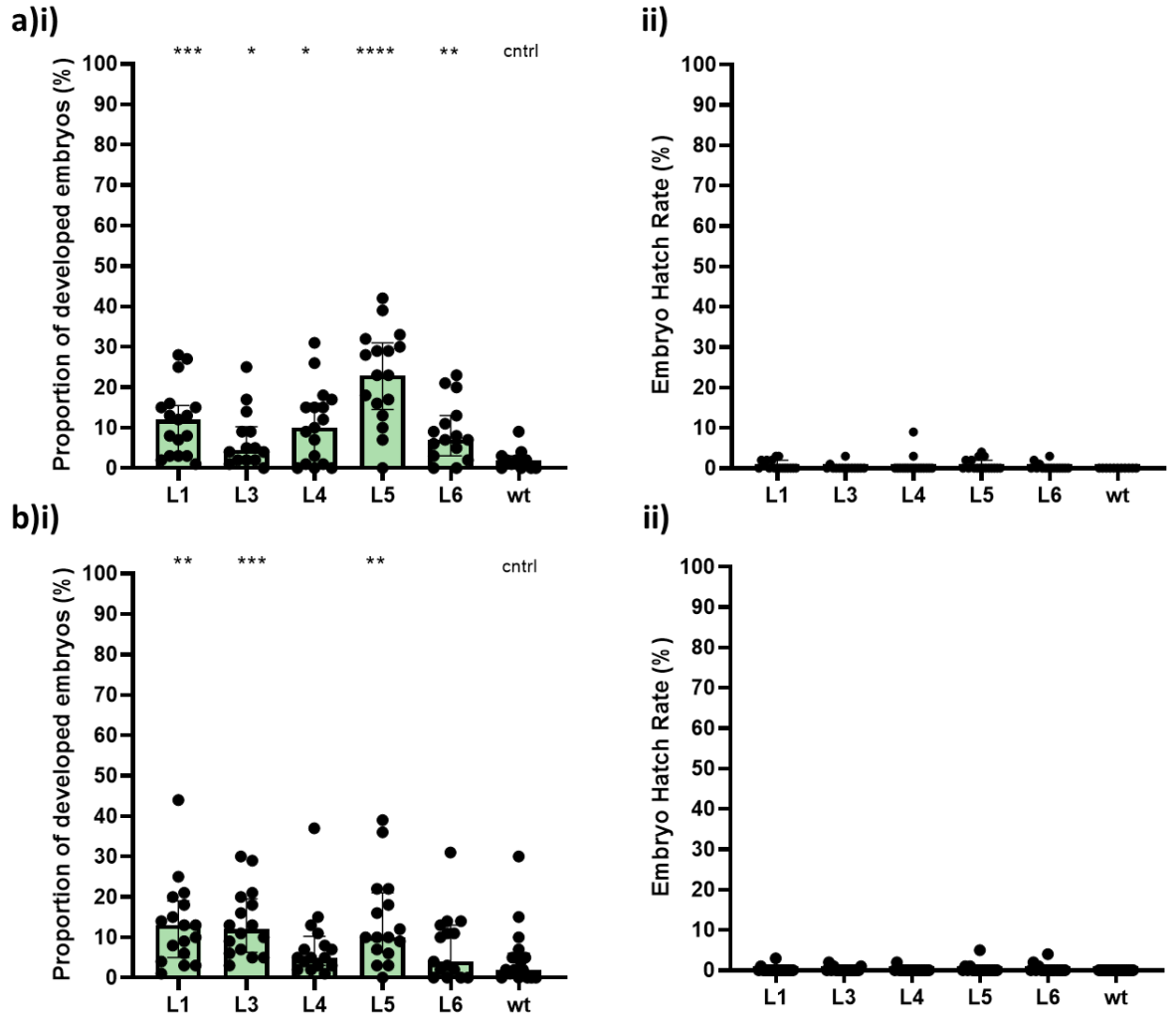


Figure 4.17: Maternal expression of *cifA*$_{wPip(TI)}$ significantly increases the proportion of developed embryos but not hatch rate in crosses with *Ø2t*-cifB*$_{wPip(TI)}$ males. Heterozygous females from five different genomic insertion *exu-cifA*$_{wPip(TI)}$ lines (L1-5) were crossed with heterozygous males from either *Ø2t*-cifB*$_{wPip(TI)}$ L1 **(a)** or *Ø2t*-cifB*$_{wPip(TI)}$ L2 **(b)**, and the proportion of developed embryos **(i)** and hatch rate **(ii)** of the resulting progeny was counted. Dots represent the proportion of developed embryos laid by one female/the resulting hatch rate, bars represent the median, error bars denote interquartile ranges, asterisks denote statistical significance. Statistical significance was determined by Mann-Whitney U-tests, ****=p<0.0001.

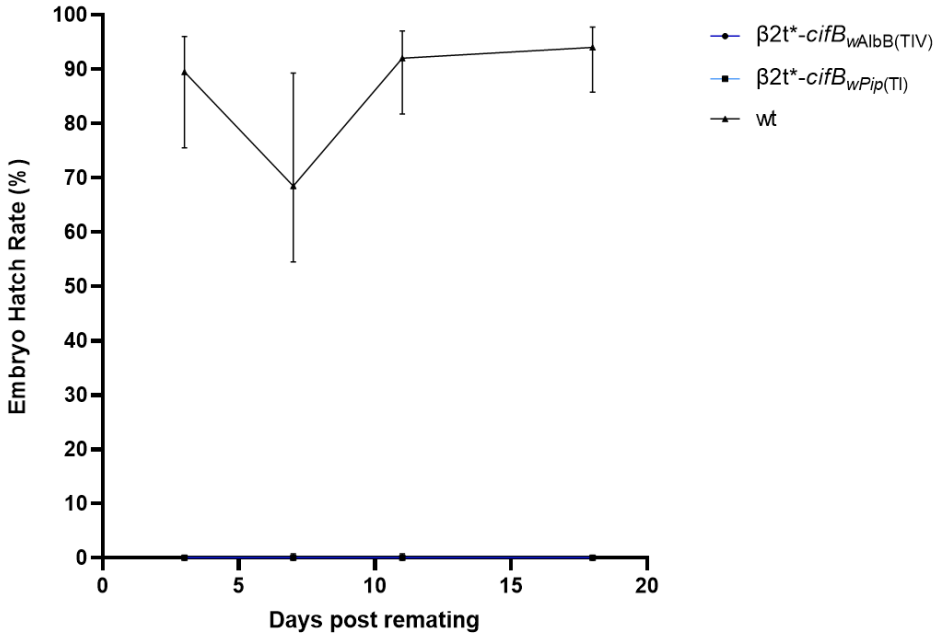
4.2.5 Lasting suppression of female fertility through paternal *cifB*$_{w\text{AlbB(TIV)}}$/*cifB*$_{w\text{Pip(TI)}}$ expression

In contrast to *D. melanogaster* which requires insemination to induce prolonged remating refractoriness in females, it was found that only the transfer of seminal fluid proteins is required in *Ae. aegypti* to prevent insemination by subsequent copulations[144–146]. As expression of *cif* genes under the control of the *β2t** promoter resulted in either the absence of sperm production (*cifB*$_{w\text{AlbB(TIV)}}$) or the production of incompatible sperm (*cifB*$_{w\text{Pip(TI)}}$), it presented an opportunity to study whether these phenotypes effect the period of post-mating refractoriness. Additionally, if the remating rate is higher for females that have previously copulated with spermless males, the absence of sperm competition would in theory drastically increase the fertilisation rate with sperm from subsequent males.

