Investigating the impact of insecticide exposure and resistance on the vector competence of *Anopheles gambiae* and *Aedes aegypti* for arboviruses

Thesis submitted in accordance with the requirements of the Liverpool School of Tropical Medicine for the degree of Doctor of Philosophy by

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Abstract

Investigating the impact of insecticide exposure and resistance on the vector competence of *Anopheles gambiae* and *Aedes aegypti* for arboviruses.

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Arboviruses spread by mosquitoes pose a major threat to global health. Despite their importance, there are limited options available for their control. There are no specific medical treatments and few safe and effective vaccines. Therefore, control focuses on the use of insecticides to target the mosquito vectors. Extensive insecticide use has resulted in widespread insecticide resistance in *Aedes* and *Anopheles* mosquito vectors of arboviruses. Insecticide resistance and exposure can have marked effects on the physiology of vectors, and there are concerns that it may be capable of altering the innate permissiveness of vectors to acquire and transmit arboviruses, known as vector competence. This thesis aimed firstly to investigate how insecticide selection pressure can alter the transcriptome of mosquitoes, including the expression of genes relating to metabolic insecticide resistance and innate immunity. Secondly, it investigated how insecticide resistance and exposure can interact with the vector competence of mosquitoes for arboviruses.

Chapter 2 investigated how a target site resistance mechanism, the L1014F knockdown resistance allele, may affect the vector competence of *Anopheles gambiae* for O'nyong nyong virus (ONNV) using a CRISPR/Cas9 gene-edited mosquito line. Data from oral infections and intrathoracic injections show that L1014F homozygosity is not associated with changes in the infection susceptibility for ONNV.

Chapter 3 investigated how different insecticide rotation scenarios influence the transcriptome of *Aedes aegypti*. Switching insecticides was associated with widespread transcriptomic changes across multiple biological domains, including the expression of genes relating to metabolic insecticide resistance and innate immunity pathways. Removal of temephos selection pressure was associated with a widespread downregulation of genes encoding antimicrobial peptides, and potential activators of the Toll immune pathway.

Chapter 4 investigated the effects of altering insecticide selection pressures on the vector competence of *Ae. aegypti* for Zika virus (ZIKV). Temephos selection was associated with slower dissemination and lower body viral titres following oral infection compared to an insecticide unselected strain. Data from intrathoracic injections potentially showed the presence of salivary barriers associated with temephos selection.

Chapter 5 investigated the effects of sublethal larval exposure to temephos on the vector competence of *Ae. aegypti* for ZIKV. Larval exposure to temephos was associated with higher salivary titres of ZIKV in a temephos susceptible mosquito strain. No other differences in vector competence were observed relating to temephos exposure.

The available evidence suggests that insecticide resistance and exposure can have impacts on the vector competence of mosquitoes for arboviruses, however, there is not a clear consensus on the direction of the effect. Changes to vector competence due to insecticide resistance and exposure have the potential to alter the vectorial capacity of mosquito populations, especially in areas where the probability of daily survival is high. As insecticide resistance permits vectors to survive contact with insecticides, small changes to vector competence could become increasingly important for arbovirus transmission.

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Before starting my PhD, I worked as a medical doctor in the NHS. I never truly felt like I was in the right place. I now know I am in the right place, doing the right thing, and am proud to call myself a scientist.

Acronyms

- **AMP** – Antimicrobial peptide
- **ArCL2** – Arthropod containment level 2
- **BL** – Basal lamina
- **Bti** – *Bacillus thuringiensis*
- **CCE** - Carboxylesterase
- **CHIKV** – Chikungunya virus
- **CPE** – Cytopathic effects
- **CYP** – Cytochrome P450 monooxygenase
- **DALY** – Disability-adjusted life year
- **DEFA** – Defensin-A
- **DEFC** – Defensin-C
- **DENV** – Dengue virus
- **DMEM** – Dulbecco's modified eagle media
- **DPI** – Days post-infection
- **EIP** – Extrinsic incubation period
- **FBS** – Foetal bovine serum
- **GO** – Gene ontology
- **GST** – Glutathione-s-transferase
- **IMD** - Immunodeficiency
- **IRS** – Indoor residual spraying
- **ISV** – Insect-specific virus
- **IT** – Intrathoracic
- **JAK/STAT** – Janus kinase signal transducer and activator of transcription
- **kdr** – knockdown resistance
- **KEGG** – Kyoto Encyclopaedia of Genes and Genomes
- **LC50** – Lethal concentration to kill 50%
- **LLIN** – Long-lasting insecticide treated bed net
- **MGEB** – Midgut exit barrier
- **MGIB** – Midgut infection barrier
- **ONNV** – O n'yong nyong virus
- **PCA** – Principal components analysis
- **PCR** – Polymerase chain reaction
- **Pfu** – plaque forming units
- **PO** – Per os
- **REC** – Recife strain of *Aedes aegypti*
- **ROS** – Reactive oxygen species
- **RNA** – Ribonucleic acid
- **RNAi** – Ribonucleic acid interference
- **SGEB –** Salivary gland exit barrier
- **SGIB** – Salivary gland infection barrier
- **SINV** – Sindbis virus
- **ULV** – Ultra low volume
- **VC** – Vectorial capacity
- **VGSC** – Voltage-gated sodium channel
- **YFV** – Yellow fever virus
- **ZIKV** – Zika virus

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Chapter 1 - Introduction

1.1 Mosquito-borne arboviruses

Arboviruses transmitted by mosquitoes pose a serious challenge to the health of humans and animals worldwide. Viruses from three main families, *Flaviviridae*, *Togaviridae*, and *Phenuviridae*, are responsible for the majority of human infections, and are spread by a wide range of mosquito vectors from the Anophelinae and Culicinae subfamilies [1-12] (**Table 1**). Dengue virus (DENV) cases number approximately 100-400 million per year, whilst chikungunya virus (CHIKV) and Zika virus (ZIKV) are thought to cause approximately 1 million cases per year [13-15]. Seroprevalence studies suggest that many cases go undiagnosed, so the true prevalence is likely to be much greater [16, 17]. These infections cause significant mortality and morbidity. Globally, CHIKV and ZIKV alone are thought to result in the loss of more than 150,000 disability-adjusted life years (DALYs) per year [18].

Symptoms of acute infection are generally similar with many arboviruses, and consist of headache, fever, joint and muscle pain, and a rash [2, 19]. However, there are several distinct sequelae of infection depending on the virus. Flaviviral infection can result in encephalitis, haemorrhagic symptoms including shock syndrome, and congenital diseases such as microcephaly [2]. Alphaviruses generally cause either an arthritic or encephalitic syndrome, both of which can have serious long term consequences [12, 20].

Despite the threat posed by arboviruses, there are limited options available for their treatment and prevention. Currently, there are no antivirals recommended for the treatment of arboviral diseases, and treatment relies solely on supportive therapies [21]. With the exception of yellow fever virus (YFV), for which there has been a highly effective vaccine for over 50 years, there are a lack of safe and effective vaccines [22]. The prospect of a widely implementable vaccine for DENV has been set back due to issues with disease enhancement following use of the Dengvaxia® vaccine in seronegative children [23]. The development of vaccines for CHIKV and ZIKV has also attracted research attention, but their epidemic nature may disincentivise large-scale investment by pharmaceutical companies [21].

1.2 Zika virus – an epidemic flavivirus

ZIKV is a single-stranded, positive-sense ribonucleic acid (RNA) flavivirus first identified in 1947 in Uganda [24, 25]. For the next 50 years less than 20 human infections were reported, most of them detected incidentally by YFV serosurveillance programmes [26]. It was not until 2007 that the first major ZIKV epidemic was recorded, occurring on Yap Island in the Federated States of Micronesia [27]. Following this, cases were seen more widely in the Pacific region, and by 2015, infections were reported in South America [28]. From January 2015 to February 2023, there had been >8.6 million confirmed cases of ZIKV in the Americas [29]. The majority of infections are asymptomatic or cause a mild, febrile illness with rash, arthralgia, myalgia and headache [30]. Rarely, neurological complications can occur including neuropathies, myelitis, and Guillain-Barré syndrome [31]. ZIKV infection during pregnancy is

associated with an increased risk of congenital complications including microcephaly, known collectively as congenital Zika syndrome [32, 33]. Transmission is primarily spread through the bite of mosquitoes, but it can also occur horizontally between people through sexual contact, blood products, and transplacentally from mother to foetus [30]. Viral RNA has been detected in numerous vectors, including species of *Anopheles* and *Culex* [3]. However, this does not necessarily imply they are capable of transmission, and the primary vectors are considered to be *Aedes aegypti* and *Ae. albopictus* [3].

1.3 O'nyong nyong virus – a neglected alphavirus

O'nyong nyong virus (ONNV) is an alphavirus from the family *Togaviridae*, first identified in Uganda in 1959 [34]. It is a member of the Semliki Forest virus complex, and is closely related to other notable alphaviruses including CHIKV [35]. It was responsible for a large outbreak in East Africa in 1959-1962 in which more than 2 million cases were observed [34]. Since then, a number of outbreaks have been reported across sub-Saharan Africa, but as diagnosis and surveillance are generally limited, the true extent of these epidemics remains unclear [34, 36]. The symptoms of infection are similar to CHIKV with rash, fever, headache, and joint pain, being commonly reported [37]. Peculiarly for arboviruses, ONNV is transmitted primarily by anophelines, with *Anopheles gambiae* and *An. funestus* likely to be the main vectors [35]. Laboratory infection data suggest that *Ae. aegypti* may also be a competent vector, however the contribution of this vector to the natural transmission cycle is unknown [38].

Table 1 – Medically important arboviruses and their mosquito vectors
Data collated from a number of sources [1-12]

Family	Genus	Species	Abbreviation	Main Vectors
Flaviviridae	*Flavivirus*	Dengue virus	DENV	*Aedes aegypti* *Ae. albopictus* Sylvatic *Aedes* spp.
		Zika virus	ZIKV	*Ae. aegypti* *Ae. albopictus* Sylvatic *Aedes* spp.
		Yellow fever virus	YFV	*Ae. aegypti* *Ae. albopictus* *Haemagogus* spp. *Sabethes* spp.
		West Nile virus	WNV	*Culex pipiens* *Cx. quinquefasciatus* *Cx. tarsalis* *Cx. modestus*
		Japanese encephalitis virus	JEV	*Cx. tritaeniorhynchus* *Cx. vishnui* *Cx. gelidus*
		Usutu virus	USUV	*Cx. pipiens* *Cx. quinquefasciatus* *Cx. modestus* *Ae. albopictus*
Togaviridae	*Alphavirus*	O'nyong nyong virus	ONNV	*Anopheles gambiae* s.l *An. funestus*
		Mayaro virus	MAYV	*Haemagogus* spp. *Ae. aegypti* *Ae. albopictus* *Anopheles* spp.
		Western equine encephalomyelitis virus	WEEV	*Cx. tarsalis* *Culiseta* spp.
		Eastern equine encephalomyelitis virus	EEEV	*Culiseta melanura*
		Venezuelan equine encephalomyelitis	VEEV	*Culex* spp.
Phenuviridae	*Phlebovirus*	Rift valley fever virus	RVFV	*Aedes* spp. *Culex* spp.

1.4 Vector control

Targeting the mosquito vectors of arboviruses remains the most effective method of controlling arboviral diseases. Insecticides constitute the mainstay of control efforts, despite recent advances in *Wolbachia* and gene drive based control methods [39-41]. Neurotoxic insecticides from three classes, the pyrethroids, organophosphates, and carbamates, are commonly used for vector control [42, 43]. Pyrethroids exert a neurotoxic effect through binding to voltage-gated sodium channels (VGSCs) which leads to prolonged channel opening, and aberrant conduction [44]. The neurotoxic activity of organophosphates and carbamates is via irreversible blocking of the acetylcholinesterase enzyme, leading to hyperexcitation via a synaptic accumulation of acetylcholine [45]. Other insecticides that are commonly used to target mosquito larvae are the insect growth inhibitors, and *Bacillus thuringiensis* (Bti). Insect growth inhibitors, such as pyriproxyfen, arrest the normal development of mosquito larvae, preventing normal emergence as adults [46]. Toxins produced by the *B. thuringiensis* bacterium have larvicidal activity through lysis of midgut epithelial cells [47].

For *Aedes* species, larviciding, insecticide space spraying, and indoor residual spraying (IRS) are the most commonly employed techniques [48]. Given the preference of *Ae. aegypti* for small water containers in the peri-domestic environment, larval sites can be identified and targeted with organophosphate insecticides, insect growth inhibitors, or Bti [49]. Larviciding can be effective at reducing the density of vectors in an area, especially if combined with other interventions targeting adults [50, 51]. Space spraying is performed in a variety of ways including fogging and ultra-low volume (ULV) spraying and are designed to target adult *Aedes* vectors [48, 52]. Though widely used, the ability of space-spraying to reduce vector populations is debated [48]. Endophilic *Aedes* vectors may be targeted through IRS, insecticide spraying of interior walls of buildings, however the evidence base for its effectiveness against *Aedes* vectors is weak [52].

Anopheles vectors of ONNV are rarely targeted specifically for arbovirus control. Because they may also be important vectors of *Plasmodium* species, control efforts for malaria are likely to reduce ONNV transmission [34]. The use of long-lasting insecticidal bed nets (LLINs) and IRS are commonly used against malaria vectors and are considered to be highly effective at reducing the incidence of disease [53].

1.5 Insecticide resistance

Resistance to insecticides is now widespread in vectors of arboviruses [54-58]. This has worrying implications for interventions which may no longer be able to maintain operational control of vector populations [59]. Insecticide resistance may increasingly influence the epidemiology of arboviral diseases, as evidenced by high resistance to pyrethroids and organophosphate insecticides in areas of high DENV transmission in Malaysia [60]. Insecticide resistance in mosquitoes is achieved via two main mechanisms: target site, and metabolic [61].

1.5.1 Target site insecticide resistance mechanisms

Target site insecticide resistance mechanisms refer to alterations to the binding site of neurotoxic insecticides. Mutations to the VGSC binding site, often referred to as knockdown resistance mutations (kdr), inhibit binding of pyrethroids and reduce the insecticidal effects. A growing number of non-synonymous point mutations affecting the VGSC have been reported in *Aedes* and *Anopheles* species [56, 62, 63]. Target site mutations to the organophosphate and carbamate binding site of the acetylcholinesterase enzyme, due to a G119S substitution, are common in *Anopheles* species [64-66]. This has been reported in *Aedes* [67], but is less likely to occur because, unlike in *Anopheles*, it requires more than one mutation to result in an amino acid substitution [56, 68].

1.5.2 Metabolic insecticide resistance mechanisms

Metabolic insecticide resistance occurs due to increased activity of insecticide detoxification enzymes. Enzymes from the cytochrome p450 monooxygenase (CYP), glutathione s-transferase (GST), and carboxylesterase (CCE) families have been associated with insecticide resistance in *Aedes* and *Anopheles* vectors [56, 57]. Increased gene expression, binding affinity, and activity of these enzymes can rapidly detoxify insecticides and allow mosquitoes to survive previously lethal doses [69]. Cytochrome P450s, particularly those from subfamilies CYP6 and CYP9, have been consistently associated with resistance to pyrethroids in *Aedes* and *Anopheles* [56, 57]. In addition, two epsilon-class GSTs (GSTE2 and GSTE7) have been implicated in deltamethrin resistance in *Ae. aegypti*, and therefore may be important in conferring resistance to pyrethroids [70]. Organophosphate resistance appears to result from a diverse and variable range of metabolic mechanisms, with enzymes from the CYP, CCE and GST classes likely to be involved in *Aedes* [56, 71]. In particular, the carboxylesterases CCEae3A and CCEae6A are commonly seen in temephos resistant populations [53].

1.6 Vectorial capacity

Given the critical role vectors play in the transmission cycles of arboviruses, it is essential that the efficiency of populations of mosquitoes to spread viruses can be determined and quantified. This allows comparisons between vector populations, can be used to highlight important vector species, permits monitoring of the effects of vector control interventions, and aids with assessing the impact of insecticide resistance. In addition, as vectors and arboviruses are increasingly spreading into new geographic ranges, it is important that the transmission risk posed in the event of novel virus-vector pairings is proactively assessed.

Vectorial capacity (VC) is a concept that summarises a number of key factors affecting pathogen transmission by mosquitoes and provides an estimate of the importance of a mosquito population as vectors [72, 73]. It aims to capture a number of human, vector, and pathogen factors to provide an estimate of the ability of a vector to spread a pathogen [74]. VC can be defined as the number of infectious bites arising in a vector population given the presence of one infected human host [73]. It was originally

formulated as a means of evaluating malaria vector control programmes [75], but has since been adapted for other pathogens, including arboviruses [74, 76-78]. The classical equation is shown in **Figure 1**. Vector density (*m*) is given as the ratio of mosquitoes to humans in a particular area. The mosquito biting rate (*a*) is an estimate of the number of bites on a human per day, which is derived from the proportion of bloodmeals which are on humans (human blood index) and the time period between blood meals (provided by the length of the gonotrophic cycle) [79]. The equation is highly sensitive to changes in the probability of daily survival of adult mosquitoes (*p*), as survival beyond the length of a parasite's extrinsic incubation period (*n*) is a prerequisite for transmission. Vector competence (*b*), which will be discussed in detail below, is given as the proportion of vectors capable of infecting a new human host during blood-feeding out of the total that fed on an infectious human host [73, 76, 77]. For arboviruses, this is usually estimated from the presence of virus in the saliva or salivary glands of mosquitoes.