An excess of wild-type males, or heterozygous males from either *β2t*-cifB*$_{w\text{AlbB(TIV)}}$ L1 or *β2t*-cifB*$_{w\text{Pip(TI)}}$ L1 were added to four sets of cages (each containing three wild-type females) and left to copulate for 2 days. The males were then removed and replaced with heterozygous *exu-cifA*$_{w\text{Pip(TI)}}$ L4 males at a 1:1 ratio with females. These males are fully fertile and half of the sperm will carry a fluorescent marker gene (eGFP) different to that of the *cifB*$_{w\text{AlbB(TIV)}}$ and *cifB*$_{w\text{Pip(TI)}}$ males (AmCyan1); the rate of resulting progeny carrying this fluorescent marker is therefore a proxy for the overall remating rate. The females were fed and eggs collected 3 days post remating, egg cones were subsequently collected at days 7, 11, and 18 with females being refed after each oviposition round. The embryo hatch rates were counted for each egg cone, and the progeny were screened using a fluorescent microscope for the presence of the *exu-cifA*$_{w\text{Pip(TI)}}$ L4 (indicating remating) which would be detected by eGFP expression.

Prior mating with *β2t*-cifB*$_{w\text{AlbB(TIV)}}$ L1 or *β2t*-cifB*$_{w\text{Pip(TI)}}$ L1 males resulted in complete suppression of wild-type female fertility for the total length of the study (18 days) despite subsequent copulation events with *exu-cifA*$_{w\text{Pip(TI)}}$ L4 males (Figure 4.18). When wild-type females were initially mated with wild-type males, the mean percentage of transgenic progeny remained at 0.00% for the majority of the study. However, one transgenic individual was observed in one batch of eggs at the final timepoint, which suggested that the refractory period might be ending (Figure 4.18). However, subsequent refeeding and egg collections could not take place as the fitness of the females was adversely affected at this age resulting in deaths. Therefore, it was concluded that the refractoriness induced by the *β2t**-

cifB$_{wAlbB(TIV)}$/ *cifB*$_{wPip(TI)}$ males was comparable to that induced by wild-type males for the period of the study. These findings reinforce the idea that insemination is not necessary to induce a long period of post-mating refractoriness in *Ae. aegypti* females and introduces the idea that males sterilised through testes-specific expression of *cifB*$_{wAlbB(TIV)}$ could potentially be used in population suppression strategies.



Day of egg collection (Days post remating)	Percentage of transgenic progeny (±SD)
3	0.00 (±0.0)
7	0.00 (±0.0)
11	0.00 (±0.0)
18	0.25 (±0.5)

Figure 4.18: Paternal expression of either *cifB*$_{wAlbB(TIV)}$ or *cifB*$_{wPip(TI)}$ induces lasting suppression of female fertility. An excess of wild-type (wt) males or males heterozygous for the *β2t*-cifB*$_{wAlbB(TIV)}$ L1 or *β2t*-cifB*$_{wPip(TI)}$ L1 insertions were allowed to mate with wt females before being replaced with heterozygous *exu-cifA*$_{wPip(TI)}$ L4 males. Graph shows the median embryo hatch rate of egg cones (from 4 replicate cages) collected at sequential days post remating, error bars denote interquartile ranges. Table shows the percentage mean of progeny displaying eGFP expression (*exu-cifA*$_{wPip(TI)}$) in egg cones collected from cages where the wt females were initially crossed with wt males before subsequent remating.

4.3 Summary and conclusions

Testes-specific expression of *cifB*$_{w\text{AlbB(TIV)}}$ was found to induce complete sterility in males. This sterility appeared to be the direct result of nuclease activity possessed by the transgene, which was visualised by TUNEL assays on testes dissected from males expressing *cifB*$_{w\text{AlbB(TIV)}}$. The microscopic analysis revealed multiple DNA breaks in cells of the testes, and this may be contributing to a stalling in the process of spermatogenesis resulting in the absence of mature sperm production. Paternal co-expression of *cifA*$_{w\text{AlbB(TIV)}}$ and *cifB*$_{w\text{AlbB(TIV)}}$ from different genomic loci was found to rescue sperm production if the expression of *cifA*$_{w\text{AlbB(TIV)}}$ was higher or comparable to that of *cifB*$_{w\text{AlbB(TIV)}}$. When sperm production did occur, mating with wild-type females still resulted in the complete reduction of embryo viability. When the females were infected with *w*AlbB embryo viability was rescued, which suggested that the sterility observed was indicative of CI induction.

Co-expression of *cifA*$_{w\text{AlbB(TIV)}}$ and *cifB*$_{w\text{AlbB(TIV)}}$ from the same locus (using the T2A peptide sequence) was found to robustly rescue sperm production. However, the rescue ability of CifA$_{w\text{AlbB(TIV)}}$ in zygotes present due to either a *w*AlbB infection or through deposition by *exu-cifA*$_{w\text{AlbB(TIV)}}$ females was impaired. Increasing the paternal expression of *cifA*$_{w\text{AlbB(TIV)}}$ was found to increase the embryo viability rate in crosses with *w*AlbB-infected females and uninfected *exu-cifA*$_{w\text{AlbB(TIV)}}$ females but not uninfected wild-type females. Therefore, additional *cifA*$_{w\text{AlbB(TIV)}}$ expression in males did not reduce CI penetrance. This contradicts a finding from the transgenic *An. gambiae* study which found that increased levels of *cifA*$_{w\text{Pip(TI)}}$ expression led to the attenuation of CI induced by CifB$_{w\text{Pip(TI)}}$[93]. It is possible that potential differences in the mechanism of CI-mediation between Type I and Type IV *cif* genes could explain this result. However, differences in insect species and promoter sequences are also potentially contributing factors.

Together, these results represent the first recapitulation of CI induction and rescue phenotypes through the transgenic expression of *Wolbachia cif* genes in *Ae. aegypti* mosquitoes and provide some insights into how the Type IV *cif* genes function in this model species. Firstly, this study confirmed the nuclease activity predicted of CifB$_{w\text{AlbB(TIV)}}$ which is expected to be how this factor modifies paternal chromatin. Secondly, based on TUNEL assays performed on *w*AlbB-infected testes, this nuclease activity is not observed during spermatogenesis which suggests that it may occur after fertilisation. This observation

supports the TA model of CI-mediation. Future work will focus on detecting CifB$_{w\text{AlbB(TIV)}}$ transfer to oocytes and whether the nuclease activity occurs in fertilised embryos. Thirdly, this study identified a role of CifA$_{w\text{AlbB(TIV)}}$ in attenuating the toxicity of CifB$_{w\text{AlbB(TIV)}}$ during spermatogenesis, which explains why co-expression of *cifA* and *cifB* is often needed to induce CI (the two-by-one model).

Similar to testes-specific expression of *cifB*$_{w\text{AlbB(TIV)}}$, expression of only *cifB*$_{w\text{Pip(TI)}}$ resulted in a near complete loss of male fertility. However, *cifB*$_{w\text{Pip(TI)}}$ expression was not found to impede spermatogenesis which indicated that although sperm was produced they were modified in such a way as to result in incompatibility. Prior studies have suggested that CifB$_{w\text{Pip(TI)}}$ does not possess nuclease activity and instead functions as a deubiquitinase [78,89,171]. TUNEL assays performed on testes from *ß2t*-cifB*$_{w\text{Pip(TI)}}$ males reinforced this hypothesis as no DNA breaks were observed in cells undergoing spermatogenesis - a result different to that observed in *ß2t*-cifB*$_{w\text{AlbB(TIV)}}$ testes. In future, deubiquitination assays will be performed to determine whether this activity occurs either during spermatogenesis or after fertilisation, and explore whether the male sterility phenotype is linked to this activity.

Male sterility induced by *cifB*$_{w\text{Pip(TI)}}$ expression alone would fit with the TA model of CI, and replicate results obtained in transgenic *An. gambiae*[93]. However, due to a lack of *Ae. aegypti* lines transinfected with the *w*Pip(Pel) strain, we are unable to confirm whether this sterility is the result of CI induction. To overcome this deficit, lines expressing *cifA*$_{w\text{Pip(TI)}}$ under the maternal promoter *exu* were generated. However, although the embryo viability rate was increased by maternal *cifA*$_{w\text{Pip(TI)}}$ expression this did not equate to a similar increase in hatch rate. This arrest in later development is often seen in incompatible crosses between infected *C. pipiens* mosquitoes [73,192,193]. This effect may be due to small polymorphisms in the binding interface of *cif*$_{w\text{Pip}}$ genes, which could reduce the binding efficiency between different cognate pairs. Partial binding might attenuate the level of CifB$_{w\text{Pip(TI)}}$ modifications, thus permitting embryos to reach a later stage of development but not to the extent of CI rescue. Alternatively, differences in the expression of cognate pairs shared by different strains of *w*Pip either through natural transcriptional differences or through differences in the number of copies may also result in this phenotype. It is possible that this later arrest in embryonic development in our transgenic system is the result of an insufficient level of *cifA*$_{w\text{Mel(TI)}}$ expression. In the *An. gambiae* study, rescue was not observed until a higher expression of

cifA$_{w\text{Pip(TI)}}$ was achieved by switching the *zero population growth (zpg)* promoter with the *vasa* promoter[93]. Although the *PUb* promoter has been shown to drive higher *cifA*$_{w\text{Mel(TI)}}$ expression in the ovaries, this did not result in higher rescue ability which was hypothesised to be the result in differences in expression patterns and deposition levels. Therefore, alternative maternal promoters will be sought and tested. Alternatively, based on the findings from the *cif*$_{w\text{AlbB(TIV)}}$ investigation, paternal co-expression of *cifA*$_{w\text{Pip(TI)}}$ and *cifB*$_{w\text{Pip(TI)}}$ might be necessary to attenuate the putative toxicity of *cifB*$_{w\text{Pip(TI)}}$ during spermatogenesis. In Future, either *ϐ2t*-cifA*$_{w\text{Pip(TI)}}$ or *ϐ2t*-cifA*$_{w\text{Pip(TI)}}$-T2A- *cifB*$_{w\text{Pip(TI)}}$ lines will be generated to investigate whether paternal co-expression of *cifA*$_{w\text{Pip(TI)}}$ and *cifB*$_{w\text{Pip(TI)}}$ from either the same or different loci will induce rescuable sterility comparable to that induced by a *Wolbachia* infection.

Chapter 5:

General conclusions and future considerations

5. General conclusions and future considerations

Ae. aeqypti mosquitoes are the primary vector of several arboviruses that impose major health and economic burdens on human populations. Presently, the best methods to reduce this burden is to control the vector population and thus arboviral transmission. The release of *Ae. aegypti* mosquitoes transinfected with varying strains of the maternally-inherited intracellular bacterium *Wolbachia*, have been shown to be an effective tool in vector control strategies [8–10,119,120,125–127]. The success of *Wolbachia*-mediated vector control is attributed to the bacteria's ability to manipulate host reproduction in a mechanism termed CI. This mechanism results from the interplay between two factors encoded by the *Wolbachia* genome known as Cifs. However, the exact method through which the *cif* genes function to elicit the CI phenotypes of induction and rescue are not yet fully characterised. Furthermore, to-date no studies have investigated the molecular functions of Cifs in *Ae. aegypti*.

An alternative method to *Wolbachia*-mediated vector control is through the creation of synthetic gene drive systems. Gene drives are composed of selfish genetic elements which distort the expected inheritance in their favour and could be used to spread alleles through a vector population which render them refractory to disease transmission. However, although successfully trialled in caged experiments[151,152] no synthetic gene drives have yet been deployed in the field due to environmental and public concerns. These synthetic systems are often modelled on naturally occurring selfish genetic elements which often employ TA principles. As *Wolbachia* Cifs are postulated to function in a TA model, it was hypothesised that the *cif* genes could be used to build gene-drive systems in various configurations. However, before a *cif*-based gene drive system designed for *Ae. aegypti* mosquitoes can be built a full characterisation of Cif function and the identification of suitable promoters in this host species is needed. This investigation represents the first study of transgenic *cif* gene expression in *Ae. aegypti* and has provided several insights on their function/dynamics and which promoters might be adequate for gene drive construction.

Firstly, this study showed that paternal expression of *cifB* alone could result in male sterility. However, in the case of the Type IV *cifB* genes encoded by *w*AlbB, *cifA/B* co-expression was found necessary to restrict DNA damage during spermatogenesis. As DNA damage does not occur during spermatogenesis in *w*AlbB-infected males, it is likely that damage could occur in zygotes. Therefore, the strength of CI would be determined by the abundance of CifB loaded

into spermatid nuclei. Accordingly, the rescue capability of *w*AlbB-infected females was found to increase when the relative expression of paternal *cifA/B*$_{wAlbB(TIV)}$ was lowered, which indicated that the dosage of CifB affected the strength of CI. As increasing the expression of *cifA*$_{wAlbB(TIV)}$ relative to *cifB*$_{wAlbB(TIV)}$ was also found to increase the rescue capability of females, it can be hypothesised that CifA must therefore reduce the paternal transmission of unbound CifB. Consistent with this result an overexpression of *cifA*$_{wMel(TI)}$ was found to reduce the penetrance of CI induced by *w*Mel-infected males. In an alternative study, a higher *cifA*$_{wPip(TI)}$ expression was also found to reduce the penetrance of CI induced by paternal *cifB*$_{wPip(TI)}$ expression[93]. Together these results contradict the HM model of CI which suggests that CifA plays an auxiliary role in the modifications imposed by CifB during spermatogenesis, and instead suggest that CifA plays an inhibitory role during spermatogenesis. Therefore, as the inhibitory role of CifA is conserved in both the induction and rescue mechanisms the Cifs can be seen to function in a TA model. It is unknown how CifA might reduce the paternal transmission of free CifB, however there are two potential mechanisms. Firstly, CifA:CifB binding might reduce the localisation of CifB to the paternal chromatin. In prior *in vitro* studies, CifB demonstrates nuclear localisation however in the presence of CifA appears to localise in the cytoplasm of cells during interphase; which provides evidence that CifA can affect the cellular localisation of CifB[88]. Secondly, CifA bound to CifB might be transferred to zygotes which might inhibit CifB activity after fertilisation. However, so far no evidence for CifA transmission has been documented[84,88]. It is important to note that although CifA was first found in a proteomic study of mated spermathecae, the females used in this study were also infected and therefore the presence of CifA might have had a maternal origin[95].

This study confirmed that CifB$_{wPip(TI)}$ does not act as a nuclease, and therefore this peptide must induce CI through another mechanism. It was originally thought that the DUB domain possessed by Type I *cif* genes might be involved in the induction of CI [78,89,171], however recent data suggests that this domain is not involved in this process and instead functions to stabilise the protein[88]. Mutation of the DUB domain resulted in a lower abundance of CifB$_{wPip(TI)}$ transmitted to zygotes, and therefore the deubiquitinase activity demonstrated by Type I CifB homologs might prevent their degradation by the host proteasome - thus maintaining high levels of paternal CifB transmission[88]. As the inactive/active PD-(D/E)XK nuclease domains are conserved between CifB homologs of all five Types, it suggests a retained function. This

function likely involves binding of CifB to the paternal chromatin and may explain why only CifB appears to be transferred to zygotes. It is possible that binding of CifB to the paternal chromatin is all that is required to result in CI, as binding might directly or indirectly obstruct the host DNA replication machinery which would result in a delay of the paternal pronuclei's mitotic progression- a key CI phenotype[88]. Expression of *cifB*$_{w\text{Pip(TI)}}$ in transgenic *Ae. aegypti* was found to result in a small number of developed embryos that did not hatch. This result has been seen in both native *C. pipiens w*Pip infections[73,192,193], as well as transgenic experiments involving the *cif*$_{w\text{Pip(TI)}}$ genes in *D. melanogaster*[88] and *An. gambiae*[93]. This difference in developmental arrest has been seen to depend on the severity of mitotic delay[73,88], which itself has been shown to depend on the dosage of CifB[88]. A higher paternal transmission is seen to result in more severe defects which results in haploid development[88]. Maternal deposition of the cognate CifA$_{w\text{Pip(TI)}}$ in this study was found to increase the proportion of developed unhatched embryos which seems to contrast these findings. It is likely that deposition of CifA$_{w\text{Pip(TI)}}$ is inhibiting the toxicity of CifB$_{w\text{Pip(TI)}}$ during the initial rounds of mitosis, however as *cifA*$_{w\text{Pip(TI)}}$ expression is not maintained during embryogenesis and CifB$_{w\text{Pip(TI)}}$ is predicted to be more stable (due to the presence of the DUB domain) the level of CifA$_{w\text{Pip(TI)}}$ might not be enough to prevent toxicity at later development stages. Therefore, either utilising a regulatory sequence which promotes maternal CifA$_{w\text{Pip(TI)}}$ deposition as well as embryonic expression, or reducing the paternal transmission of CifB either through i) lowering the paternal *cifB*$_{w\text{Pip(TI)}}$ expression or ii) co-expressing both *cifA*$_{w\text{Pip(TI)}}$ and *cifB*$_{w\text{Pip(TI)}}$ is expected to increase the capacity of transgenic females to rescue CI.