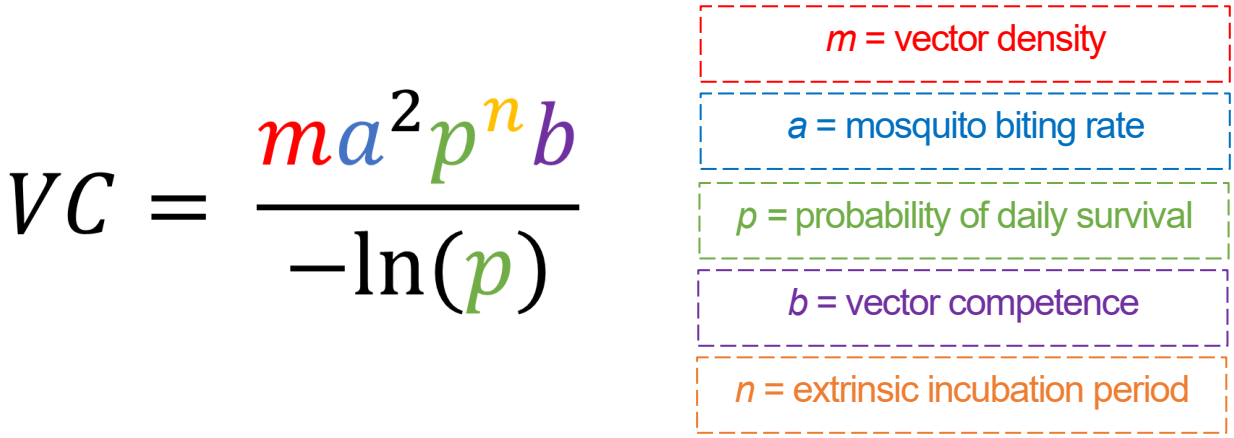


Figure 1 – The vectorial capacity equation
The vectorial capacity equation estimates the number of potentially infectious bites arising from all the vectors in an area given the presence of a single infected human host. Vector density (m) is the ratio of mosquitoes to humans in an area. Mosquito biting rate (a) is estimated from the length of the mosquito gonotrophic cycle and the human blood index. The probability of daily survival (p) estimates the likelihood of a mosquito surviving through one day. Vector competence (b) is simply the proportion of mosquitoes developing salivary infection out of the total feeding on an infectious human host. The extrinsic incubation period (n) is the length of time between ingestion of a virus by a mosquito, and development of salivary titres that are sufficient to infect a human host.

1.7 Vector competence

Vector competence is an important component of the vectorial capacity calculation. For arboviruses, it represents the proportion of mosquitoes that feed on an infectious, viraemic host, that will consequently develop sufficient quantities of virus in their saliva to infect a human upon blood feeding [76]. This seemingly straightforward metric is in reality the summation of a complex, multifaceted relationship between 1) the vector, 2) the environment, 3) endosymbionts residing in the vector, and 4) the virus [80].

There are a wide range of factors which contribute to the observed vector competence of a mosquito. These can be categorised as intrinsic, genetic, and extrinsic factors [81].

1.7.1 Intrinsic factors

1.7.1.1 Tissue barriers to infection

Unlike extracellular parasites, arboviruses must infect and replicate within mosquito cells. In order for a population of arboviruses to exploit a mosquito host, they must successfully establish infection in the vector, replicate, and disseminate to the mosquito saliva where they can be transmitted to a new host (**Figure 2**) [82]. This process is highly complex and involves successful infection and replication in a range of diverse tissue types, and involves overcoming a number of tissue barriers to infection.

1.7.1.1.1 Midgut barriers

Blood containing viruses is deposited in the midgut lumen of a mosquito during blood feeding on a viraemic host. The initial site of viral entry is at the epithelial surface of microvilli within the mosquito midgut, where viruses enter cells via poorly understood mechanisms [82]. Arboviruses face a number of obstacles to successful infection of midgut epithelial cells, which are collectively termed the midgut infection barrier (MGIB) (**Figure 2**). The presence of digestive enzymes and midgut microbiota may present a hostile environment for successful viral entry into cells [83]. Furthermore, viruses must exit the midgut lumen prior to the formation of the peritrophic matrix, a chitinous membrane which encapsulates the blood meal within 4-12 hours after feeding [82]. Given these obstacles, only a small number of midgut epithelial cells may be initially infected [82]. Smith et al [84] showed that an average of 28 *Aedes taeniorhyncus* midgut cells were successfully infected with Venezuelan equine encephalitis virus (VEEV). This has also been reported for flaviviruses, with ≤15 midgut cells initially becoming infected with replication-incompetent West Nile virus particles (WNV) in *Culex quinquefasciatus* [85]. It has been shown that there is a significant bottleneck in viral genetic diversity following midgut infection, which is thought to reflect the small number of successfully infected cells [86]. It is likely that the number of infected midgut cells and the degree of bottleneck is at least partially a function of the viral titre of the ingested blood meal [87].

Following cell entry, viruses undergo replication at the membrane of the endoplasmic reticulum, prior to beginning the process of dissemination to other tissues [82]. The midgut escape barrier (MGEB) constitutes the second major obstacle, and limits viral exit from the midgut epithelium. Midgut epithelial cells are attached by their basolateral membranes to a basal lamina (BL) which, via poorly understood mechanisms, viruses must pass through to disseminate beyond the midgut. It is thought that remodelling of the BL following blood feeding, and/or microperforations sustained as the midgut distends to accommodate the blood meal, may provide routes for viral escape [87, 88]. This is supported by the observation that *Ae. aegypti* taking a secondary, uninfected blood meal 3-days after infection with ZIKV virus, had significantly higher dissemination of virus than controls [89]. Despite this, the MGEB constitutes

a second severe bottleneck for the viral genetic diversity of flaviviruses and alphaviruses [90-93]. Once virions have successfully traversed the BL and entered the haemocoele, secondary replication occurs in a variety of tissues including the fat body, haemocytes, neurons, and muscle cells [83].

1.7.1.1.1 Salivary gland barriers

Infection of the acinar cells of the salivary gland is required for secretion of virus into the saliva of mosquitoes. The salivary gland infection barrier (SGIB) reflects the obstacles to successful infection of the salivary glands. The presence of a SGIB and has been demonstrated in a number of vector-virus models where mosquitoes with established midgut infection did not develop infected salivary glands [94-96]. The salivary glands are surrounded by a BL which has been suggested to contribute to the SGIB [97], but the exact mechanisms of the SGIB have not been fully determined [82]. The presence of a salivary gland escape barrier (SGEB), where salivary glands but not saliva is infected, has previously been reported in a number of vector-virus pairings [98-100]. The mechanisms underpinning these observations remain unclear. However, these barriers impose a bottleneck on viral diversity at the salivary glands, but the severity is lower than that observed at the midgut [86, 87, 92].

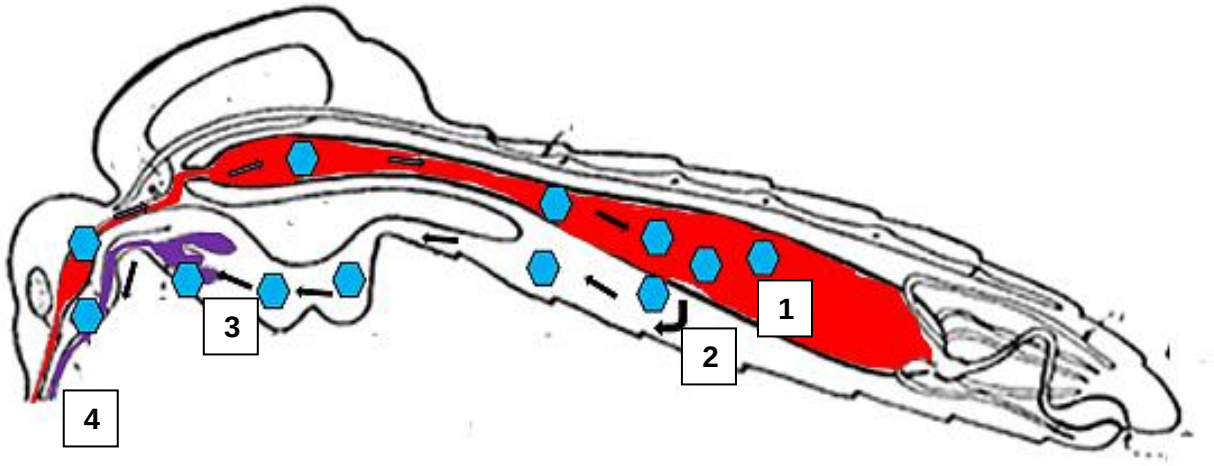


Figure 2 – Viral replicative cycle in a competent mosquito vector
Following ingestion of a blood meal, viruses must successfully infect midgut epithelial cells. There are numerous barriers to initial infection, collectively termed the midgut infection barrier (1). Following successful infection of midgut cells, replication occurs. Viruses must than transcend the basal lamina and enter the haemocoele, known as the midgut escape barrier (2). Viruses then infect a number of secondary tissues including haemocytes, muscle, and neurons. For transmission to occur, viruses must overcome salivary gland infection barriers (3) and establish infection in the cells of the salivary gland. Viruses must then overcome salivary gland exit barriers (4) to be secreted into the saliva. Figure adapted from Franz et al [82]. Reproduced under Creative Commons Attribution (CCBY) License (http://creativecommons.org/licenses/by/4.0/)

1.7.1.2 Innate antiviral immune pathways

Mosquitoes are not passive vehicles for arboviruses and mount a robust innate immune response to infection. There are a number of innate immune responses to viral infection in mosquitoes [101].

1.7.1.2.1 Haemocytes

Haemocytes are mosquito blood cells that play an important role in the cellular immune response to arboviruses [101]. The antiviral roles of haemocytes are pathogen recognition, phagocytosis, and antimicrobial peptide (AMP) production [101]. However, a number of arboviruses exhibit tissue tropisms for haemocytes, which form an important replicative site in the haemocoele [102-104]. A subclass of haemocytes, the granulocytes, have been shown to be important in limiting the dissemination of DENV and ZIKV beyond the midgut in *Aedes aegypti* [105]. The less abundant oenocytoid granulocytes, elicit the phenoloxidase (PO) cascade in response to infection, which has been shown to be an important component of the antiviral response to Semliki Forest virus in *Ae. albopictus* [106].

1.7.1.2.2 Lipid droplets

Lipid droplets are increasingly thought to be an important component of the cellular antiviral immune response in mosquitoes [107-109]. Activation of immune signalling pathways by DENV and Sindbis virus (SINV) has been shown to lead to increases in lipid droplets in the mosquito midgut, presumably to inhibit infection [107]. As lipids are also essential components for viral replication, it still remains unclear whether this is a manipulation by an infecting virus, or a host-mediated immune response [110]. Further research is required to determine the extent of the role lipid droplets play in the cellular immune response of mosquitoes to viruses.

1.7.1.2.3 RNA interference pathway

The RNA interference (RNAi) pathways are a group of highly conserved humoral immune responses in insects [111]. They are triggered in response to the presence of intracellular double-stranded RNA (dsRNA), which leads to the production of small RNAs that inhibit viral replication [112]. Different small RNAs are produced by each RNAi pathway including small interfering RNAs (siRNAs), Piwi-interacting RNAs (piRNAs), and microRNAs (miRNAs). Whilst piRNAs and miRNAs are involved in the antiviral immune response of mosquitoes, the siRNA pathway is considered the primary antiviral immune response [101, 112]. During the replication of arboviruses with RNA genomes, dsRNA is generated as intermediates of replication [113]. The dsRNA is cleaved into siRNAs of 21-33 nucleotides in length by the Dicer2 (Dcr2) RNAse enzyme and its associated r2d2 protein (**Figure 3**) [101, 114]. The RNA induced silencing complex (RISC) binds these siRNAs and degrades complementary viral RNA using the host endonuclease enzyme, Argonaute-2 (Ago-2) [101, 111].

The importance of this antiviral pathway has been well established. Silencing of Dcr2 in *Ae. aegypti* was associated with a reduction in the extrinsic incubation period and a marked increase in DENV titres, indicating the important role of this pathway in controlling infection [115]. Its importance was also

demonstrated in *An. gambiae*, where silencing of Ago-2 led to 16-fold increases in the salivary titres of ONNV [116]. The RNAi pathways act to limit viral replication systemically, but also act locally at important sites of virus infection and replication [117]. Knockdown of Ago2 and Dcr2 increases the replication of alphaviruses and flaviviruses in the midguts of *Ae. aegypti* [118-120]. It is therefore possible that RNAi contributes to the MGIB [121].

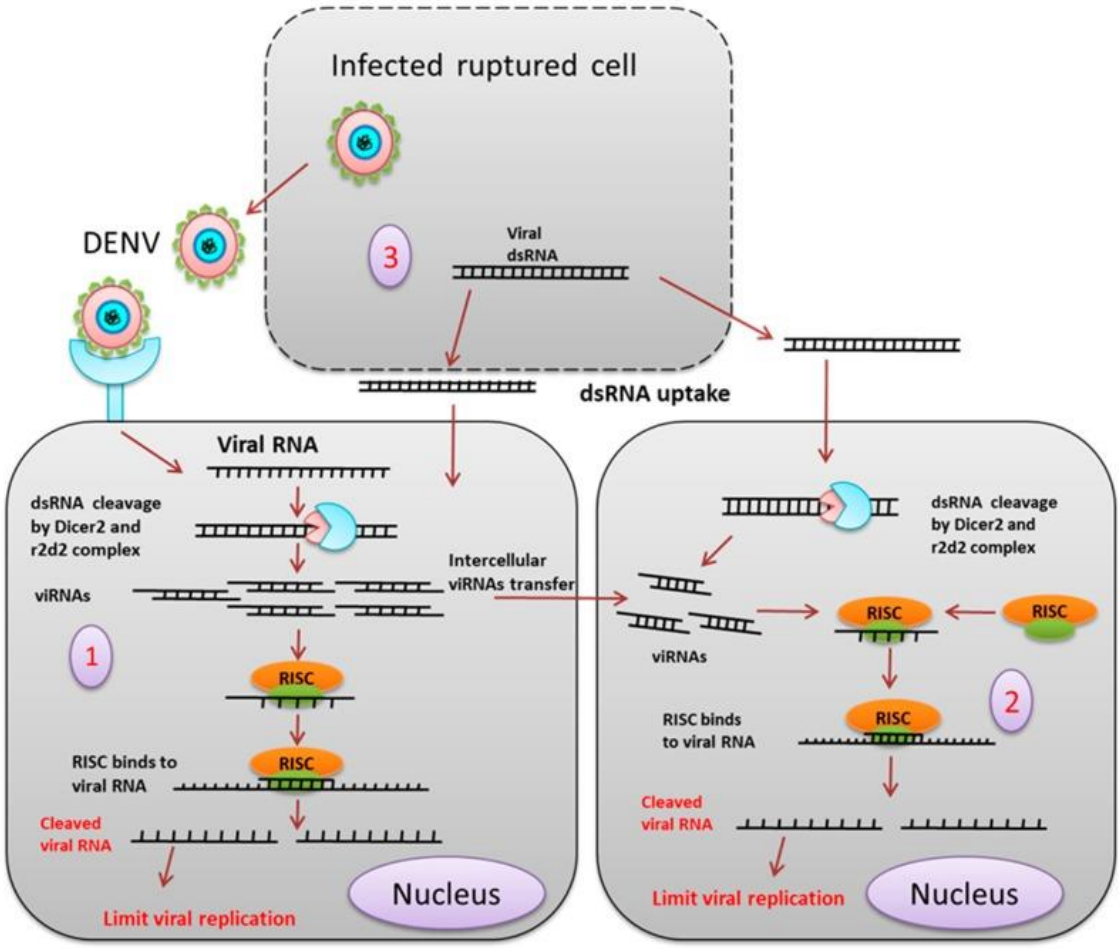


Figure 3 – RNA interference pathway
(1) Virus-derived dsRNA is bound by the Dcr2-r2d2 complex and cleaved into siRNAs. These are bound by the RNA induced silencing complex (RISC) which binds complementary viral RNA sequences and degrades them. (2) siRNAs can be passed to neighbouring cells (3) rupturing of cells due to viral replication releases dsRNA which can be assimilated by other cells. Figure from Mukherjee et al [114], reproduced under Creative Commons Attribution (CCBY) License (http://creativecommons.org/licenses/by/4.0/)

1.7.1.2.4 The JAK/STAT pathway

The JAK/STAT pathway is a signal transduction pathway that activates the transcription of antimicrobial effector molecule genes [114, 122]. The pathway is activated through ligand binding of the monomeric Dome pathway receptor, which leads to its activation and dimerization (**Figure 4**) [123]. This causes phosphorylation of the associated enzyme Hopscotch (Hop), which in turn phosphorylates the Dome

receptor [122]. Inactive STAT monomers in the cytoplasm are activated through binding to the phosphorylated Dome receptor, which leads to their dimerization [123]. After being transported to the nucleus, the STAT dimers bind to promoters for the expression of target genes [122]. Like the RNAi pathway, it is considered to have an important role in limiting midgut infection and has been shown to be important in limiting the ONNV midgut titres in *An. gambiae* [117, 124]. Silencing of Dome and Hop led to increased titres in the midguts of *Ae. aegypti* [125].