Now that a preliminary study of Cif dynamics in transgenic *Ae. aegypti* mosquitoes has been conducted, these findings can be used to inform future gene drive construction. As Cifs are expected to function according to TA principles - *Wolbachia*-mediated CI closely mirrors a hypothetical drive system termed *Semele*[164]*.* This system consists of two components: a toxin expressed by males which renders females infertile, and an antidote expressed by females that rescues infertility. In 2011 (before discovery of the *cif* genes), mathematical modelling of the *Semele* drive system predicted a threshold frequency of 36.4% in the absence of transgene insertion-associated fitness costs[164]. After the *cif* gene discovery and recapitulation of their phenotypes in transgenic organisms, an independent study mathematically modelled a 'cifAB drive system' consisting of both *cifB* (and if required the cognate *cifA*) regulated by a

male germline promoter and *cifA* regulated by a female germline promoter[165]. This drive system was predicted in the absence of transgene insertion-associated fitness costs to have an invasion threshold of ~36% – a similar finding to the *Semele* study[164,165].

Based on the findings from this thesis, a drive construct aimed at targeting *Ae. aegypti* mosquitoes and consisting of the Type IV *cif* genes from *w*AlbB would require a higher paternal expression of $cifA_{wAlbB(TIV)}$ relative to $cifB_{wAlbB(TIV)}$, to function. This could be achieved in two ways: i) *β2t** promoter regulated $cifA_{wAlbB(TIV)}$ expression and $cifB_{wAlbB(TIV)}$ expression regulated by the *topi* promoter, and ii) expression of $cifA_{wAlbB(TIV)}$ under the control of the *PUbt* promoter and $cifB_{wAlbB(TIV)}$ expression regulated by the *β2t** promoter (Figure 5.1). The former example would consist of three components, as maternal $cifA_{wAlbB(TIV)}$ expression under the *exu* promoter would be needed for rescue. However, the *PUbt* promoter was found to regulate high expression of the transgene in both ovaries and testes (and in one insertion line was found to promote comparable rescue to that of the *exu* promoter) and therefore the later construct example would consist of only two components. The number of genetic components inserted into mosquitoes likely affects their fitness and therefore it would be beneficial to keep the number low. However, unrestricted somatic expression (as observed with the *PUb*/*PUbt* promoters) might likewise increase fitness costs. Indeed, several genomic insertion lines of *PUb*/*PUbt*- $cifA_{wMel(TI)}$ lines displayed high mortality rates. Therefore, an alternative promoter that drives expression restricted to both male and female germlines should be sought.

To limit the drive temporally as well as spatially, the male CI-inducing component and the female CI-rescuing component could be split to resemble a killer-rescue system (Figure 5.1b)[165]. Males possessing *cifB* (the killer gene) would possess no advantage over wild-type males and therefore this component would be lost unless *cifA* (the rescue gene) is fixed in the population. As long as *cifB* is present at a high level within the population, females possessing *cifA* will possess a reproductive advantage. Once *cifB* is lost, *cifA* females will no longer maintain an advantage and if *cifA* (or its linked cargo/effector gene) has a fitness cost it will eventually be lost from the population. The spread of $cifA_{wMel(TI)}$ through a *w*Mel-infected *Ae. aegypti* population also represents a self-limiting drive[179]. Due to the ability of $cifA_{wMel(TI)}$ to rescue CI-induced by *w*Mel the transgene would spread through the population whilst the number of infected individuals would decrease (if environmental conditions resulted in

imperfect maternal transmission). Furthermore, as a *PUbt-cifA*$_{wMel(TI)}$ line was shown to both rescue and attenuate the penetrance of CI-induction, this drive would be expected to result in a quicker decrease of the *Wolbachia* infection frequency. Once *Wolbachia* had been lost from the population the fitness costs associated with *cifA*$_{wMel(TI)}$ or the linked effector gene expression would result in the loss of this drive construct from the population.

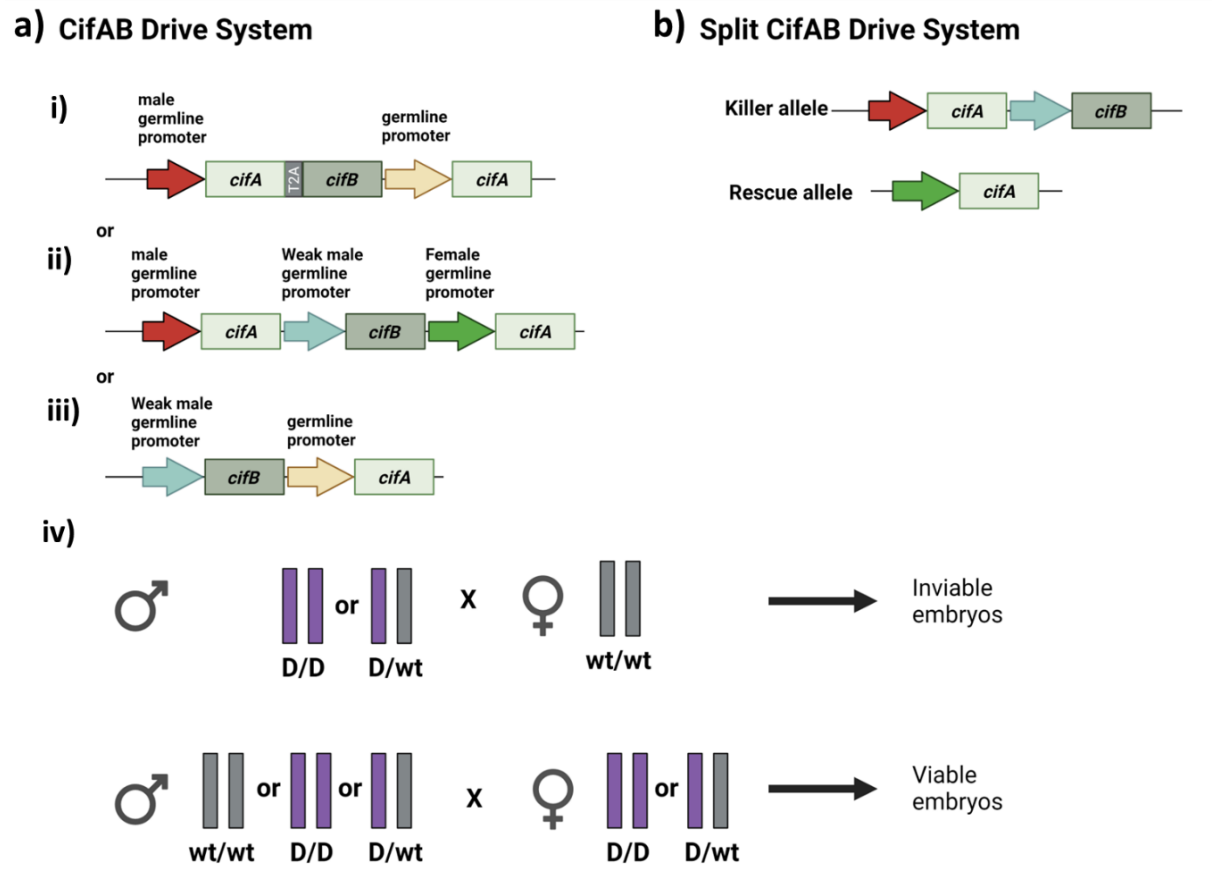


Figure 5.1: Schematic representation of a single locus or split CifAB drive system. a) The hypothesised single locus CifAB drive could be comprised of **i)** a male germline promoter driving both cognate *cif* genes and a germline specific promoter driving *cifA,* or **ii)** *cifA* and *cifB* expression in the male germline regulated by a stronger and weaker promoter respectively, and *cifA* expression regulated by a strong maternal promoter, or **iii)** a weak male promoter driving *cifB* expression and a germline specific promoter driving *cifA* expression in both the male and female germlines. **iv)** Schematic illustration of key CifAB drive system crosses: males carrying the drive allele (D) and wild-type (wt) females results in incompatibility, which is rescued by drive carrying females. **b)** Schematic representation of a hypothesised split CifAB drive system comprised of killer and rescue alleles. The killer allele is composed of strong and weak male germline promoters driving *cifA* and *cifB* expression

respectively, males that inherit this allele will produce inviable offspring unless the female carries the rescue allele composed of a strong maternal promoter driving *cifA* expression.