1.7.1.2.5 The Toll pathway

The Toll pathway is an important antifungal and antibacterial immune pathway that also has antiviral activity [126, 127]. However, the mechanisms by which this pathway is activated in response to viral infection remain poorly understood [122, 123]. Activation of the Spätzle ligand causes binding and activation of the Toll receptor (**Figure 4**). The activated toll receptor leads to the phosphorylation through binding to two adapter proteins (MyD88 and Tube), and the Pelle kinase [101]. This leads to degradation of the negative regulator Cactus which is bound to the transcription factor Rel1 [127]. Proteasomal degradation of Cactus allows Rel1 to travel to the nucleus and promote the transcription of antimicrobial peptide genes [117, 122]. The Toll pathway is known to be important in regulating DENV and ZIKV infection in *Ae. aegypti* [126, 128]. Several flaviviruses and alphaviruses exhibit inhibitory effects on the Toll pathway, suggesting it exerts antiviral effects [124]. However, its role in alphavirus infections remains to be fully elucidated, as Toll-mediated antiviral activity in systemic ONNV infection has been reported in *An. gambiae* [124], but stimulation of the Toll pathway did not limit CHIKV replication in an *Ae. aegypti* cell line (Aag2) [129].

1.7.1.2.6 The IMD pathway

The IMD pathway is crucial in antibacterial and anti-*Plasmodium* immune responses, but is also considered to have a role in antiviral immunity [122, 130]. Viral activation of the IMD pathway occurs via binding an unknown receptor mechanism that leads to activation of the IMD molecule (**Figure 4**) [114, 123]. The transcription factor Relish (Rel2) is activated via two separate signalling pathways [127]. Activated Rel2 translocates to the nucleus where it promotes the transcription of IMD associated AMPs, diptericin and cecropin [114]. The signalling molecule Vago is also produced, which exhibits antiviral properties through activation of the JAK/STAT pathway [131]. Some studies have suggested that this pathway is important in the antiviral immune response, with silencing of components associated with increases in viral titre [132], and DENV infection associated with induction of IMD components in *Ae. aegypti* salivary glands [133]. However, other studies have observed no changes in viral titres following

IMD knockout [134]. Further research is required to fully elucidate the role of the IMD pathway in antiviral immunity to arboviruses in mosquitoes.

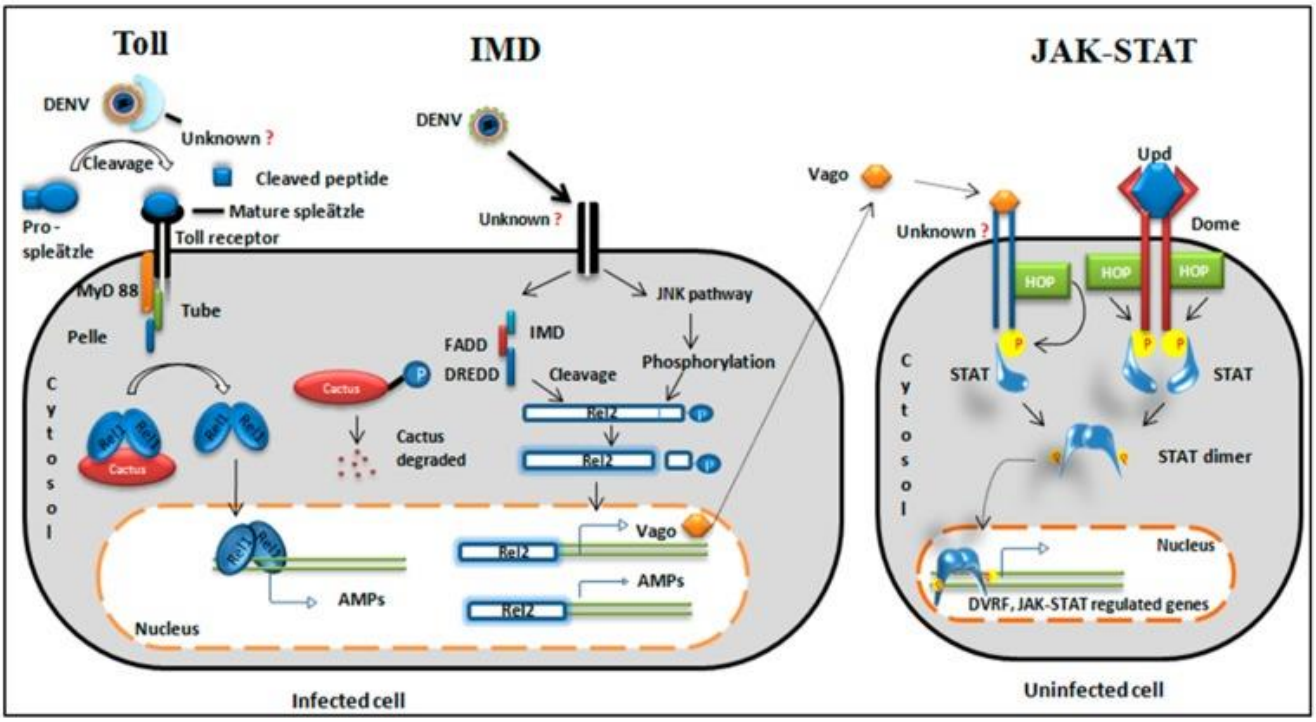


Figure 4 – The Toll, IMD and JAK/STAT innate immunity pathways
There are three main signal transduction pathways in mosquitoes that are involved in the antiviral immune response: the Toll pathway, the IMD pathway, and the JAK-STAT pathway. Figure from Mukherjee et al [114], reproduced under Creative Commons Attribution (CCBY) License (http://creativecommons.org/licenses/by/4.0/)

1.7.1.2.7 Antimicrobial peptides

Antimicrobial peptides are the effector molecules of the innate immune response, and are produced following activation of innate immunity pathways [114]. They are produced by the fat body, epithelial cells, and haemocytes, and are present in the haemolymph and various organs [114]. They are widely induced following arbovirus infection in mosquitoes, suggesting an important role in antiviral immunity, however their mechanisms of action are poorly elucidated. Defensins are the main family of AMPs present in *Aedes* species, and are produced in response to a wide range of pathogens including viruses [135]. Defensin-A (DEFA) and defensin-C (DEFC) have been shown to be upregulated to different levels in *Ae. aegypti* depending on whether they are infected with CHIKV or ZIKV [136]. Furthermore, DENV has been shown to inhibit the activity of defensins, suggesting they have important antiviral activities [137]. There is also evidence that defensins may be exploited by Japanese encephalitis virus (JEV) to facilitate access into mosquito cells [138]. Cecropins are another important AMP produced in response to viral infection. They have been shown to have antiviral activity against DENV and CHIKV [139]. Furthermore, induction of cecropins in the salivary glands of *Ae. aegypti* has been reported in response to DENV infection [133]. Other AMPs involved in the antiviral response are attacin and gambicin [101].

1.7.1.3 Microbiome

Another intrinsic factor affecting vector competence is the microbiome, which comprises the bacterial, fungal, protozoal and viral endosymbionts residing within mosquitoes. To date, the bulk of research attention has been directed towards the bacterial microbiome. A diverse range of bacterial species inhabit the mosquito midgut, salivary glands, and other tissues [140]. These have complex relationships with vector competence, which remain to be fully elucidated. The normal constitution of the microbiome has been shown to be an important determinant of vector competence. *Ae. aegypti* strains with different DENV susceptibilities had marked differences in microbiome constitution [141]. However, there is no clear direction of effect. For example, removal of the microbiome was associated with increased DENV midgut titres in *Ae. aegypti* that were treated with antibiotics [126]. Conversely, reduction of the midgut flora in *An. gambiae* resulted in significantly reduced infection with ONNV [124].

Furthermore, introduction of different bacteria has been associated with alterations in vector competence. A bacterium from the *Chromobacterium* genus, found in the midguts of wild *Ae. aegypti*, was introduced via a sugar meal to a laboratory Rockefeller *Ae. aegypti* strain, and was associated with significant reductions in susceptibility to DENV infection [142]. Similar results have also been observed with bacteria from the *Paenibacillus* and *Proteus* genera [80, 140]. Conversely, *Serratia odorifera* has been associated with increased susceptibility for both CHIKV and DENV when reinfected into antibiotic-treated mosquitoes [143, 144].

Another important, but understudied component of the mosquito microbiome is the viriome, consisting of insect-specific viruses (ISVs). Though poorly understood, ISVs have been linked with alterations in the vector competence of mosquitoes for arboviruses [145]. A recent study reported that the presence of two mosquito-specific viruses, Humaita Tubiacanga virus (HTV) and Phasi charoen-like virus (PCLV), was associated with a marked increase in the likelihood of DENV infection in wild-caught *Ae. aegypti* [146]. The presence of a newly identified ISV called Wiesbaden virus (WBDV) was associated with a significant increase in viral titre for CHIKV, but not ZIKV in *Ae. koreicus* [147]. However, ISVs have also been associated with decreased vector competence, and even complete blocking of transmission, for a number of arboviruses [148-150].

The microbiome is thought to modulate vector competence via a number of different mechanisms. Endosymbiotic bacteria and viruses may modulate the immune systems of mosquitoes for arboviruses through activation of innate immune pathways [151]. The non-specific nature of the Toll, JAK/STAT and IMD pathways means that immune system activation in response to endosymbiotic bacteria and viruses would potentially have collateral effects on arboviruses [151]. The observation that aseptic *Ae. aegypti* had lower levels of AMP expression than those with a normal microbiome, supports the view that the microbiome may be important in determining the baseline level of immune response [126]. A further mechanism of interaction between the microbiome and vector competence relates to competition for

resources. Endosymbionts may directly compete for resources required by arboviruses for replication, such as lipids, thereby limiting viral spread [140].

1.7.2 Genetic factors

In addition to intrinsic factors, there are a number of vector and parasite genetic factors that contribute to vector competence. It is well established that mosquitoes from the same species may have markedly different vector competence for the same viral strain [152-154]. Likewise, a particular vector can have significant differences in vector competence for different viral strains of the same species [155, 156]. It is becoming increasingly clear that these complex genotype x genotype interactions are critical for determining vector competence. This was demonstrated in a study testing the vector competence of three isofemale lines for three different strains of DENV, where none of the vector lines were most permissive to all viruses, and none of the virus strains were most successful in all of the vector lines [157]. Quantitative trait loci mapping has been used to identify genetic loci associated with vector competence, and has demonstrated that the loci correlating with the vector competence phenotype can vary by the infecting viral strain [158].

1.7.3 Extrinsic factors

A range of environmental or extrinsic factors are also important for determining vector competence. Increased larval rearing densities have been shown to increase the proportion of disseminated DENV infections in *Ae. albopictus* [159]. Part of this effect may be due to increased competition for resources between larvae. The nutritional status of larvae has been reported to influence the competence of adult *Aedes aegypti* for ZIKV, with mosquitoes that were undernourished as larvae demonstrating higher viral dissemination [160]. However, this effect has not been consistently observed in all vector-virus pairings [161].

Carbohydrate meals taken by adult mosquitoes also appear to influence vector competence. Adult *Cx. pipiens* infected with West Nile virus (WNV) that were maintained on low sucrose meals were significantly more likely to have virus in saliva, than mosquitoes maintained on 10% sucrose [162]. Furthermore, ingestion of a sugar meal has been shown to lead to increased expression of antiviral immune genes in the midguts of *Ae. aegypti*, and this was associated with decreases in vector competence for arboviruses [163]

Vector competence is also influenced by both environmental temperature during larval development [164], and following ingestion of an infected blood meal [165, 166]. Moreover, there is evidence to suggest that the diurnal temperature range can be as important as the average daily temperature in determining vector competence [167]. It is likely that many of these extrinsic factors may interact, demonstrated by the fact that larval density has different impacts on competence depending on the larval rearing temperature [164].

1.7.4 Determining vector competence experimentally

1.7.4.1 Per os (PO) infection route

Vector competence is assessed through experimental infection of wild-caught or laboratory mosquito strains, and follows a broadly similar experimental design [168]. Mosquitoes from different treatment groups can be infected by providing a blood meal containing virus,. To capture different stages of the viral replication in mosquitoes, at pre-determined timepoints post-infection, mosquitoes are sampled to determine the spread and titre of virus within their tissues. This sampling usually involves dissection of the mosquito into different parts. To determine the presence of virus in saliva, samples can be collected using forced salivation techniques. Salivary glands are often collected rather than saliva, due to the labour intensive nature of forced salivation [169]. The gold standard technique for determining the presence of infectious virus in saliva is to allow mosquitoes to feed on susceptible animal hosts. However, due to logistical constraints, this is rarely performed [170].

The presence and titre of virus in these samples can be assessed using a number of virological and molecular methods. Molecular methods such as polymerase chain reaction (PCR) are high throughput, but as they measure viral RNA, are likely to overestimate the titre of infectious viruses. Virological approaches such as plaque assays and cytopathic effects assays, though lower throughput, have the advantage of only detecting infectious viruses [171].

The proportion of different samples that are positive for virus is used to determine a number of key metrices of vector competence. In this thesis, the following metrices are determined following infection via the PO route:

1) **Infection prevalence** – the proportion of blood fed mosquitoes that develop an infection in their midgut, which is estimated by the presence of virus in the body (thorax and abdomen)
2) **Dissemination prevalence in mosquito heads** – the proportion of mosquitoes with infection in their bodies that also have infection present in their heads
3) **Dissemination prevalence to mosquito legs** – the proportion of mosquitoes with infection in their bodies that also have infection present in their legs
4) **Transmission prevalence** - the proportion of mosquito with infection in their heads that also have virus present in their saliva

1.7.4.2 Intrathoracic inoculation

Mosquitoes may also be infected with viruses through intrathoracic injection of virus directly into the haemocoele [172]. This is clearly not a natural route of infection, as it bypasses the midgut stage, which is a critical determinant of vector competence. However, it can be used to estimate a number of factors which can contribute to the observed vector competence.

1) **Infection prevalence from IT injections (IPIT)**– the proportion of injected mosquitoes with virus present in different body parts of mosquitoes.
2) **Transmission prevalence from IT injections (TPIT)** – the proportion of injected mosquitoes with virus present in saliva. This is not equivalent to the transmission prevalence following infection via the PO route.

The IPIT reflects the ability of a virus to replicate in the tissues of a mosquito following direct inoculation into the haemocoele. The transmission prevalence from IT injections (TPIT) can help determine whether there are salivary gland infection and/or escape barriers present.

1.8 The impacts of insecticide resistance on vectorial capacity and vector competence

1.8.1 Target site insecticide resistance

Target site insecticide resistance mechanisms are thought to be capable of altering the vectorial capacity of mosquitoes [173, 174]. Mutations to voltage-gated sodium channel and acetylcholinesterase binding sites have been associated with differences in a number of life history traits including fertility, fecundity, development times and feeding behaviours in *Aedes*, *Anopheles*, and *Culex* [174-179]. The broad scope of the vectorial capacity input variables makes it sensitive to fitness costs associated with target site insecticide resistance. Effects on vector longevity have the potential to drastically alter vectorial capacity, as shortened lifespan may prevent completion of the EIP of a virus, and increased lifespan permits transmission over a longer period. However, the direction of the effect of *kdr* on longevity is unclear with some studies reporting increases [174, 175] or decreases [173, 176] in longevity. It is important to note however, that in the presence of insecticide, longevity is likely to be increased by a target-site mutation, compared to an insecticide susceptible counterpart.

In addition to effects on vectorial capacity, there is evidence to suggest target site mutations may alter the vector competence of mosquitoes for arboviruses. However, the direction of any effect remains unclear. The presence of *kdr* alleles (L1016 and C1534) in field caught *Ae. aegypti* was associated with decreased vector competence for DENV [180]. Similarly, isofemale lines from field caught mosquitoes with the F1543C *kdr* allele had lower viral dissemination rates than controls with wildtype VGSC [181]. Conversely, laboratory strains of *Ae. aegypti* with F1534C and V1016I mutations that had undergone permethrin selection, had higher dissemination of DENV [175]. However, it is not possible to determine that the effects seen in these studies were directly attributable to *kdr* genotype, as other genetic differences between vectors were not accounted for. Studies designed to better control for extraneous alleles that could influence vector competence have shown that vectors may be rendered more permissive to arboviral infection by target site insecticide resistance mechanisms. Introgression of V1016I and F1534C alleles into a susceptible strain of *Ae. aegypti* was associated with higher infection prevalence and dissemination of ZIKV [182]. Similar techniques were used to introduce the ace-1 G119S

allele into susceptible *Cx. pipiens*, and this was associated with increased dissemination of WNV beyond the midgut, and higher prevalence of salivary infection than wildtype controls [183].

It remains unclear why point mutations on mosquito VGSC or acetylcholinesterase enzymes would be capable of altering vector competence for arboviruses. One possibility relates to neurohormonal control of immune gene transcription, which could be altered by mutations to these target sites [184-186]. Another possibility is that other alleles linked to arboviral competence are in linkage disequilibrium with target site mutations, thereby the effects seen are not direct pleiotropic effects of the target site mutations in question [187-189]. Further investigation is required to determine whether the effects seen are directly related to target-site insecticide resistance mechanisms and determine how this could interact with vector competence.