If *cifA* expression under a zygotic promoter was able to rescue CI induced by transgenic males this would greatly reduce the introduction threshold frequency. However, it is unknown whether this can occur. Instead, shifting the stage at which CifB-induced modifications occur to a later developmental stage might allow embryonic expression of *cifA* to rescue. Utilisation of a Tet-Off conditional expression system showed that embryonic expression of *cifB*$_{w\text{Mel(TI)}}$ (activated by *PUbt*-tTAV) resulted in complete lethality, a result not observed when both *cifA*$_{w\text{Mel(TI)}}$ and *cifB*$_{w\text{Mel(TI)}}$ were expressed in embryos possessing the *PUb-cifA*$_{w\text{Mel(TI)}}$-T2A-*cifB*$_{w\text{Mel(TI)}}$ insertion. This result provides a proof-of-principle for embryonic lethality/rescue mediated by *cif* genes. Consequently, an engineered underdominance system comprising of two independently inherited transgene insertions can be conceived (Figure 5.2). One insertion would consist of a toxin *cifB* gene variant and a non-cognate antidote *cifA* gene, whilst the other would encode the corresponding cognate antidote or toxin genes. In this configuration, when individuals possess both insertions the toxicity imposed by the CifB variants can be suppressed by their cognate CifA peptides. However, individuals inheriting just one insertion will be rendered inviable. Similar two-locus underdominance systems have been modelled mathematically and are expected to drive when transgene frequencies exceed ~27%[201]. This presents a more achievable release number than that of the CifAB drive, however the construction of two-locus underdominance systems could prove challenging. To negate the toxicity of each construct alone, both could be co-injected into embryos resulting in a few individuals possessing independent insertions of both, thus leading to suppression. A similar strategy was employed to generate *zpg-cifB*$_{wPip(TI)}$ lines in *An. gambiae* when it was found that *cifB*$_{w\text{Pip(TI)}}$ expression by itself was toxic[93].

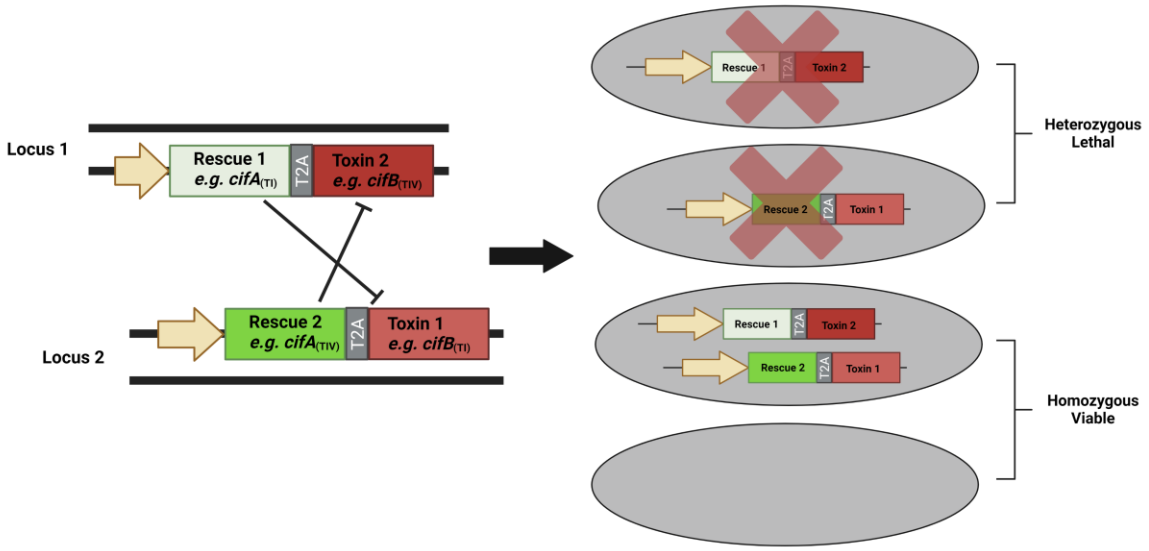


Figure 5.2: Schematic illustration of the engineered two-locus underdominance system. Two transgene insertion alleles at different genomic loci (Locus 1 & 2) generate a mutually repressing TA system. Embryonically active promoters (yellow arrows) drive expression of *cifA* and *cifB* variants configured such that inheritance of only one of the transgene alleles is lethal (red cross).

One key concern to the efficacy of *cif*-based vector control strategies is the development of resistance to the drive component. As the mechanism of CI likely targets ubiquitous host targets (CI is observed in a wide host range) at key developmental stages, it is expected that the development of resistance to the CI mechanism itself would be very rare. Indeed, resistance to Cif function has not been documented in *Wolbachia* infected/transinfected hosts. Although unlikely, if the host did evolve resistance to CI-mediated by one set of *cif* genes, then this could be addressed through the release of a drive allele comprising a divergent *cif* gene pair - as divergent *cif* pairs are expected to induce incompatibility through different mechanisms. Instead, the breakdown of the drive would most likely occur through mutations obtained in the *cif* coding sequences. As CifA provides a selective advantage due to the rescue mechanism, mutations in *cifA* that would perturb this process would be selected against. Likewise, mutations in the CifA:CifB binding interface domains of *cifB* would also be lost, as this would prevent rescue. However, mutations that inhibit the toxicity of CifB would

be selected for and lead to drive breakdown. The selection pressure for these mutations would be strongest at the start of the releases and would ease as the drive allele nears fixation. To account for this, releases should target small populations that are highly confined (limited migration from neighbouring populations). As the *cif*-based gene drives are inherently confinable, this system of targeted release is not seen as a disadvantage as they would be aimed to target select populations in disease hot spot areas. Another factor that could limit the breakdown of the drive before it reaches fixation is to add extra toxin domains to *cifB*. Type V *cifA* genes retain a similar size to that of other Types, however Type V *cifB* genes are considerably larger than other *cifB* homologs and can contain multiple putative toxin domains[82] – which might suggest that additional synthetic toxin domains could potentially be attached to CifB and not interfere with the Cif binding dynamics.

The major benefit to a *cif*-based gene drive system is that there is an exhaustive range of cognate-specific TA *cif* pairs to be exploited which is advantageous for subsequent releases when a former drive (using one set) has lost functionality. Or alternatively, as multiplexing sgRNAs is used to overcome drive resistance in HGDs[152], *cif*-based drive alleles could potentially consist of multiple *cif* pair variants which would slow the rate of drive breakdown. Another key advantage to these gene drive designs is that because *Wolbachia*-mediated CI phenotypes have been documented in numerous insect species the Cifs are expected to function in most vector species independent of variable host factors. Furthermore, [82]although *Wolbachia*-mediated vector control has proven successful in the field, factors which affect *Wolbachia* density such as long periods of quiescence, high temperatures and larval crowding can impact the penetrance of CI, conditions which are not expected to impact the transgenic mediation of CI.