1.8.2 Metabolic insecticide resistance

Metabolic insecticide resistance can result in profound changes in the physiology of vectors. As such, it has been associated with a number of fitness costs to longevity, energy stores, reproductive success, and host-seeking behaviour [190-194]. Given the multifactorial nature of vectorial capacity, there is clearly potential for it to be affected by metabolic insecticide resistance [58]. There is also evidence to suggest metabolic insecticide resistance may interact with vector competence, but the direction of any effect remains unclear. There was higher dissemination and transmission of WNV in *Cx. pipiens* that were homozygous for the esterase^2 SA2 locus [183]. Similarly, dissemination of DENV was greater in *Ae. aegypti* that had been selected with permethrin for target site and metabolic resistance mechanisms than susceptible controls [175]. Conversely, deltamethrin resistance in *Ae. aegypti* has been associated with decreased dissemination of CHIKV and DENV [181, 195]. More research is required to determine the nature of the interaction between metabolic insecticide resistance and vector competence, and elucidate the mechanisms for any interaction observed. However, there are a number of possible mechanisms.

The presence of a resource trade-off between insecticide detoxification and innate immune responses to arboviral infection, could explain alterations in vector competence [58]. Increased activity and overexpression of insecticide detoxification enzymes are metabolically costly for mosquitoes, in particular for reserves of lipids [191]. Lipids are required for many biological processes, but are particularly important in the innate antiviral immune response of mosquitoes [58]. As such, diversion of lipids for insecticide detoxification may impair the effectiveness of these immune responses and lead to increases in vector competence. However, the availability of lipids for phospholipid synthesis, the main component of cell membranes, is also crucial in the replicative cycle of flaviviruses [196]. This could provide a mechanism for decreased vector competence if prioritisation of resources for insecticide detoxification depletes those required for viral replication [58].

Another putative mechanism relates to interaction between insecticide detoxification and innate immunity pathways. Different classes of enzymes involved in insecticide detoxification have opposing actions on

the levels of reactive oxygen species (ROS), with cytochrome P450 enzymes increasing, and GSTs reducing ROS levels [58]. ROS can directly activate the innate immune responses via the Toll pathway, which could in turn lead to reductions in vector competence via increased AMP production [197]. Populations of *Anopheles* and *Culex* with metabolic resistance to insecticides have been shown to have higher constitutive expression of genes encoding antimicrobial peptides (AMPs) than susceptible controls [198, 199]. Furthermore, alteration of insecticide selection regimens has been associated with changes in immune gene expression in *Ae. aegypti* (see **Chapter 3**). In addition, there are notable disparities in immune gene transcription in response to Zika virus (ZIKV) infection between insecticide resistant and susceptible strains [200]. A third mechanism could be mediated by changes in the microbiome of insecticide resistant mosquitoes, which have been reported to have different microbiome compositions to susceptible controls [201-205]. The microbiome is an important modulator of the innate immune response, so such changes relating to insecticide resistance, may explain the differences seen in vector competence [80].

1.9 The impacts of insecticide exposure on vectorial capacity and vector competence

Insecticide-based vector control methods aim to result in rapid mortality of mosquitoes by delivering a lethal dose of insecticide. However, there are situations where mosquitoes may receive a sublethal dose of insecticide. Insecticide resistance can allow vectors may survive contact with a dose of insecticide that would be sufficient to rapidly kill susceptible mosquitoes. Avoidance behaviours can reduce the time a mosquito spends on an insecticide treated surface, reducing the dose they receive [206-208]. Furthermore, degradation of insecticides in the environment can result in reduced amounts of active ingredients on treated surfaces [209, 210]. Whilst insufficient to cause mortality, numerous effects of sublethal insecticide exposure on life history traits have been reported including behaviour, fecundity, longevity, and development [211-217]. These effects, especially impacts on vector behaviour, and longevity could clearly have an impact on the vectorial capacity of mosquitoes. In addition, there is evidence that sublethal insecticide exposure may also affect the vector competence of mosquitoes for arboviruses.

Some studies have shown that sublethal insecticide exposure may enhance viral infection and dissemination from the midgut. Bifenthrin-exposed *Ae. albopictus* had a higher prevalence of disseminated infection for ZIKV than unexposed [218], and malathion exposure was associated with higher prevalence of disseminated infection of Sindbis virus to the heads of *Ae. aegypti*, but interestingly not *Ae. albopictus* [219]. Sublethal exposure to the *Bacillus thuringiensis* (Bti)-based larvicide was associated with significantly higher infection and dissemination prevalence for ZIKV in *Ae. aegypti* [220]. This effect has also been noted for the infection and dissemination prevalence of certain strains of DENV, but there was no significant effect on chikungunya virus (CHIKV) competence [221]. Knecht et al [222]

reported that mosquito age appeared to interact with sublethal bifenthrin exposure in modulating competence of *Ae. albopictus* for Zika virus (ZIKV). Older mosquitoes that were exposed to bifenthrin had higher dissemination and viral titres than unexposed, but these effects were not seen in younger mosquitoes [222].

However, the evidence base is not unanimous. No differences were detected in infection prevalence for DENV between *Ae. albopictus* that had been fed bifenthrin in sugar meals, but body titres were significantly lower in exposed mosquitoes at 14 days post-infection (dpi). Furthermore, sublethal exposure of *Ae. aegypti* to malathion prior to infection with dengue virus (DENV) via blood feeding was not associated with significant differences in infection prevalence [223].

A variety of mechanisms have been suggested for an interaction between sublethal insecticide exposure and vector competence, ranging from detoxification pathways, pervasive midgut damage, immune modulation, and effects on the microbiome. Increased activity of CYP and GST enzymes in response to sublethal insecticide exposure can lead to changes in the levels of ROS [58]. Innate immunity pathways can be directly triggered by ROS, thereby raising the possibility that insecticide exposure could influence innate immune gene expression [197]. It is also possible that cellular immune responses may be affected by insecticide exposure. Sublethal organophosphate and organochlorine exposure of a non-vector Reduviid *Rhynocoris kumarii* caused profound changes in the total number and ratios of several haemocyte subclasses [224]. Haemocytes are known to be important components of the mosquito immune response to viruses, therefore such changes may be capable of altering vector competence.

The normal structure and function of the mosquito midgut often presents a significant barrier to infection and dissemination of viruses, therefore pervasive midgut damage caused by larval exposure to insecticides could potentially affect vector competence [82]. Sublethal exposure has been implicated in causing a number of structural midgut abnormalities in mayfly larvae (*Callibaetis radiatus*) exposed to deltamethrin [225], and *Ae. aegypti* larvae to either Spinosad [226] or imidacloprid [227]. This midgut damage transcended metamorphosis and persisted into the adult forms [226, 227].

Another unresearched area is the potential for sublethal insecticide exposure to interact with the microbiome of mosquitoes. Not only has permethrin selection been associated with alterations in the microbiome of *Ae. aegypti* [202], but experimental microbiome alterations have also been implicated in the insecticide resistance phenotype of *An. arabiensis* [228]. Furthermore, there is evidence to suggest that gut microbiota of insects may be directly involved in the degradation pathways of some insecticides [229]. It remains to be established what effects sublethal insecticide exposure may have on mosquito microbiomes, and how this may influence vector competence.

1.10 Rationale

Arboviruses spread by mosquitoes present a serious challenge to global health, causing significant morbidity, mortality, and economic cost globally [14, 18, 230, 231]. In many cases, their impact is increasing, both in the incidence of infections, and the geographic range of their spread [230, 232]. Despite their importance, control and treatment options remain very limited. There are no specific medical treatments, and there is a critical lack of safe and effective vaccines for the majority of arboviral diseases [233]. Whilst novel vector control techniques such as *Wolbachia* and gene drive technologies provide promise for the future, current control relies heavily on the use of insecticides [39, 234, 235].

Extensive insecticide use has contributed to the dramatic rise of insecticide resistance in mosquito vectors of arboviruses [55, 56, 236, 237]. This has been linked with profound physiological alterations resulting in fitness costs and changes to life history traits [58, 174, 188, 190-194]. The vector competence of mosquitoes is a complex summation of biological and environmental factors, therefore there is particular concern that insecticide resistance may be associated with alterations in the ability of a vector to acquire and transmit pathogens. Whilst there has been little research conducted to date, there is evidence that insecticide resistance can influence vector competence for arboviruses [181-183, 195]. In addition, contact with sublethal doses of insecticide may alter vector competence. Whilst insufficient to cause rapid mortality, sublethal insecticide exposure has been associated with a range of impacts on vector behaviour, development times, fecundity, and longevity [211-217]. There is also evidence to suggest that vector competence for arboviruses may be altered by sublethal exposure to insecticides, however this has not been thoroughly investigated [218, 219, 222].

Developing a better understanding of the interactions between insecticide resistance and exposure, and vector competence is important for planning control interventions. Regular rotation of insecticides is recommended as a means to maintain operational control in areas with resistant vector populations [238, 239]. This is likely to produce complex insecticide resistance profiles and exposure patterns that may have effects on vector competence. Arboviruses are increasingly spreading into new geographic areas and exploiting novel vectors. As such, understanding how insecticide resistance patterns in local vector populations may interact with novel and emerging arboviruses, should form an important part of planning for future outbreaks.

1.11 Aims

The aims of this thesis are to:

1. Investigate how insecticide selection pressures alter the transcriptome of mosquitoes, with a focus on metabolic insecticide resistance and innate immunity genes
2. Investigate how insecticide resistance and exposure may alter the vector competence of mosquitoes for arboviruses

Each of the following objectives will be addressed separately in the four experimental chapters of this thesis.

1.12 Objectives

1. Investigate the impacts of the L1014F knockdown resistance allele on the vector competence of *Anopheles gambiae* for O'nyong nyong virus
2. Determine the effects of switching insecticide selection pressures, compared to the maintenance of temephos selection, on the transcriptome of *Aedes aegypti* in each of the following scenarios:
    a. Switching to malathion
    b. Switching to permethrin
    c. Removal of insecticide
3. Determine the effects of these insecticide selection scenarios on the vector competence of *Aedes aegypti* for Zika virus
4. Investigate the impacts of sublethal temephos exposure on vector competence for Zika virus in two strains of *Aedes aegypti* with differing insecticide susceptibilities

Chapter 2 – Knockdown resistance allele L1014F introduced by CRISPR/Cas9 is not associated with altered infection susceptibility for o'nyong nyong virus in *Anopheles gambiae*

2.1 Abstract

Knockdown resistance (*kdr*) alleles conferring resistance to pyrethroid insecticides are widespread amongst vector populations. Previous research has suggested that these alleles are associated with changes in the vector competence of mosquitoes for arboviruses and *Plasmodium*, however non-target genetic differences between mosquito strains may have had a confounding effect. Here, to minimise genetic differences, the laboratory *Anopheles gambiae* colony Kisumu (Kis) was compared to a CRISPR/Cas9 homozygous kdr L1014F mutant Kisumu-kdr (Kis-kdr) line in order to examine associations with vector competence for o'nyong nyong virus (ONNV). Mosquitoes were infected using either blood feeds or intrathoracic microinjections. There were no significant differences in the prevalence of virus in mosquito bodies or saliva between *kdr* mutant and wildtype lines from either oral or intrathoracic injection routes. The ONNV titre was significantly higher in the legs of the wildtype Kis strain at 7dpi following intrathoracic microinjection, but no other significant differences in viral titre were detected. ONNV was not detected in the saliva of mosquitoes from either strain. Our findings suggest that the *kdr* L1014F allele is not associated with altered infection susceptibility for ONNV.

2.2 Introduction

Pyrethroid insecticides are widely used to target mosquito species that transmit human pathogens such as arboviruses and *Plasmodium*. They exert insecticidal effects through binding to receptor sites on voltage-gated sodium channels (VGSC) of mosquito neurones, resulting in prolonged channel opening, repeated nerve discharges and disrupted neurological functioning [44]. Mutations to the pyrethroid binding sites of the VGSC can prevent the binding of pyrethroids, which may result in reduced phenotypic sensitivity to pyrethroids known as knockdown resistance (kdr) [44]. To date, several amino acid substitutions have been associated with knockdown resistance in *Anopheles* species, including substitutions of leucine at codon position 1014 for phenylalanine (L1014F) or serine (L1014S) [44]. These mutations, and many others, are now commonly found across sub-Saharan Africa [240]. In addition to their ability to confer insecticide resistance, concerns have been raised that these mutations may influence the vector competence of mosquitoes for human pathogens. Vector competence describes the ability of a vector to acquire, incubate, and transmit a pathogen, and is a complex summation of many

interlinked determinants including vector and pathogen genetics, microbiome, and environmental factors [241-243].

Given that *kdr* are simple amino acid substitutions in the VGSC of mosquito neurones, it is not immediately apparent how this modest alteration could feasibly influence vector competence. Several putative mechanisms have been suggested, ranging from pleiotropic effects of *kdr*, to indirect effects relating to broader changes in physiology. It has been speculated that a mutated VGSC may be directly capable of altering the immune response of vectors to pathogens through modifying the neurohormonal control of immune function. For example, the gene expression of 20-hydroxyecdysone is under neurohormonal control and has been shown to increase immune gene transcription and alter *Plasmodium berghei* infection rate in *Anopheles gambiae* [244-246]. Less direct mechanisms have also been proposed. The presence of *kdr* has been linked to multiple fitness costs in mosquito vectors including development times, longevity, reproduction success, and feeding behaviours [174, 188, 247, 248]. Given that vector competence is a complex balance of multiple known and unknown determinants, it is conceivable that mutated VGSCs could indirectly influence vector competence through such physiological disturbances. A further possibility is that the evolutionary conditions favouring the emergence and spread of *kdr*, may also co-select for alleles on local or distant loci that can impact vector competence [187, 188]. For example, the *kdr* mutation occurs in a haplotype that contains a gene coding for the serine protease *ClipC9*, which has been linked with anti-*Plasmodium* immunity [249, 250]. It has also been shown in genome wide association studies with *Aedes aegypti* that there are single nucleotide polymorphisms commonly associated with *kdr* in distant loci of the genome [189].

Several studies have investigated the potential impacts of *kdr* on vector competence to date, but findings have been varied and conflicting, likely due to the range of vector-parasite models used, and significant differences in study design. Whilst there are relatively few studies that have focused on the potential impacts of *kdr* on arbovirus transmission, inferences may also be drawn from several field and laboratory investigations conducted using *Plasmodium* parasites and their mosquito vectors. The presence of the L1014S allele was associated with a higher prevalence of *P. falciparum* sporozoites in field-caught *An. gambiae s.s.* from Tanzania [251] but similar studies have not detected any significant effect [252, 253]. Infection prevalence data from field caught mosquitoes are vulnerable to the confounding effects of insecticide use in the study area. *Kdr*-carrying mosquitoes may be more likely to survive the extrinsic incubation period (EIP) of a parasite in areas where insecticide use is common and may therefore be more likely to harbour malaria sporozoites than those with wildtype VGSC. Conversely, *Plasmodium*-infected mosquitoes with *kdr* alleles may be more vulnerable to the effects of pyrethroids that their uninfected counterparts, making survival through a parasite's EIP less likely in areas of insecticide use [254].

Experimental infection of field mosquitoes in a laboratory can address some of these issues. Ndiath et al [255] reported that *An. gambiae s.l.* carrying the L1014F or L1014S alleles, had significantly higher prevalence and density of oocysts and sporozoites than wildtype controls, but this effect was not seen in a similar study with a field-derived *Anopheles* line [256]. Experimentally infected field strains of *Ae. aegypti* from Florida with differing *kdr* genotypes (L1016 and C1534) had different infection and transmission rates for dengue virus, with the strains with the highest proportion of *kdr* demonstrating the lowest vector competence indices [180]. Similar results were observed in isofemale lines derived from field populations, whereby strains with the highest prevalence of 1534C alleles had the lowest virus dissemination rate at 7dpi, and viral loads were not significantly different to the wildtype strains [257]. However, the main limitation of all studies using field-caught mosquitoes is that despite the thorough characterisation of strains by *kdr* genotype, they are not able to account for any potential effects of other genetic differences that could alter vector competence.

This problem is partially addressed by developing laboratory strains of mosquitoes with differing *kdr* profiles. Chen et al [175] developed a permethrin resistant mosquito line from a parental field-caught *Ae. aegypti* strain using insecticide selection. The resistant strain had both a significantly higher prevalence of mutated F1534C and V1016I, and a higher dissemination rate of dengue virus to legs at 14 days post-infection (dpi) than the unselected strain [175]. However, the use of insecticide selection here may also have led to unintended genetic differences between resistant and susceptible strains which could affect vector competence [242]. As such, introgression of *kdr* alleles into susceptible mosquito populations has been used to investigate the impacts on vector competence. L1014F was introgressed into the wildtype *An. gambiae s.s.* Kisumu strain, and *kdr* genotype was associated with increased infection prevalence at both the oocyst and sporozoite stages compared to the wildtype Kisumu strain [245]. One of the few arbovirus-related studies utilised backcrossing of insecticide resistant and susceptible laboratory mosquito strains to investigate the impacts of V1016I and F1534C alleles on the competence of *Ae. aegypti* for Zika virus [182]. The results show that *Ae. aegypti* with these alleles had greater infection rates and higher viral dissemination to the legs than wildtype mosquitoes at multiple timepoints post-infection [182]. Whilst introgression is a more targeted way of introducing alleles of interest into wildtype lines compared to insecticide selection, it is possible that confounding alleles are also inadvertently introgressed in the process.