In addition to using Cifs to create gene drives for vector population replacement strategies, the *cif* genes could also be used to suppress populations. As testes-specific expression of *cifB* rendered transgenic males infertile, and females that mated with these males did not have a reduced mating refractory time, the release of these males could be used to reduce *Ae. aegypti* population sizes. To permit the mass rearing of infertile males, the germline-specific expression of *cifB* would be controlled in a tetracycline repressible system (Figure 5.3). In the absence of a tetracycline analogue, expression of *cifB* in both the female and male germlines would be expected to render both sexes infertile[93]. Although unfertile females would be

unable to transmit the transgene into the targeted population/ increase the population size, they would still be capable of transmitting disease.

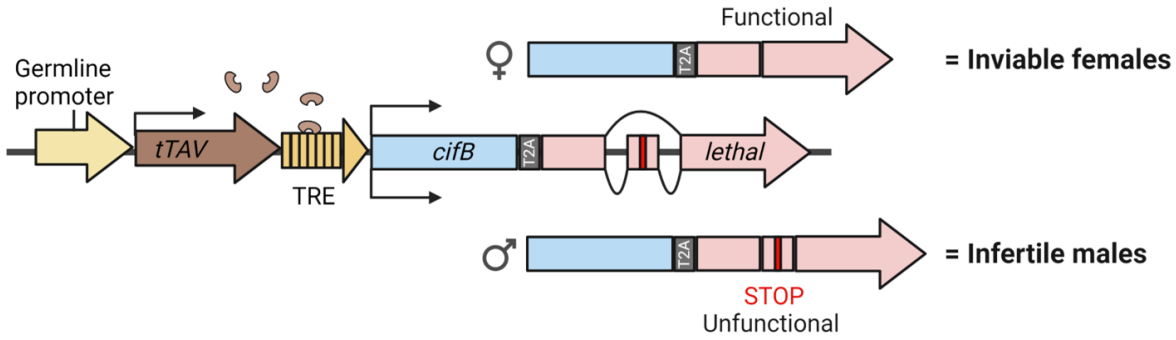
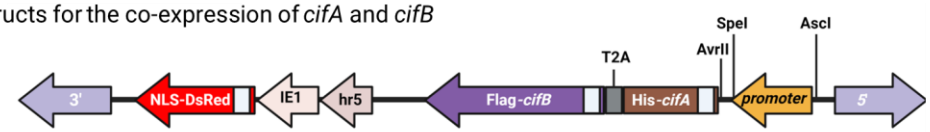


Figure 5.3: Schematic illustration of a construct for reducing vector populations through CifB-induced sterility. In the absence of tetracycline, tTAV expressed in germline cells activates expression of a toxin cassette consisting of *cifB* linked to a lethal allele via a T2A peptide sequence. The expression of *cifB* would sterilise both sexes. Sex-specific alternative splicing results in a truncated/non-functional lethal gene product in males, due to a premature stop codon (red) permitting the production of males. The functional lethal gene product in females would result in inviable females.

Therefore, a female-specific lethal allele could be linked to *cifB* expression which would facilitate the release of infertile males only (Figure 5.3). The fsRIDL has been generally approved as a tool for population suppression, however as it involves the transmission of transgenic elements into wild populations this might prevent its global acceptance. Although the *cif*-based suppression system would involve the release of genetically modified (GM) mosquitoes, the targeted populations would not inherit this genetic alteration which would address the publics concerns regarding GM releases. Together, the advantages of a *cif*-based vector control strategies for interrupting disease transmission mean that further study of transgenic *cif* gene expression in mosquito species is warranted.

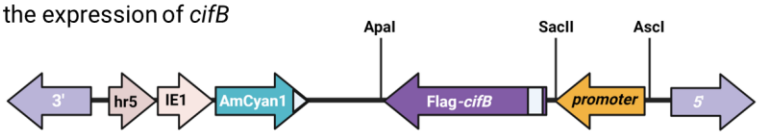
Appendices

A) Constructs for the co-expression of *cifA* and *cifB*



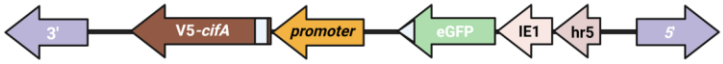
Donor plasmid	Cloning method	Backbone plasmid	Primer Name: sequence (5'-3')	Insert (plasmid)	Primer Name: sequence (5'-3')
*β2t**-*cifA*$_{wMel(TI)}$-T2A-*cifB*$_{wMel(TI)}$					
topi-*cifA*$_{wMel(TI)}$-T2A-*cifB*$_{wMel(TI)}$					
*β2t**-*cifA*$_{wMel(TI)}$-T2A-*cifB*$_{wMel(TI)}$	Restriction cloning (AscI & SpeI)	*topi*-*cifA*$_{wMel(TI)}$-T2A-*cifB*$_{wMel(TI)}$		*β2t** (*β2t**-DsRed2)	*β2t** AscI F: ATGATGGCGCGCCCGAAGATCATTCTTGGTTTTAGTGG *β2t** SpeI R: TCAGAGAACTAGTCATCCTGGAGCACTTCTAGC
prot-*cifA*$_{wMel(TI)}$-T2A-*cifB*$_{wMel(TI)}$	Restriction cloning (AscI & SpeI)	*topi*-*cifA*$_{wMel(TI)}$-T2A-*cifB*$_{wMel(TI)}$		prot (prot-DsRed2)	*prot* AscI F: ATGATGGCGCGCCCTAACAGGCGATCAGCAATAATTAGC *prot* SpeI R: TCAGCGAGACTAGTCCATCTTGATCCAATAAGTGTGTAAAGTGG
PUb-*cifA*$_{wMel(TI)}$-T2A-*cifB*$_{wMel(TI)}$	Restriction cloning (AscI & SpeI)	*topi*-*cifA*$_{wMel(TI)}$-T2A-*cifB*$_{wMel(TI)}$		*PUb* (PUb-AaHyPiggyBac)	*PUb* AscI F: GGCGCGCCATCTTTACATGTAGCTTGTGC *PUb* AvrII R: CTACCTAGGGTTGAAATCTCTGTTGAGCAG
*β2t**-*cifA*$_{wAlbB(TIV)}$-T2A-*cifB*$_{wAlbB(TIV}$	Hifi cloning	*β2t**-*cifA*$_{wMel(TI)}$-T2A-*cifB*$_{wMel(TI)}$	t2A-cifBalbB F: GAATCAGTAAGCGGCCTAAGATACATTG t2A-CifBalbB R: CCTTGTAATCTGGTCCTGGGTTTTCTTC	*cifB*$_{wAlbB(TIV)}$ (pUC-*cifB*$_{wAlbB(TIV)}$)	cifBalbB-t2A F: CCCAGGACCAGATTACAAGGATGATGATGATAAG CifBalbB-t2A R: CTTAGGCCGCTTACTGATTCACGGCTCG
	Hifi cloning	*β2t**-*cifA*$_{wAlbB(TIV)}$-T2A-*cifB*$_{wMel(TI)}$	t2A-cifAalbB F: ACACTCGAAGGGATCGGGAGAAGGACGTG t2A-CifAalbB R: GTCCCGATTCGTGGTGATGGTGATGATGC	*cifA*$_{wAlbB(TIV)}$ (pUC-*cifA*$_{wAlbB(TIV)}$)	cifAalbB-t2A F: CCATCACCACGAATCGGGACTGGATCACAAC CifAalbB-t2A R: CTCCCGATCCCTTCGAGTGTCCCAGCGAC