In this study, to minimise confounding genetic differences, vector competence of an L1014F homozygotic line of *An. gambiae*, produced using CRISPR/Cas9, was compared to its parent VGSC wildtype strain. Both strains were experimentally infected with o'nyong nyong virus (ONNV) via infected blood meals or intrathoracic injections. ONNV is the only known pathogenic arbovirus primarily vectored by *Anopheles* species in sub-Saharan Africa, and has a broadly overlapping distribution with mosquito populations carrying *kdr* [240, 258].

2.3 Methods

2.3.1 Virus and mosquito strains

ONNV UgMp30 (BEI Resources, NR-51661) was obtained from colleagues at the Liverpool School of Tropical Medicine (LSTM) and passaged once in Vero CCL81 cells for the intrathoracic injection experiments. For the oral infections, ONNV UgMp30 was passaged twice in Vero cells. Two mosquito lines were used in this study: the insecticide susceptible laboratory Kisumu strain of *An. gambiae* (hereafter Kis) with non-mutated VGSC; and a CRISPR/Cas9 gene-edited L1014F *kdr* homozygous Kisumu line (hereafter Kis-kdr). The Kis-kdr line was developed by colleagues at LSTM, and the methods are described in detail elsewhere [176]. Briefly, Kisumu eggs were injected with donor plasmids containing a red fluorescent protein (RFP) marker. RFP-expressing G_0 larvae were backcrossed with Kisumu to obtain a G_1 line that was screened for the L1014F mutation. Progeny of G_1 females positive for L1014F by locked nucleic acid (LNA) polymerase chain reaction (PCR) were backcrossed with Kisumu to obtain greater numbers of individuals with the *kdr* allele. Several generations of intercrossing of heterozygous individuals achieved a 100% homozygotic line for L1014F [176].

2.3.2 Insecticide susceptibility testing

Both strains of mosquitoes (Kis n=40, Kis-kdr n=60) were exposed at 3-5 days old to the discriminating dose of permethrin (0.75%) in WHO tube assays for 1 hour [259]. Two tubes of 20 mosquitoes were used for KIS, and three tubes of 20 females were used for KDR. They were transferred back into holding tubes with access to 10% sucrose, and knockdown was recorded. At 24 hours post-exposure, mortality rates were determined. Controls of both mosquito strains (n=20) were exposed to insecticide negative papers in WHO tube assays.

2.3.3 Per os (PO) infections

Mosquitoes aged 5-7 days were housed in 350mLcardboard soup cups and sugar starved for 24 hours with access to water. ONNV stocks were diluted 1:1 with human blood to achieve a final viral titre of $1x10^6$ pfu/mL. The phagostimulant ATP was added at a concentration of 900µM. Under Arthropod Containment Level 2 conditions, mosquitoes were allowed to blood feed via the Hemotek feeding system (Hemotek Ltd) for 1 hour. Unfed and partially fed mosquitoes were discarded, and fully fed mosquitoes were incubated until processing with access to 10% sucrose ((27˚C (+/- 1), 80% relative humidity (+/- 5), and 12:12 hours light:dark).

2.3.4 Intrathoracic (IT) microinjections

Non-blood fed females aged 3-5 days were briefly cold anaesthetised at -20˚C for 30 seconds and transferred to a cold plate. Each mosquito was intrathoracically inoculated with 100nL ONNV ($1x10^5$ pfu/ml) at a rate of 50nL/second using a Nanoject III (Drummond Scientific). Following IT injection, mosquitoes were transferred to cardboard soup cups with access to 10% sucrose and incubated until further processing, as above.

2.3.5 Dissections and forced salivations

For IT injections, a cohort of mosquitoes were sacrificed at 5, 7, or 10dpi, and head, body (thorax + abdomen), legs, and saliva were collected. For oral (PO) infections, mosquitoes were sampled at 0 and 7dpi. For forced salivation, mosquitoes were cold anaesthetised, legs and wings removed, and the proboscis placed in a 20µL pipette tip containing mineral oil. Mosquitoes were allowed to salivate for 15 minutes, then dissected into head and bodies. Saliva was ejected into a tube containing 100 µL infection media (Dulbecco's Modified Eagle Media (DMEM) + 2% foetal bovine serum (FBS), 1:1000 v/v 50mg/mL gentamicin, 1:200 v/v 10mg/mL Fungin (Invivogen)). Body parts were placed into a Safelock tube containing 300µL infection media and a 5/32" diameter stainless steel ball bearing (Dejay Distribution Limited). Samples were immediately stored at -80˚C until further processing.

2.3.6 Plaque assays

For IT injection samples, 24-well tissue culture plates were seeded with 500µL of $2.5x10^5$ cells/ml Vero CCL81 cells in growth media (DMEM + 10% FBS + 1:1000 v/v 50mg/mL gentamicin) and incubated overnight (37˚C, 5% CO_2). For PO infection samples, Vero cells were used, and seeded as above. Body part samples were defrosted and homogenised at 26Hz for 5 mins using a TissueLyser (Qiagen). Homogenised samples were centrifuged for 5 mins to pellet debris. Each sample was serially diluted in infection media, and 100uL inoculum was added in duplicate to the confluent cell monolayer in the cell culture plates. These were incubated for 1hr (37˚C, 5% CO_2) prior to adding a 0.4% agarose overlay. After 48 hours of incubation (37˚C, 5% CO_2), the plates were fixed with formaldehyde and stained using a 0.25% v/v crystal violet solution. Plaques were counted, and the mean of both replicates was used to calculate the viral titre of each sample.

2.3.7 Cytopathic effects assays

Cytopathic effects (CPE) assays were performed on saliva samples to determine whether ONNV was present in saliva. 96-well cell culture plates were seeded with 100µL Vero CCL81 at a density of $2.5x10^5$ cells/mL in growth media and incubated overnight. After incubation the growth media was removed and replaced with 75µL infection media. Saliva samples were vortexed for 15s and 25µL was added in duplicate to the cells. The plates were incubated for 72 hours (37˚C, 5% CO_2) before being scored by microscopy for the presence of CPE. If either of the replicates for each sample were positive, the saliva sample was considered to contain viable ONNV. The limit of detection of the CPE assays was established at approximately $1.9x10^2$ pfu/mL, which is <5 pfu per 25µL added to each CPE assay replicate (**Supplementary Data 4**).

2.3.8 Statistical analysis

Fishers' exact tests were used to determine significance of differences in infection and dissemination prevalence, and knockdown and mortality rates following insecticide exposure. This was justified due to the small sample size for each timepoint. For viral titre data of mosquito samples and ingested blood meals, normality was determined using Shapiro-Wilks test and separate ANOVA were performed for each timepoint, using viral titre and mosquito strain as grouping variables. If the viral titre data were non-Normally distributed, Kruskal-Wallis tests were used. All statistical analyses were performed using base functions in R (version 4.2.1) (Code available in **Appendix 1**).

2.4 Results

2.4.1 Permethrin susceptibility testing

Exposure to 0.75% permethrin in WHO tube assays revealed a significant difference in susceptibility between strains. There was 95.0% ([95% CI 83.1-99.4] n=40) knockdown following 1hr exposure of Kis, compared to 3.3% ([0.4-11.5] n=60) of Kis-kdr (Fishers' exact p≤2.2e-16). Mortality at 24hrs revealed a similar picture with Kis showing high susceptibility (100.0% mortality [91.2-100.0] n=40), and Kis-kdr

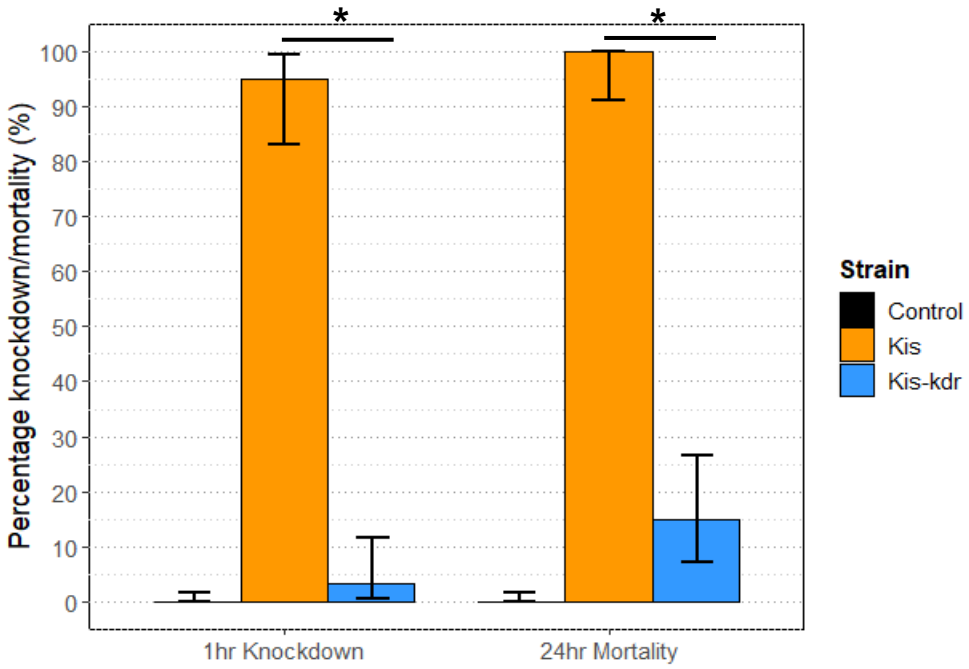


Figure 1 – Susceptibility to 0.75% permethrin in WHO tube assay
Kis (n=40) and Kis-kdr (n=60) were exposed in WHO tube assays to 0.75% permethrin for 1 hour. 2 tubes of 20 females, and 3 tubes of 20 females were used for KIS and KDR, respectively. 20 controls of both strains were mock-exposed to non-insecticide treated filter papers. Percentage knockdown at 1hr, and mortality at 24hr are indicated by the coloured bars. 95% Wilson confidence intervals are marked in black. There was significantly higher (Fisher's exact p≤2.2e-16) knockdown at 1 hour in Kis (95.0% [95% CI 83.1-99.4]) than Kis-kdr 3.3% [0.4-11.5]). Mortality at 24 hours was significantly higher in Kis (100.0% [91.2-100.0] than Kis-kdr (15.0% [7.1-26.6]) (Fisher's exact p≤2.2e-16). There was no knockdown or mortality in negative controls of either strain.

showing a significantly lower mortality (15.0% [7.1-26.6] n=60) (Fishers' exact p≤2.2e16) (**Figure 1**). There was no knockdown or mortality seen in the unexposed control groups for either strain.

2.4.2 Oral infections

Homogenisation of mosquitoes immediately following blood feeding revealed a similar range of inoculum titres, spanning from approximately 3.2 to 4.2$\log_{10}$ pfu/mosquito, in both strains. The mean inoculum titres were not significantly different between strains (Kis 3.77 [95% CI 3.59-3.95] n=15; Kis-kdr 3.67 [3.52-3.82] n=23; ANOVA p=0.40) (**Figure 2**).

Establishment of infection via infected blood meal was achieved in both mosquito strains. The infection prevalence was not significantly different between Kis (45.0% [23.1-68.5] n=20) and Kis-kdr (40.0% [19.1-64.0] n=20) (Fishers' exact p=1). At the 7dpi timepoint, there was very limited dissemination of virus to other body parts in both mosquito strains, with only a single head sample testing positive by plaque assay, and no positive leg samples. These differences were not significantly different between strains (Fishers' exact p=1). Of the mosquitoes with established infection at 7dpi, the mean body ONNV titre was higher in Kis-kdr (2.30 $\log_{10}$ pfu/body) than Kis (1.99 $\log_{10}$ pfu/body), but this was not significant by ANOVA (p=0.57) (**Figure 3**). Notably, the ONNV titres of the mosquito bodies were markedly lower at 7dpi (~1.3-3.5$\log_{10}$ PFU/body) than at 0dpi (~3.2-4.2$\log_{10}$ PFU/body). The only mosquito with a disseminated infection to the head was of the Kis strain and had a body ONNV titre of 2.41 ($\log_{10}$ pfu/body) which was the highest body titre of any Kis mosquito assayed, but the saliva sample was negative for virus. Interestingly there were three Kis-kdr mosquito bodies which had higher titres, but did not exhibit disseminated infections to their heads by 7dpi. Unfortunately, there were insufficient numbers of mosquitoes surviving beyond 7dpi to allow for further timepoints to be conducted.

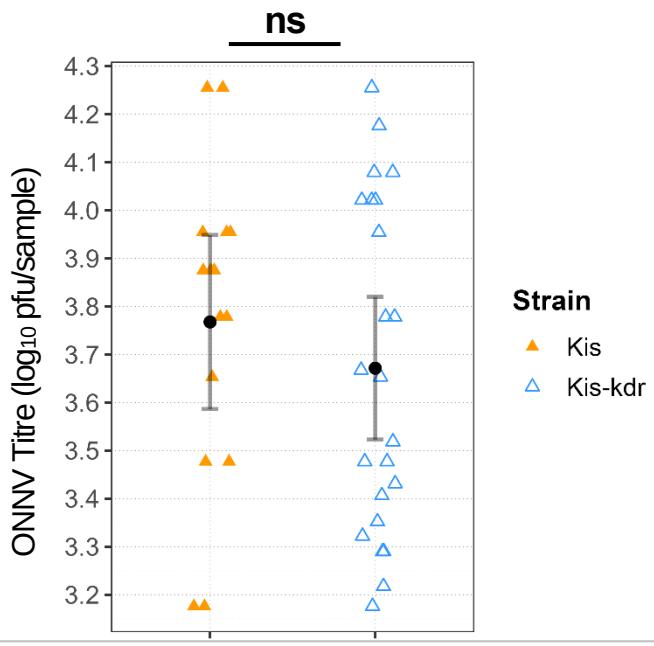


Figure 2 – ONNV titre in mosquito bodies immediately post-feeding
Kis (n=15) and Kis-kdr (n=23) were homogenised immediately post-blood feeding and the viral titre established by plaque assay. Samples were collected from a single blood feed. The data were normally distributed (Shapiro-Wilks p>0.5). The mean values and 95% Wilson confidence intervals are marked in black. There was a similar range of viral titres observed in both strains and the mean ONNV titres were not significantly different (Kis 3.77 [95% CI 3.59-3.95], Kis-kdr 3.67 [3.52-3.82], ANOVA p=0.40).

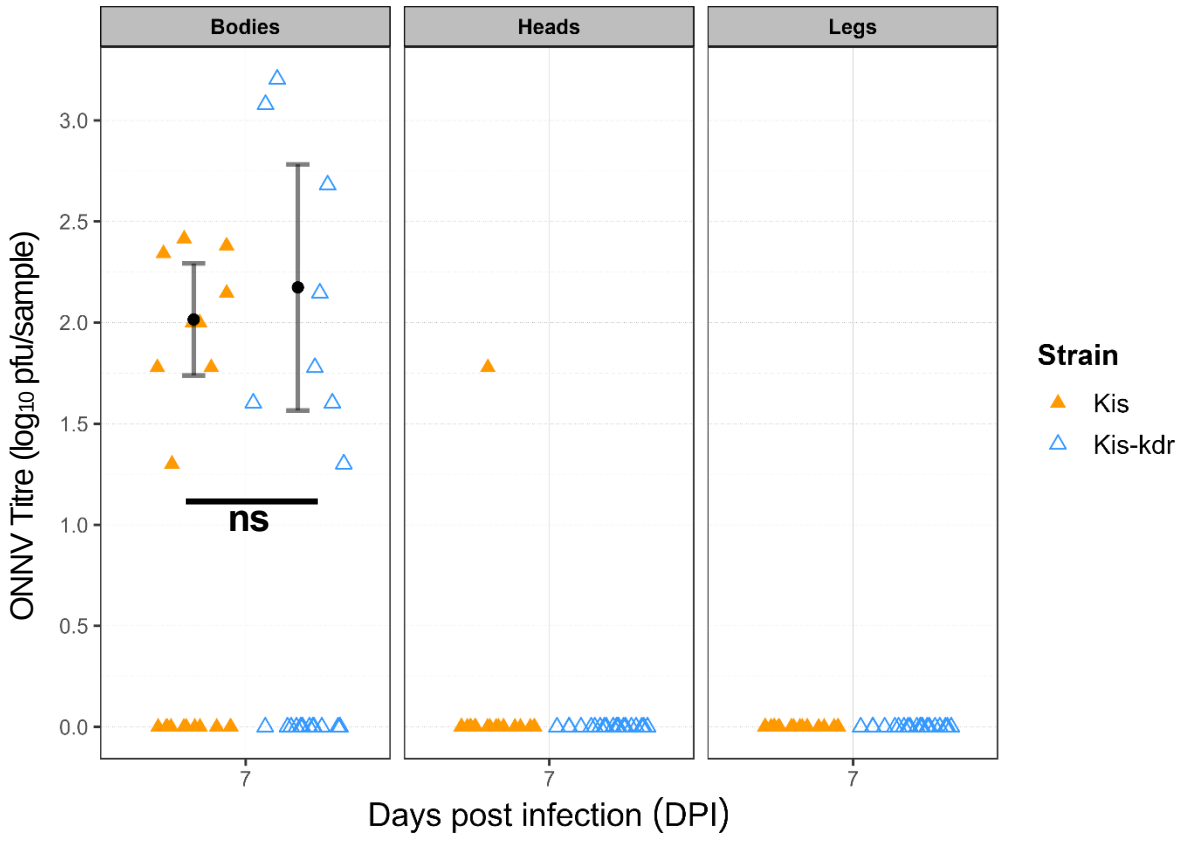


Figure 3 – ONNV titre in mosquito body parts following oral infection

ONNV titres of mosquito bodies, heads, and legs at 7dpi were established by plaque assay for Kis (n=20) and Kis-kdr (n=20) strains. Data were collected from a single blood feed. Mosquitoes without established infection were excluded, and the titre data for infected mosquitoes were normally distributed (Shapiro-Wilks p>0.5). 95% Wilson confidence intervals are shown in black. The mean body ONNV titres were not significantly different between strains (Kis 1.99 $\log_{10}$ pfu/sample; Kis-kdr 2.30; ANOVA p=0.57). Only one Kis mosquito had a disseminated infection to its head at 7dpi. No virus was found in the legs of either mosquito strain at 7dpi.

2.4.3 Intrathoracic injections

For the intrathoracic injection experiments, a sample size of >20 mosquitoes per strain was achieved for all timepoints, except Kis-kdr at 10dpi. The infection prevalence from intrathoracic injections in the bodies and heads of mosquitoes was 100% at all tested timepoints for both mosquito strains (**Supplementary data 1 & 2**). The infection prevalence following IT injection in the mosquito legs was also high in both strains at all timepoints tested (5dpi Kis 90.9% [95% CI 70.8-98.8], Kis-kdr 95.5% [77.2-99.9]; 7dpi Kis 100%, Kis-kdr 100%; 10dpi Kis 95.7% [78.1-99.9], Kis-kdr 92.9% [66.1-99.8]) (**Supplementary data 3**). None of these differences were significantly different (Fishers' exact p=1). The mean viral loads in each tissue at each timepoint followed the same broad pattern for each mosquito strain (**Figure 4**). Following inoculation of virus into the haemocoele there was rapid amplification of virus in the bodies of mosquitoes. By 7dpi, the viral load in the majority of bodies had surpassed the initial titre delivered by microinjection. Rapid amplification of virus in the heads and legs of mosquitoes was also seen, however there was a plateauing of viral titre in bodies and legs by the 10dpi timepoint. The viral titre in the heads of mosquitoes

continued to increase up to 10dpi. None of the titres in body parts were significantly different between strains at any timepoint except legs at 7dpi (Kis mean titre 3.46 [3.32-3.60]; Kis-kdr 3.22 [3.01-3.42]; ANOVA p=0.049) (**Table 1**; **Figure 4**). This significant difference was no longer observed at 10dpi. CPE analysis of saliva samples revealed no positive samples at any timepoint in either strain.

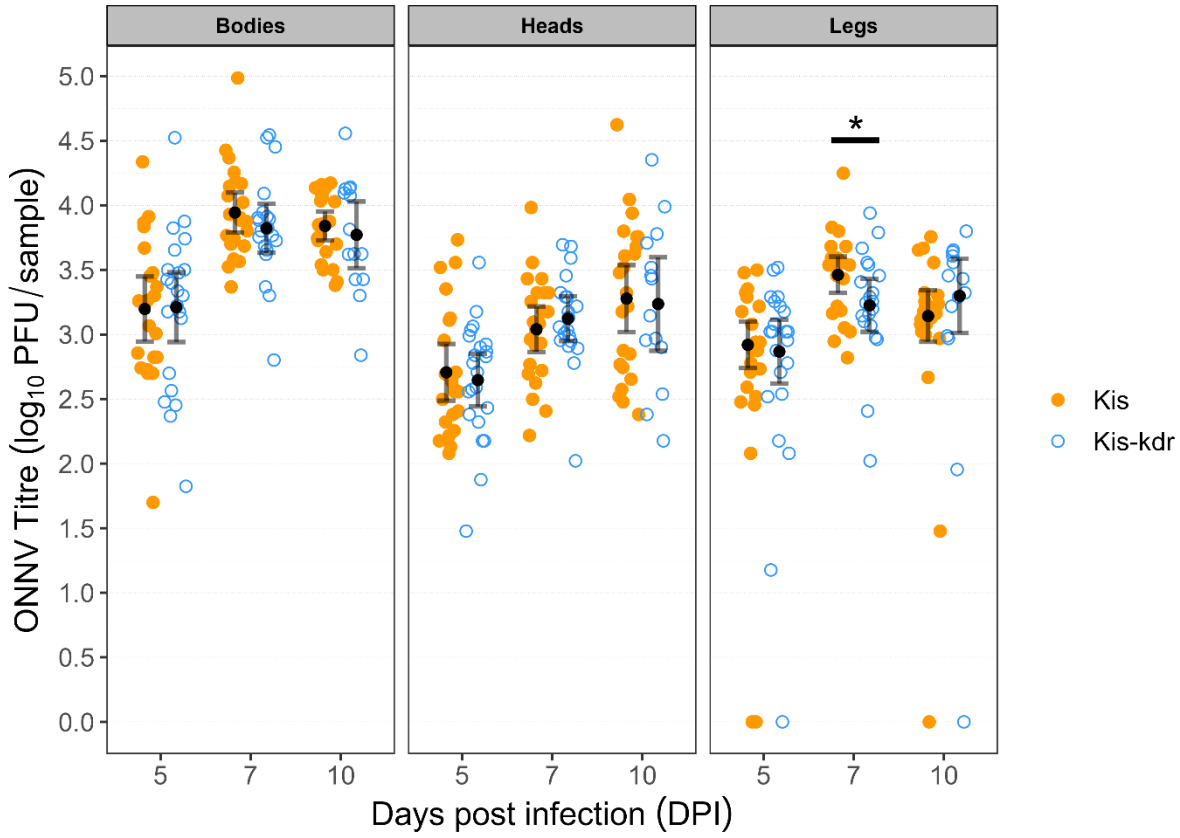


Figure 4 – ONNV titre in mosquito body parts following intrathoracic microinjection
Mosquitoes were injected intrathoracically with 100nL of 10^5 pfu/mL ONNV. Mean viral titre and 95% Wilson CI shown in black (virus negative samples excluded from calculations). Mosquitoes were sampled at 5, 7 or 10 dpi and dissected into heads, bodies (thorax and abdomen), and legs. Sample sizes for each strain x timepoint are shown in **Table 1**. There was significantly higher viral titre in the legs of Kis than Kis-kdr at 7dpi (Kis 3.46pfu/mL [3.32-3.60]; Kis-kdr 3.22pfu/mL [3.01-3.42]; ANOVA p=0.049). No other comparisons were statistically significant.

Table 1 – Mean ONNV titres following intrathoracic microinjection				
Part	**Strain**	**Mean ONNV Titre (log_{10} pfu/sample)** [95% CI]		
		5dpi	**7dpi**	**10dpi**
Body (thorax + abdomen)	Kis	**3.20** [2.95-3.45] n=22	**3.94** [3.79-4.10] n=23	**3.84** [3.73-3.95] n=23
	Kis-kdr	**3.21** [2.94-3.48] n=22	**3.82** [3.63-4.01] n=20	**3.77** [3.51-4.03] n=14
Head	Kis	**2.71** [2.49-2.93] n=22	**3.04** [2.86-3.22] n=23	**3.28** [3.02-3.54] n=23
	Kis-kdr	**2.65** [2.44-2.85] n=22	**3.12** [2.95-3.30] n=20	**3.24** [2.87-3.60] n=14
Legs	Kis	**2.92** [2.74-3.10] n=20	**3.46** [3.32-3.60] n=23	**3.14** [2.94-3.34] n=22
	Kis-kdr	**2.87** [2.62-3.11] n=21	**3.22** [3.02-3.43] n=20	**3.30** [3.01-3.59] n=13

Table 1 – Mean ONNV titres following intrathoracic microinjection
Cohorts of mosquitoes were sampled at 3 timepoints post-infection via intrathoracic microinjection with ONNV. Mean titres and 95% Wilson confidence intervals shown for each body part sample at 3 different timepoints post-infection. Virus negative samples were not included in these calculations. Viral titres were determined by plaque assay.

2.5 Discussion

Mutations to voltage-gated sodium channels, associated with resistance to pyrethroid insecticides, have previously been correlated with alterations in vector competence for *Plasmodium* spp. and some arboviruses [175, 180, 182, 245, 251, 255, 257]. Here we investigated the potential impacts of the L1014F allele on the vector competence of *Anopheles gambiae* for ONNV. Susceptibility testing to permethrin demonstrated a significant phenotypic difference between wildtype and *kdr*-mutant lines, which corroborates previous evaluation of these mosquito lines, and supports the role of the L1014F in causing permethrin resistance [176].

Our data show that there was no impact of the L1014F allele on the ONNV infection prevalence in the bodies of mosquitoes following PO infection. Parker-Crockett et al [182] reported a higher infection prevalence for ZIKV in *Ae. aegypti* carrying the 1016 and 1534 *kdr* alleles following introgression. Our data suggest that this is not the case with the L1014F allele in this *Anopheles*-ONNV model. Previous investigations with laboratory and field mosquito strains have reported that the L1014F allele does not lead to alterations in vector competence [252, 253, 256, 260]. Our results may support this conclusion,

but due to a lack of dissemination of ONNV from the midgut following PO feeds in both Kis and Kis-kdr strains, we cannot exclude any potential effects of the L1014F allele on transmission. The almost universal lack of dissemination of virus from the midgut of mosquitoes following oral infection, irrespective of their *kdr* genotype, suggests that the Kisumu strain is not a highly competent vector for this strain of ONNV. It is possible that further incubation time beyond the 7dpi tested may be required for dissemination from the midgut to occur, or dissemination may be inhibited by the presence of a midgut escape barrier [82]. Furthermore, as the dissemination prevalence of many arboviruses is heavily influenced by the viral titre in the ingested blood meal, higher PO doses of ONNV than those conducted here may have produced greater dissemination [261]. Unfortunately, despite serial passaging of ONNV stocks in both mammalian and insect cell lines, and attempts at concentration of viral stocks using filtration, it was not possible to produce higher titre stocks for PO feeds than those used here. Furthermore, the use of antibiotics during the propagation of virus stocks may have altered the microbiome of the mosquito midgut following ingestion in the blood meal, and may have led to reduced dissemination of virus from the midgut.

Data from IT injections showed that both strains were able to support viral replication in the haemocoele, and other body parts, when the midgut was bypassed. Excluding a transiently lower mean ONNV titre in the legs of the KDR strain at 7dpi following IT injection, viral titres were not significantly different between strains. We did not detect viable ONNV in saliva samples from either mosquito strain at any timepoint post-IT injection. Alphaviruses, including ONNV, can be difficult to isolate via forced salivation, presumably due to lower salivary viral loads than with other genera of arboviruses [262-265]. It is not clear here whether additional incubation time post-infection was required for virus to enter the saliva, or whether this finding represents the presence of salivary gland infection and/or escape barriers which could have excluded ONNV from the saliva [266]. If this barrier is dose-dependent, then use of higher doses of ONNV for IT injections may have been required to permit virus to enter the saliva [94]. Furthermore, the use of gentamicin in the inoculum for IT injection may have altered the composition of the salivary gland microbiome, which could have unforeseen consequences on viral entry and infection of the salivary glands [267, 268].

In contrast to our findings, a number of investigations have reported that *kdr* is associated with altered vector competence indices [175, 180, 182, 245, 251, 255, 257]. There are several reasons for these discordant findings. Genetic differences were minimised between *kdr*-mutant and wildtype strains through the use of a gene edited *kdr*-mutant line in our study. Though the CRISPR/Cas9 technique is not completely immune to the introduction of unintended genetic changes through off-target activity and genetic drift [269, 270], it limits the introduction of confounding alleles compared to backcrossing, insecticide selection, or comparisons between sympatric field strains. The effects observed in previous investigations employing these techniques may have been due to other genetic differences rather than *kdr* genotype.

In summary, our findings from PO infections suggest that the *kdr* 1014F allele, introduced by CRISPR/Cas9, is not associated with alterations to ONNV infection prevalence, a key component of vector competence. Further investigations should isolate the potential effects of *kdr* by aiming to minimise potentially confounding genetic differences between mosquito strains. Whilst aspects of vector competence for ONNV may not be significantly modified by the introduction of the L1014F allele in these *An. gambiae* strains, this does not preclude potential alterations to broader aspects of vectorial capacity. Previous evaluation of the Kis-kdr strain noted a number of fitness costs associated with introduction of the L1014F allele in the absence of insecticide pressures, including a significantly reduced average lifespan [176]. This could reduce the probability of a mosquito successfully surviving the EIP and being capable of transmitting a pathogen. Conversely, in the presence of insecticide, *kdr*-carrying mosquitoes may be more likely to survive the duration of the EIP and could have a higher resulting vectorial capacity than non-mutant populations.

2.6 Acknowledgements

The contributions of Dr Linda Grigoraki of LSTM in providing the mosquito strains used in this experiment, and Ruth Cowlishaw and Sara Elg (both of LSTM) for their help and guidance with mosquito rearing, are gratefully acknowledged.

2.7 Supplementary data

Supplementary Table 1 – Prevalence of infection in mosquito bodies following intrathoracic microinjection		
Days post-infection (dpi)	**Kis** (% infection) [95% CI]	**Kis-kdr** (% infection) [95% CI]
5	100% [84.6-100] N=22	100% [84.6-100] N=22
7	100% [85.2-100] N=23	100% [83.2-100] N=20
10	100% [85.2-100] N=23	100% [76.8-100] N=14

Supplementary Table 2 – Prevalence of infection in mosquito heads following intrathoracic microinjection		
Days post-infection (dpi)	**Kis** (% infection) [95% CI]	**Kis-kdr** (% infection) [95% CI]
5	100% [84.6-100] N=22	100% [84.6-100] N=22
7	100% [85.2-100] N=23	100% [83.2-100] N=20
10	100% [85.2-100] N=23	100% [76.8-100] N=14

Supplementary Table 3 – Prevalence of infection in mosquito legs following intrathoracic microinjection		
Days post-infection (dpi)	**Kis** (% infection) [95% CI]	**Kis-kdr** (% infection) [95% CI]
5	90.9% [70.8-98.8] N=22	95.5% [77.2-99.9] N=22
7	100% [85.2-100] N=23	100% [83.2-100] N=20
10	95.7% [78.1-99.9] N=23	92.9% [66.1-99.8] N=14

2.7.1 Cytopathic effects assay sensitivity analysis

Cytopathic effects (CPE) assays provide a high throughput, non-quantitative method for determining whether virus is present in mosquito body parts and saliva. This method is used to screen out virus-negative samples to avoid performing unnecessary plaque assays. The sensitivity and limit of detection (LOD) of this assay was determined for ONNV UgMp30 virus in Vero cells.

2.7.1.1 Method

A dilution series was prepared from stocks of ONNV UgMp30 and inoculated onto Vero cells from tandem CPE and plaque assay analysis. CPE and plaque assays were performed as described in the main methods section of the manuscript. Duplicates for each dilution were performed on plaque assay. Each dilution had 7 replicates on the CPE assay. Plaque assays were incubated for 48h prior to fixing, staining and counting of plaques. CPE assays were incubated for 72h and scored by microscopy.

2.7.1.2 Results

The sensitivity analysis using paired CPE and plaque assays show that CPE assays down to very low titres. Cell death and other cytopathic effects (e.g. cell rounding, detachment, and sloughing) were observed in 100% of CPE replicates down to a titre of $1.9x10^3$ PFU/mL which equates to approximately 4-5 PFU per 25µL inoculum added to the assay. Even at titres as low as 25 PFU/mL (<1 PFU on average per 25µL inoculum), at least 1 CPE replicate remained positive (**Supplementary Table 4**).

2.7.1.3 Discussion

These results show that CPE assays are a sensitive method for screening mosquito samples for ONNV. At least 1 CPE replicate remained positive down to <1 PFU on average per 25µL inoculum added to the assay, meaning it is likely that the CPE assay will be positive if a single PFU is added in the inoculum. In order to avoid misidentifying samples as virus negative, the presence of a single positive CPE replicate should prompt further analysis by plaque assay. As such, it seems the CPE assay used here is a highly sensitive screening method.

Supplementary Table 4 – Cytopathic effects assay sensitivity analysis for ONNV UgMp30 in Vero cells

Dilution number	1	2	3	4	5	6	7	8	9	10	11
Mean number of plaques	28.5	18	25	17	27	21	19	18	5.5	2.5	0
Titre (PFU/mL)	2.9×10^5	1.8×10^5	2.5×10^4	1.7×10^4	2.7×10^3	2.1×10^3	1.9×10^3	1.8×10^2	55	25	8.3*
Percentage of CPE replicates positive (%)	100	100	100	100	100	100	100	71.4	42.9	28.6	0
PFU in 100µL inoculum for plaque assay	2.9×10^4	1.8×10^4	2.5×10^3	1.7×10^3	270	210	19	18	5.5	2.5	0.8
PFU in 25µL inoculum for CPE assay	7.1 $\times 10^3$	4.5 $\times 10^3$	625	425	67.5	52.5	4.75	4.5	1.4	0.6	0.2

Supplementary Table 4 – Cytopathic effects assays sensitivity analysis for ONNV UgMp30 in Vero cells

Paired plaque assays and cytopathic effects (CPE) assays were performed on a viral dilution series. Each dilution had 7 replicates for CPE assay and the percentage that were positive for virus is shown. All CPE replicates were positive down to a ONNV titre of 1.9×10^3 which is approximately 4 virions per 25µL sample added to a CPE assay. 28.6% CPE replicates were positive when the titre was only 25 pfu/mL. This equates to 0.6 virions on average per CPE assay. This shows that the assay remains sensitive to extremely low quantities of virus, especially if the presence of virus in any replicate is taken to suggest the sample if virus positive.