B) Constructs for the expression of *cifB*



Donor plasmid	Cloning method	Backbone plasmid	Primer Name: sequence (5'-3')	Insert (plasmid)	Primer Name: sequence (5'-3')
TRE-*cifB*$_{wMel(TI)}$	Restriction cloning (SacII & ApaI)	TRE-CHIKV		*cifB*$_{wMel(TI)}$ (*β2t-cifA*$_{wMel(TI)}$-T2A-*cifB*$_{wMel(TI)}$)	cifB SacII F: TACATATGCACCGCGGCCACCATGGATTACAAGGACGACGATGATAAGGATG cifB ApaI R: ACGATTCATAGGGCCCTTAACGCGATCCACGTCCGTTC
prot-*cifB*$_{wMel(TI)}$	Restriction cloning (AscI & SacII)	TRE-*cifB*$_{wMel(TI)}$		*prot* (*prot-cifA*$_{wMel(TI)}$-T2A-*cifB*$_{wMel(TI)}$)	
*β2t**-*cifB*$_{wMel(TI)}$	Restriction cloning (AscI & SacII)	TRE-*cifB*$_{wMel(TI)}$		*β2t** (*β2t**-*cifA*$_{wMel(TI)}$-T2A-*cifB*$_{wMel(TI)}$)	
TRE-*cifB*$_{wAlbB(TIV)}$	Restriction cloning (SacII & ApaI)	TRE-CHIKV		*cifB*$_{wAlbB(TIV)}$ (pUC-*cifB*$_{wAlbB(TIV)}$)	
*β2t**-*cifB*$_{wAlbB(TIV)}$	Restriction cloning (AscI & SacII)	TRE-*cifB*$_{wAlbB(TIV)}$		*β2t** (*β2t**-*cifA*$_{wMel(TI)}$-T2A-*cifB*$_{wMel(TI)}$)	
*β2t**-*cifB*$_{wPip(TI)}$	HiFi cloning	*β2t**-*cifB*$_{wMel(TI)}$	β2t-cifBpip F: ATCGCGATAAACGTCTTCGAAACTAGTG β2t-cifBpip R: CTCCGTTCGACTTATCATCGTCGTCCTTG	*cifB*$_{wPip(TI)}$ (pUC-*cifB*$_{wPip(TI)}$)	cifBpip-B2t F: CGATGATAAGTCGAACGGAGATGGACTG cifBpip-B2t R: TCGAAGACGTTTATCGCGATCCTCGCGAG

C) Constructs for the expression of *cifA*



Donor plasmid	Cloning method	Backbone plasmid	Primer Name: sequence (5'-3')	Insert (plasmid)	Primer Name: sequence (5'-3')
exu-cifA$_{wMel(TI)}$					
PUb-cifA$_{wMel(TI)}$	HiFi cloning	*exu-cifA*$_{wMel(TI)}$	cifA-PUb F: AGATTTCAACAATGGGAAAGCCGATCCC cifA-PUb R: GTAAAGATAGTTAATTAACTCGCGTTAAG ATACATTG	*PUb* (*PUb-AaHyPiggyBac*)	PUb-cifA F: AGTTAATTAACTATCTTTACATGTAGCTTGTG PUb-cifA R: CTTTCCCATTGTTGAAATCTCTGTTGAGC
PUbt-cifA$_{wMel(TI)}$	HiFi cloning	*exu-cifA*$_{wMel(TI)}$	cifA-PUb F: GAGATTTCCGATGGGAAAGCCGATCCCG cifA-PUb R: TTGGCGCGCCTTAATTAACTCGCGTTAAG ATACATTGATGAG	*Pubt* (*PUbt*-DsRed2)	PUb-cifA F: AGTTAATTAAGGCGCGCCAAATCAGGTAC PUbt-cifA R: GCTTTCCCATCGGAAATCTCTGTTGAGCAGAAAAAG
TRE-*cifA*$_{wMel(TI)}$	Restriction cloning (AscI & SacII)	*exu-cifA*$_{wMel(TI)}$	CifA SacII F: ATCCGCGGGGAAAGCCGATCCCGAACCC CifA AscI R: TTGGCGCGCCCTCGCGTTAAGATACATTG A	TRE (TRE-CHIKV)	
β2t-cifA*$_{wMel(TI)}$	HiFi cloning	*exu-cifA*$_{wMel(TI)}$	cifA-B2t F: GTGCTCCAGGAATGGGAAAGCCGATCCC cifAmel-B2t R: ACGATAAATTTTAATTAACTCGCGTTAAG ATACATTG	*β2t** (*β2t**-DsRed2)	B2t-cifAmel F: GAGTTAATTAAAATTTATCGTTAGATTTTTTGTTTAAGTAT TTCG B2t-cifA R: CTTTCCCATTCCTGGAGCACTTCTAGCG
β2t-cifA*$_{wAlbB(TIV)}$	HiFi cloning	*exu-cifA*$_{wAlbB(TIV)}$	cifA-B2t F: GTGCTCCAGGAATGGGAAAGCCGATCCC cifAalbB-B2t R: AACGATAAATTTAATTAACTCGCGTTAAG ATACATTG	*β2t** (*β2t**-DsRed2)	B2t-cifAalbB F: GAGTTAATTAAATTTATCGTTAGATTTTTTGTTTAAGTATT TCGAAG B2t-cifA R: CTTTCCCATTCCTGGAGCACTTCTAGCG
exu-cifA$_{wAlbB(TIV)}$	HiFi cloning	*exu-cifA*$_{wMel(TI)}$	exu-cifAalbB F: TCGAAGTAACCTAGAATGAATCGTTTTTA AAATAAC exu-cifAalbB R: GCTTTCCCATTTTTCACTCTGTAGACAAA AG	*cifA*$_{wAlbB(TIV)}$ (pUC-*cifA*$_{wAlbB(TIV)}$)	cifAalbB-exu F: AGAGTGAAAAATGGGAAAGCCGATCCCG cifAalbB-exu R: TTCATTCTAGGTTACTTCGAGTGTCCCAGC
exu-cifA$_{wPip(TIV)}$	HiFi cloning	*exu-cifA*$_{wMel(TI)}$	exu-cifApip F: AACAAGTAACCTAGAATGAATCGTTTTTA AAATAAC exu-cifApip R: GCTTTCCCATTTTCACTCTGTAGACAAAA G	*cifA*$_{wPip(TIV)}$ (pUC-*cifA*$_{wPip(TI)}$)	cifApip-exu F: CAGAGTGAAAATGGGAAAGCCGATCCCG cifApip-exu R: TTCATTCTAGGTTACTTGTTTCCCGACAGG

Figure A1: The *piggyBac donor* plasmids used in this thesis. Schematic representation of the constructs used to investigate the expression of both *cifA* and *cifB* (A), *cifB* alone (B), or *cifA* alone (C) - restriction sites utilised in cloning are indicated. Tables list each construct used for transgenesis and detail how they were generated, those shaded in grey were not micro-injected into *Ae. aegypti* embryos. The *β2t-cifA*$_{wMel(TI)}$-T2A-*cifB*$_{wMel(TI)}$, *topi-cifA*$_{wMel(TI)}$-T2A-*cifB*$_{wMel(TI)}$, *β2t**-DsRed2, *prot*-DsRed2, *PUb-AaHyPiggyBac*, and TRE-CHIKV plasmids were donated from collaborators in Prof. Luke Alphey's lab.