*The titre of the lowest dilution was not quantifiable by plaque assay due to the low titre. The titre is therefore implied from the dilution series.

Chapter 3 – Altering insecticide selection pressure is associated with changes in the expression of metabolic insecticide resistance and immune genes in *Aedes aegypti*

3.1 Abstract

Insecticide rotations are recommended to maintain operational vector control in areas of high insecticide resistance. Insecticide selection pressures are known to have multiple effects on the physiology and life history traits of mosquitoes; however, the impacts of changing insecticide selection pressures are not well characterised. We hypothesised that alterations in insecticide selection pressures would have widespread effects on the transcriptome of *Aedes aegypti*, including effects on the expression of genes encoding detoxification enzymes and innate immunity genes. The temephos resistant Recife strain of *Aedes aegypti* was split into 3 sub-colonies based on different insecticide rotation scenarios: 1) removal of temephos; 2) switching to malathion; and 3) switching to permethrin. After 10 generations of these selection regimens, the transcriptome of 2-day old adult mosquitoes from each of these strains were compared to a strain that had been maintained under temephos selection. Differential expression analysis revealed the transcriptome was reactive to changes in insecticide selection pressure, with limited overlap in gene expression profiles between scenarios. Differences in the expression of cytochrome p450 monooxygenases, glutathione-s transferases, and carboxylesterases were associated with changes in the observed insecticide resistance phenotypes. A widespread downregulation of genes associated with innate immunity, including antimicrobial peptides and potential activators of the Toll pathway, was associated with the removal of temephos. This could have important implications for vector competence and warrants further investigation.

3.2 Introduction

The widespread use of insecticides has contributed to the high levels of insecticide resistance observed in *Aedes* populations globally [54, 271]. The decline in efficacy of insecticides used in vector control can be due to different insecticide resistance mechanisms. Target site resistance mechanisms arise from mutations affecting the neuronal binding sites of insecticides [272], whereas metabolic insecticide resistance is due to the increased activity of detoxification enzymes such as cytochrome P450 monooxygenases (CYPs), glutathione S-transferases (GSTs), and carboxylesterases (CCEs) [57]. In order to maintain operational control of *Aedes* vectors, which transmit a number of important arboviruses, regular insecticide rotation is recommended [238, 239]. Insecticide rotation involves replacing the insecticide used to control vectors in an area with an insecticide of a different class, and a different mechanism of action [273]. It is theorised that regular switching reduces the opportunity for resistance

alleles to arise, and may allow the restoration of susceptibility to the substituted insecticide [274]. There are some examples where this technique has restored operational vector control [273, 275, 276], however, in some cases, high levels of resistance to pyrethroids and organophosphates have persisted in populations of *Aedes* for several years after the removal of these insecticides [277, 278]. This may be due to the lack of a refugia of susceptible alleles, and difficulties in controlling the exposure of mosquitoes in the field to other xenobiotics and agricultural insecticides, which may preserve pathways involved in the detoxification of insecticides used for vector control [273, 279]. Whilst findings in the field are mixed, the removal of insecticide in laboratory studies has been shown to lead to a reversion of the resistance phenotype of *Aedes aegypti* for temephos [280-283], and permethrin [284], within a relatively small number of generations. Concerningly, switching classes of insecticide, from the organophosphate temephos to the pyrethroid permethrin, led to the development of high levels of resistance to the new insecticide within a small number of generations in the laboratory [283].

Target site insecticide resistance mutations may be detected using simple molecular methods [285], but metabolic resistance mechanisms are harder to identify and monitor. There are few validated molecular markers of metabolic insecticide resistance in *Aedes*, and similar insecticide resistance phenotypes often have highly divergent underlying mechanisms [57, 286, 287]. As such, RNA sequencing provides a useful way of exploring the underlying genetic determinants of the observed resistance phenotype. This technique has been used to identify candidate genes responsible for metabolic resistance by comparing resistant and susceptible populations of *Anopheles* [288, 289], *Culex* [290], and *Aedes* [291, 292]. It has also been used to characterise changes in the expression of detoxification enzymes in response to alterations in insecticide selection pressures in *Culex* [293] and *Aedes* [280, 286, 287, 294]. Strode et al [280] reported the cytochrome P450 CYP6N12 was downregulated in response to removal of temephos from a resistant strain of *Ae. aegypti*, and this corresponded with a decrease in the observed resistance to temephos. A widespread upregulation of epsilon class GSTs was reported in *Ae. aegypti* selected with temephos [286], whereas permethrin selection was primarily associated with upregulation of cytochrome P450s [287]. Development of different insecticide resistant lines from a susceptible parent strain of *Ae. aegypti* by David et al [295] demonstrated that the transcriptome is highly reactive to changes in insecticide selection pressure, with effects seen across multiple biological domains.

Alterations in insecticide selection pressure, due to rotation or removal of an insecticide, may have broader pleiotropic effects beyond expression of genes encoding detoxification enzymes. Fitness costs associated with metabolic resistance mechanisms in *Aedes* are well established [192, 193, 296], but the effects of altering insecticide selection pressures on life history traits has attracted less research attention. Changing insecticide selection regimens can result in alterations in life history traits that are apparent even in the absence of insecticide exposure. Gleave et al [190] reported alterations in longevity, fertility, and fecundity after removing or switching insecticides in a temephos resistant strain of *Ae. aegypti*. Even following a change in selection regimen from temephos to malathion, insecticides of the same class but

acting on different life stages, can produce marked reductions in longevity, but increased reproductive success [190]. In another study, glycogen reserves and longevity were lower in a temephos selected *Ae. aegypti* line compared to those reared in the absence of insecticide [282]. Furthermore, selection over 15 generations with deltamethrin was shown to lead to reduced longevity and reproductive success [297]. These data suggest that alterations in insecticide selection pressure can have profound effects on the physiology of vectors. The potential for pleiotropic effects of insecticide rotations on the innate immune responses of vectors for human pathogens, is of particular concern.

A potential mechanism for altered vector competence is through effects on immune gene expression. RNA sequencing has been used to compare immune gene expression in insecticide resistant compared to susceptible strains. Insecticide resistant *Anopheles gambiae* were reported to have a higher constitutive expression of the antimicrobial peptides (AMPs) defensins and cecropins than susceptible counterparts [298]. Cecropins have been shown to have antiviral activity against both flaviviruses and alphaviruses [133]. Similarly, isogenic organophosphate resistant *Culex pipiens* were reported to have a higher baseline expression of defensins compared to susceptible controls [299]. Furthermore, the immune gene transcriptomic response of *Ae. aegypti* following ingestion of Zika virus in a blood meal, is different in permethrin susceptible and resistant strains [300]. To our knowledge, RNA sequencing analysis of constitutive immune gene expression has not been investigated following alterations in insecticide selection pressure.

We hypothesised that alterations in insecticide selection pressure, in an already temephos resistant strain of *Ae. aegypti*, would have profound effects on the transcriptome, including the expression of detoxification enzymes, and innate immunity genes. The baseline Recife strain used is a field-derived strain with increased activity of insecticide detoxification enzymes from multiple families, and no identified target site insecticide resistance alleles [301]. It provides a good model of a mosquito population that has acquired metabolic insecticide resistance due to insecticide use for vector control, and would likely require the use of insecticide rotation in order to maintain adequate control in the field.

The aim of this study is to understand the impacts of switching insecticide selection on the gene expression of *Ae*. *aegypti*, with a focus on the transcription of genes relating to antiviral immunity and metabolic insecticide resistance. Three different insecticide rotation scenarios were explored in comparison to maintaining temephos selection: 1) switching to malathion, an organophosphate adulticide; 2) switching to permethrin, a pyrethroid adulticide; and 3) removing temephos selection pressure.

3.3 Methods

3.3.1 Mosquito strains & selection regimens

The Recife (REC) strain of *Ae. aegypti* is a temephos resistant strain that was originally colonised in Brazil [294]. The baseline REC colony (REC-B) was split into four separate strains using different insecticide selection regimens at the Liverpool School of Tropical Medicine (LSTM). The methods are described in detail by Thornton et al [283] but are outlined here. To maintain a temephos resistant REC R strain, larvae were selected every three generations to 0.5mg/L temephos in continuation of the existing selection regime undertaken by Melo-Santos et al [294]. Groups of L3 larvae were exposed every three generations to temephos for 24 hours in plastic dishes, and surviving larvae were transferred to fresh water and allowed to pupate and emerge. To investigate the effects of switching to an adulticide organophosphate, 2-5 day old female mosquitoes were exposed every generation to malathion for 1 hour using WHO tube assays [302]. For the first 6 generations, 0.4% malathion was used, and this was increased to 1.5% for the subsequent 3 generations due to a decrease in observed mortality to <25%. The permethrin selected strain REC-P was created by exposing 2-5 day old female mosquitoes to 0.4% permethrin for 1 hour in WHO tubes for the first 6 generations. This dose was increased to 0.75% permethrin for the subsequent 3 generations (**Figure 1**). The lethal concentration to kill 50% (LC50) of the mosquitoes was determined for each strain at generation 45.

To minimise non-target transcriptomic differences between mosquito strains, standard rearing conditions were used throughout. Adults and larvae from all strains were maintained in the same insectary at LSTM at a temperature of 26˚C ±2, a relative humidity of 75% ±20, and a photoperiod of 12h:12h light:dark. Larvae were reared in 1L distilled water in plastic trays, and fed with Brewer's yeast.

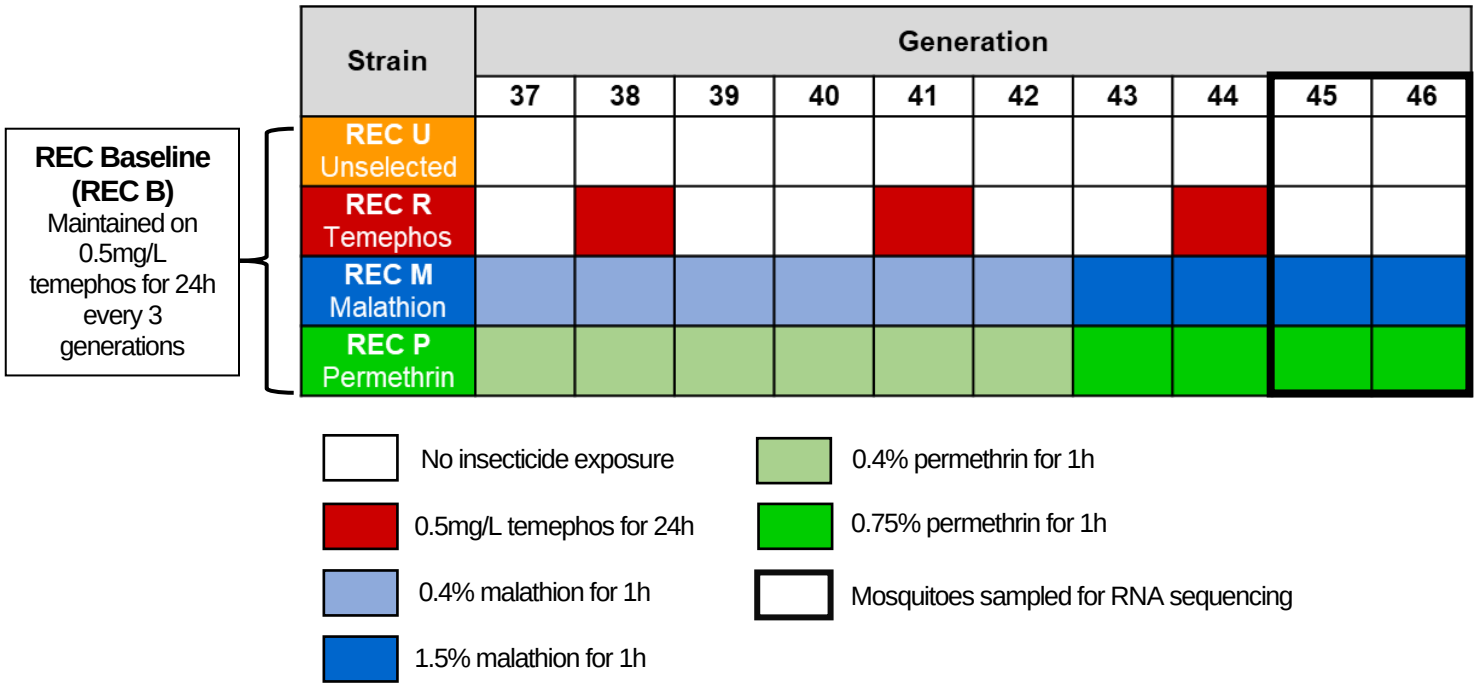


Figure 1 – Insecticide selection regimens
All four insecticide selected strains were derived from the Recife Baseline strain (REC B). REC R was maintained on the same temephos selection regimen as the original REC B strain. REC M was switched to malathion exposure every generation, and REC P was switched to permethrin exposure every generation in World Health Organisation tube assays. Detailed methods are available in Thornton et al [280].

3.3.1.1 Insecticide resistance genotypes

Previous evaluation demonstrated that the Recife baseline strain used here had multiple metabolic resistance mechanisms conferring resistance to temephos [294]. Significantly increased activity of GSTs, both α- and β-esterases, and CYPs are believed to contribute to the resistance phenotype seen [280, 301]. Importantly, target site mutations to the acetylcholinesterase enzyme or voltage gated sodium channels are not present in the Recife strain [301].

3.3.1.2 Insecticide resistance phenotypes

Previous data for the insecticide resistance phenotypes of the Recife strains were reported by Thornton et al [283], and are presented here for reference (**Figure 2**). The lethal concentration to kill 50% of mosquitoes (LC50) for temephos was significantly lower in REC U (0.15mg/L) than the other strains at generation 45, which all had minor differences in temephos resistance (REC R 0.40mg/L [95% CI 0.39-0.42]; REC M 0.42 [0.40-0.44]; REC P 0.40 [0.38-0.41]). For malathion, REC R and REC M exhibited similar LC50 values (1.18% [1.08-1.27] and 1.23% [1.12-1.35], respectively), whereas REC U exhibited a lower phenotypic resistance (0.75% [0.68-0.82]). Nine generations of permethrin exposure produced a marked increase in LC50 for permethrin in REC P (0.91% [0.84-0.98]). REC R demonstrated a higher

LC50 for permethrin (0.18% [0.12-0.25]) than REC U (0.08% [0.07-0.09). The LC50 of REC P to malathion, and REC M to permethrin, were not assessed.

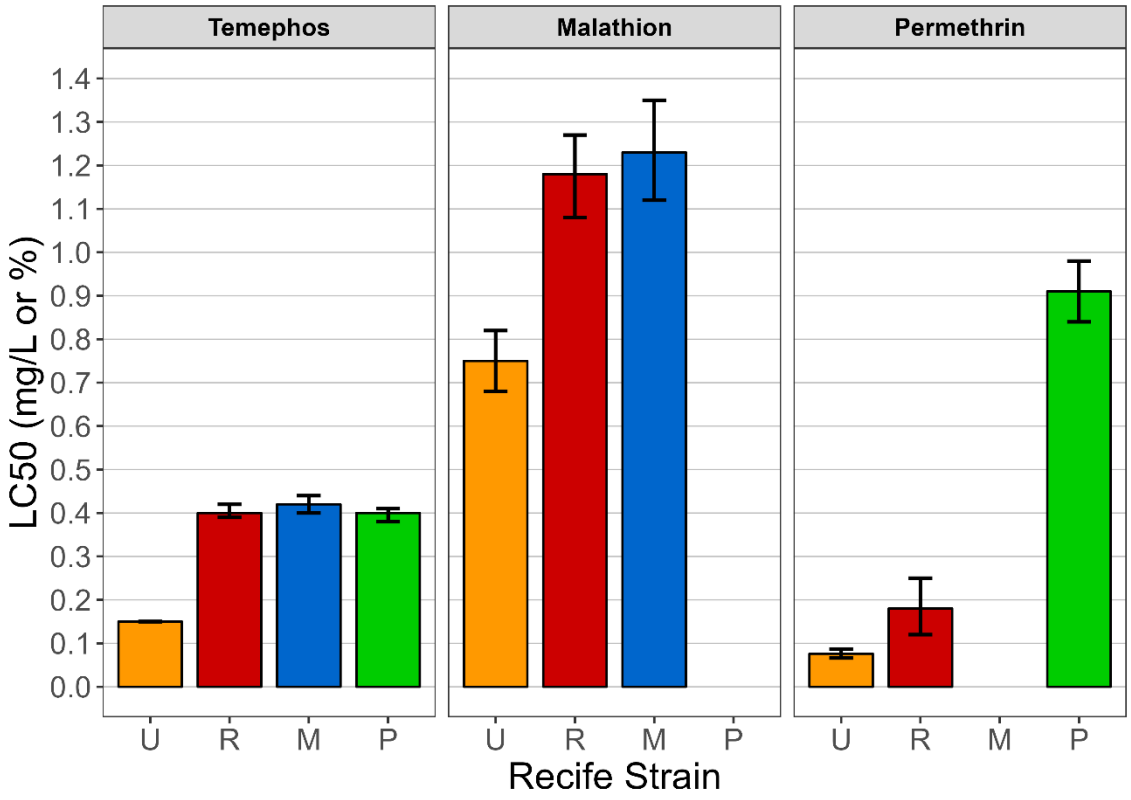


Figure 2 – LC50 for the Recife strains to temephos, malathion and permethrin
Removal of temephos was associated with a decline in temephos LC50 for REC U (0.15mg/L) compared to REC R (0.40mg/L). REC M (0.42) and REC P (0.40) maintained a similar level of temephos resistance to REC R, after 9 generations of selection with malathion and permethrin, respectively. REC R and REC M demonstrated similar resistance phenotypes to malathion (LC50 1.18% and 1.23%, respectively), whereas REC U showed greater susceptibility (0.75%). Only REC P showed high resistance to permethrin (0.91%), with much lower LC50 values seen in REC R (0.18%) and REC U (0.08%). The LC50 of REC P to malathion, and REC M to permethrin were not assessed.