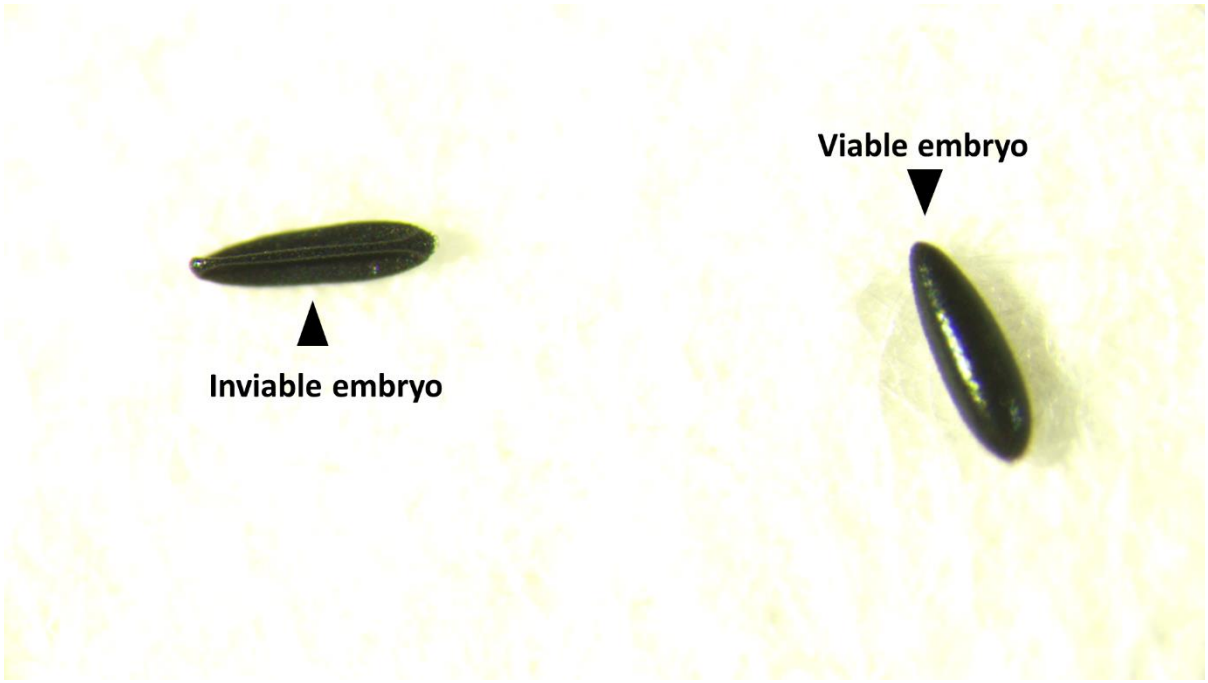


Figure A2: Morphological differences between viable and inviable *Ae. aegypti* embryos. Inviable embryos display egg collapse which signals an early arrest in development. Viable Embryos remain turgid indicating embryo development. In crosses involving males *β2t-cifA*$_{wPip(TI)}$ the resultant embryos can appear viable based on these morphological differences, however, do not hatch- which suggests a later stage of developmental arrest.

Table A1: Results from the microinjection of wild-type embryos with *piggyBac* constructs.

Construct	Embryos injected	survived to 4th instar	Transient G_0s (G_0s tested for germline insertion)	Independent genomic insertion lines generated
β2t-cifA$_{w\text{Mel(TI)}}$-*T2A-cifB*$_{w\text{Mel(TI)}}$	240	23	14 (14)	4
topi-cifA$_{w\text{Mel(TI)}}$-*T2A-cifB*$_{w\text{Mel(TI)}}$	~340	21	18 (18)	3
β2t-cifA*$_{w\text{Mel(TI)}}$-*T2A-cifB*$_{w\text{Mel(TI)}}$	~300	18	14 (14)	5
prot-cifA$_{w\text{Mel(TI)}}$-*T2A-cifB*$_{w\text{Mel(TI)}}$	~350	71	43 (14)	2
PUb-cifA$_{w\text{Mel(TI)}}$-*T2A-cifB*$_{w\text{Mel(TI)}}$	~400	22	15 (15)	5
prot-cifB$_{w\text{Mel(TI)}}$	~300	54	37 (17)	7
β2t-cifB*$_{w\text{Mel(TI)}}$	~400	70	61 (19)	9
β2t-cifA*$_{w\text{Mel(TI)}}$	~700	81	39 (21)	3
PUb-cifA$_{w\text{Mel(TI)}}$	~300	23	15 (12)	3
PUbt-cifA$_{w\text{Mel(TI)}}$	~300	38	17 (17)	3
exu-cifA$_{w\text{Mel(TI)}}$	~500	31	17 (17)	4
TRE-cifB$_{w\text{Mel(TI)}}$	~740	46	21 (17)	5
TRE-cifA$_{w\text{Mel(TI)}}$	~400	50	28 (22)	1
β2t-cifB*$_{w\text{AlbB(TIV)}}$	~600	239	134 (48)	3
β2t-cifA*$_{w\text{AlbB(TIV)}}$	~540	31	30 (30)	3
β2t-cifA*$_{w\text{AlbB(TIV)}}$-T2A-*cifB*$_{w\text{AlbB(TIV)}}$	~380	43	37 (37)	4
topi-cifA$_{w\text{AlbB(TIV)}}$-T2A-*cifB*$_{w\text{AlbB(TIV)}}$	~150	34	30 (18)	4
exu-cifA$_{w\text{AlbB(TIV)}}$	~300	72	44 (44)	3
β2t-cifB*$_{w\text{Pip(TI)}}$	~500	54	47 (38)	2
exu-cifA$_{w\text{Pip (TI)}}$	~320	75	63 (43)	6

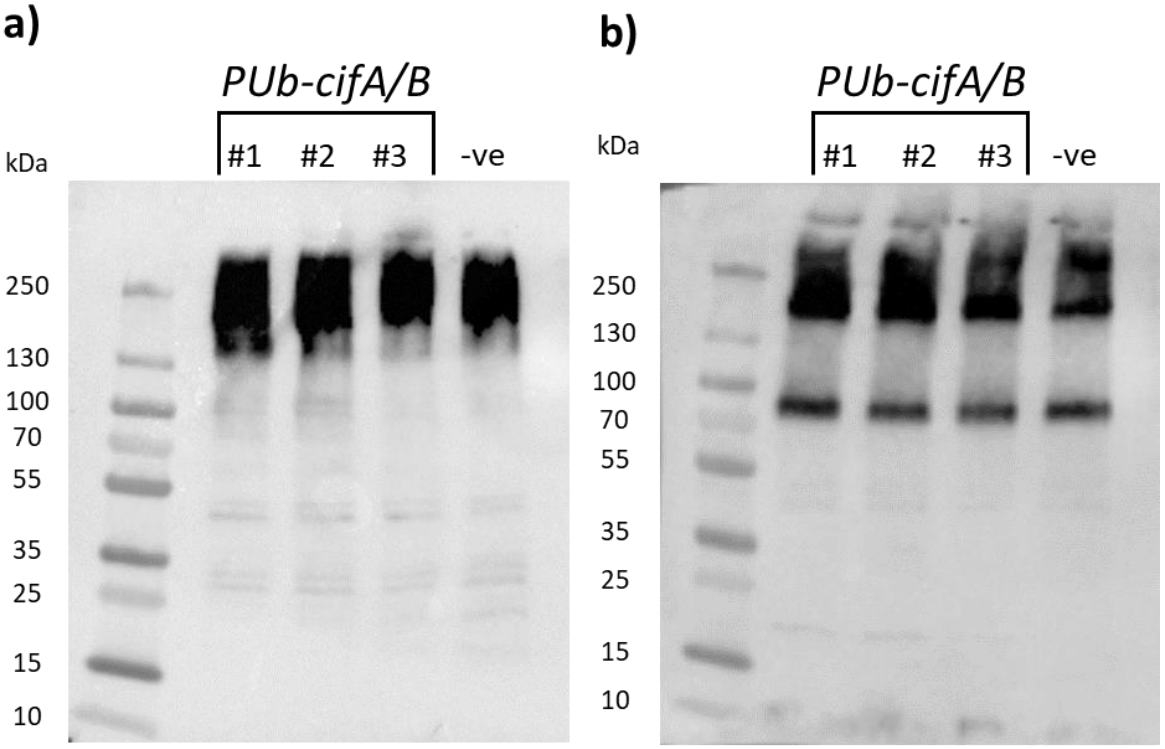


Figure A3: Western blots reveal unspecific binding of the anti-His and anti-Flag antibodies. Western blots were run using **a)** anti-Flag or **b)** anti-His antibodies on protein extracts from Aa23 cells transfected with either *PUb-cifA*$_{w\text{Mel(TI)}}$-*T2A-cifB*$_{w\text{Mel(TI)}}$ donor plasmid (3 replicates) or a mock transfection control (-ve). Expected size of Flag-*cifA*$_{w\text{Mel(TI)}}$ = 134 kDa, expected size of His-*cifA*$_{w\text{Mel(TI)}}$ = 54kDa.

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