3.3.2 RNA extraction and sequencing

Five 2-day old females were sampled for REC U, REC R, REC P and REC M from generations 45 or 46 (9-10 of insecticide selection). The baseline REC B strain was sampled in the same way at generation 37 and samples were stored at -80˚C until processing. Total RNA was extracted from pools of female mosquitoes using a Quick-RNA™ MiniPrep extraction kit (Zymo). RNA sample purity was established using a Nanodrop spectrophotometer and integrity and quantification was determined using an Agilent Bioanalyzer RNA 6000 Nano assay (Agilent). Samples that were sufficient in quality for Illumina RNA sequencing (Total RNA: ≥150-1000ng in ≤50µL; Purity ≥1.80 for both Nanodrop $A_{260/230}$ and $A_{260/280}$

ratios) were sent to the Centre for Genomic Research at the University of Liverpool for library preparation and sequencing.

3.3.3 Data analysis

Adapter sequences were trimmed from the raw Fastq files using Cutadapt (version 1.2.1). Further trimming with a minimum window quality score of 20 was performed using Sickle (version 1.200). If only one of a read pair passed this threshold, it was included in the R0 file. Trimmed sequences were analysed using the RNA-Seq-Pop workflow developed by colleagues at LSTM [303]. Briefly, paired end reads were aligned to the reference *Aedes aegypti* Liverpool AGWG genome using Kallisto [304], and gene level differential expression was performed using DESeq2 [305]. Gene counts were normalised for sequencing depth. Principal components analysis was performed on all genes and Pearson's correlation was performed for genes in each comparison of treatment groups. REC R was used as the comparator strain for REC U, REC M and REC P, as this line was maintained on the same temephos selection regimen as the original REC B strain. In addition, to determine any effects on the transcriptome of relocating the colonies into our laboratories at LSTM, REC R was compared to REC B. These data are available in **Supplementary data** (section 3.7.1). Genes were considered to be differentially expressed compared to REC R if there was a $\log_2$ fold-change of ≥2 and a Bonferroni adjusted p-value (p value multiplied by the number of tests) of ≤0.05. Gene annotation, gene ontology (GO) terms, and pathways analysis were retrieved for differentially expressed gene sets using Vector Base. Volcano plots and heatmaps were produced using the EnhancedVolcano, and ComplexHeatmap functions within the Bioconductor software (v3.16) in the R-studio environment (v4.2.2). Hierarchical clustering was used to construct heatmaps and dendrograms within the ComplexHeatmap function.

3.4 Results

3.4.1 Sequencing and alignment quality

Illumina sequencing produced approximately 983 million reads from the 20 biological replicates. Less than 1% of reads were removed during trimming due to poor sequence quality, potential adapter contamination, or lack of paired reads. All biological replicates produced >99% valid paired reads (R1/R2). Alignment to the *Ae. aegypti* Liverpool AGWG reference genome was achieved for >90% paired reads (**Table 1**).

Table 1 – Illumina sequencing and alignment quality

Strain	Replicates	Untrimmed	Trimmed	Pair R1/R2	Total aligned	Aligned (%)
REC U	5	253,979,202	252,898,877	125,916,217	114328221	90.80
REC R	5	254,543,648	253,064,147	125,800,265	113,832,514	90.49
REC M	5	236,298,002	235,229,233	117,084,451	106149966	90.66
REC P	5	238,504,954	237,563,213	118,315,390	106933029	90.38
Total	**20**	**983,325,806**	**978,755,470**	**487,116,323**	**441,243,730**	**90.58**

Table 1 – Illumina sequencing and quality
Five biological replicates were collected for each Recife strain. A total of approx. 983 million reads were mapped to the reference *Aedes aegypti* Liverpool AGWG genome. >90% paired R1/R2 reads were aligned. The sequencing and alignment quality data for REC B is shown in the **Supplementary Table 1**.

3.4.2 Differential expression analysis

Principal components analysis (PCA) demonstrated a degree of clustering of replicates from each treatment groups (**Figure 3**). As the Recife strains were closely related, and derived from the same parent strain, highly distinct clustering of treatment groups was not expected. The principal components 1 explained 0.184 of observed variance between strains. A total of 411 genes were over-expressed in all strains relative to REC R, however, only 29 of these were common in all 3 strains (**Figure 4**). There were 47 genes uniquely over-expressed in REC U, 120 in REC M, and 154 in REC P versus REC R. The total number of genes that were under-expressed in the four strains relative to REC R was 206, of which, 20 were common to all four strains. REC P had the most uniquely under-expressed genes compared to REC R (52), followed by REC M (47), and REC U (36). These findings are reflected in the hierarchical clustering heatmap showing all genes that were significantly differentially expressed in at least one strain compared to REC R (**Figure 5**). This shows that the transcriptome is reactive to changes in insecticide selection pressure and shows that different insecticides produce different transcriptomic responses. There are large clusters of genes that are over-expressed in REC P, M and U compared to

REC R. These appear relatively distinct to each comparison, with minimal overlap of over-expressed genes in other comparisons. There are also smaller, distinct clusters of under-expressed genes in the three strains relative to REC R.

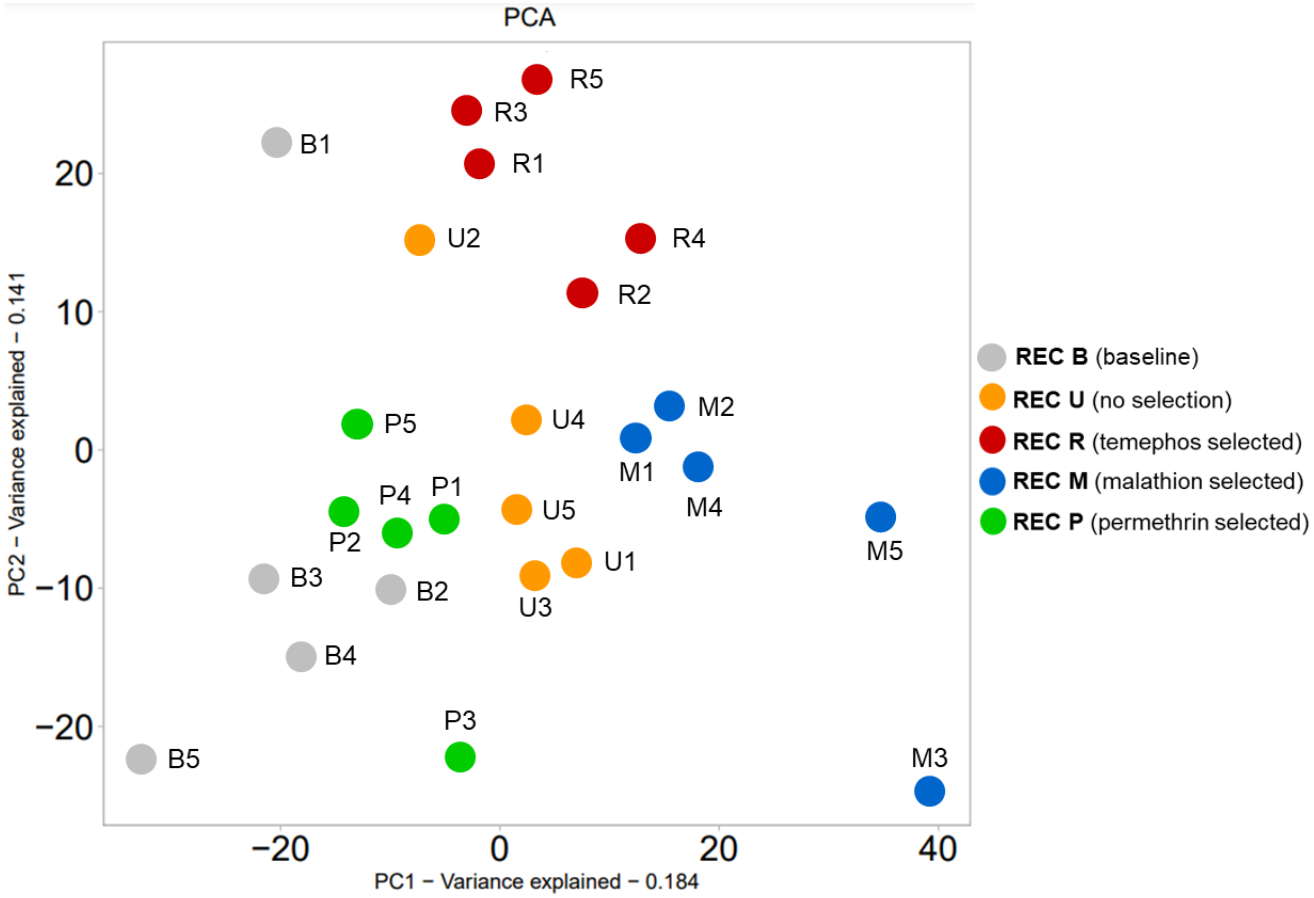


Figure 3 – Principal components analyses
Principal components analyses are shown for all the Recife strains, including the baseline REC B strain (see Section **3.7 Supplementary Data** for further analysis conducted with REC B. Numbering of samples refers to the biological replicates that were sequenced for each strain.

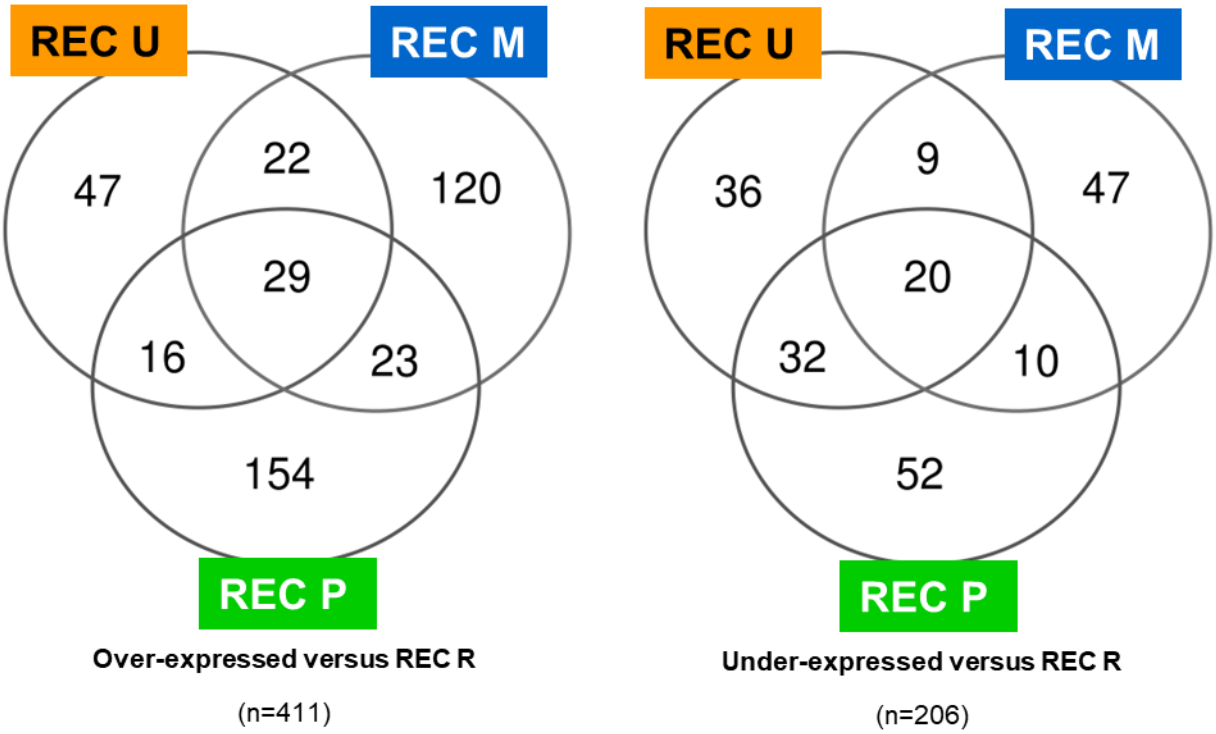


Figure 4 – Overlap in differentially expressed transcripts in Recife strains relative to REC R
There is limited overlap in the genes that are differentially over- and under-expressed in the Recife strains compared to REC R ($Log_2FC \geq 2$ and adjusted p-value ≤0.05).

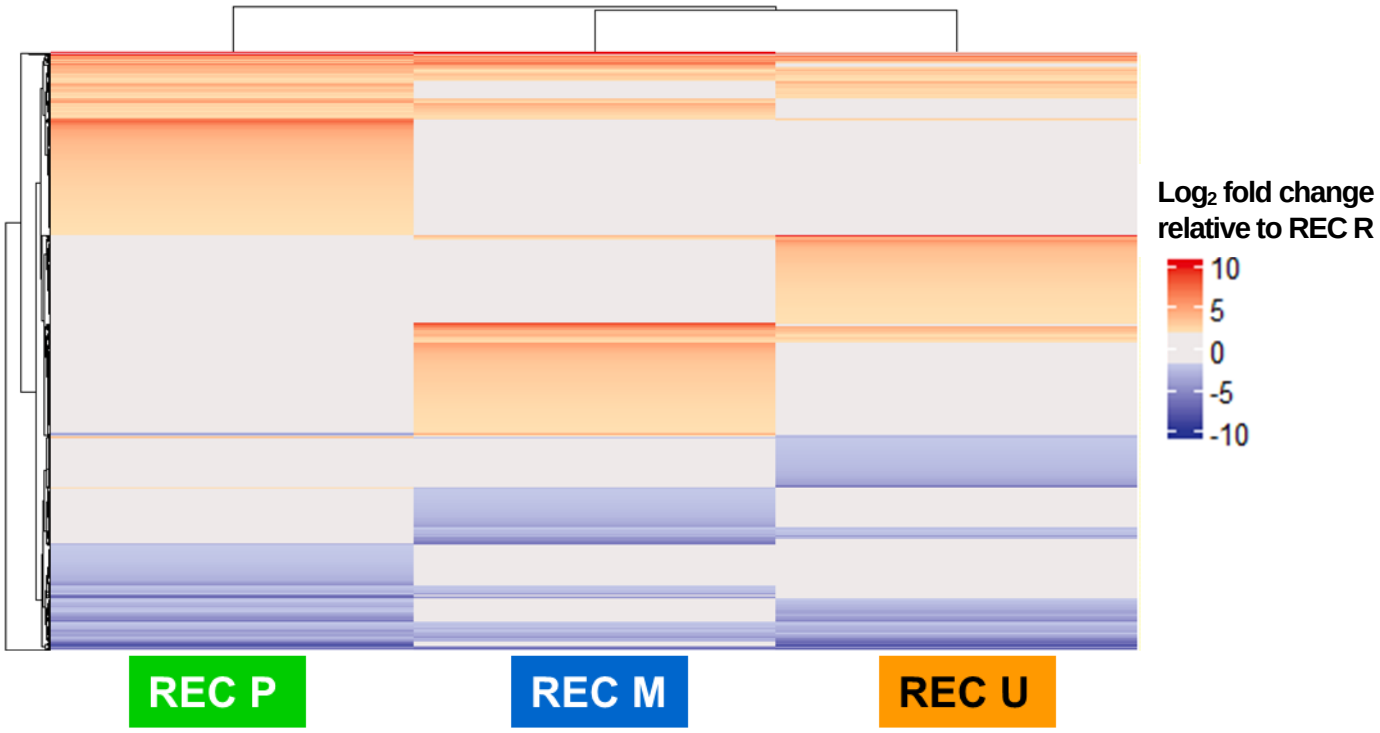


Figure 5 – Hierarchical clustering heatmap of differentially expressed transcripts in ≥1 comparison
Log2 Fold change in REC U, M and P relative to REC R. Values are shown for all genes that are significantly differentially expressed in any of 3 strains versus REC R ($log_2FC \geq 2$ and adjusted p value ≤0.05). Grey areas indicate that a gene was not significantly differentially expressed in that strain compared to REC R.

3.4.2.1 The transcriptomic effects of stopping temephos exposure – REC U versus REC R

To investigate the transcriptomic impacts of the removal of insecticide, the unselected REC U strain was compared to the temephos selected REC R strain. A total of 211 genes were differentially expressed in REC U compared to REC R (114 over-expressed, 97 under-expressed) (**Figure 6**). Annotations were available for 53 of the gene transcripts (**Supplementary Table 5**). The most over-expressed gene in REC U was a gamma-secretase subunit (AAEL002389). The serine/threonine protein phosphatase 2A activator (AAEL020244) was a highly under-expressed gene in REC U compared to REC R.

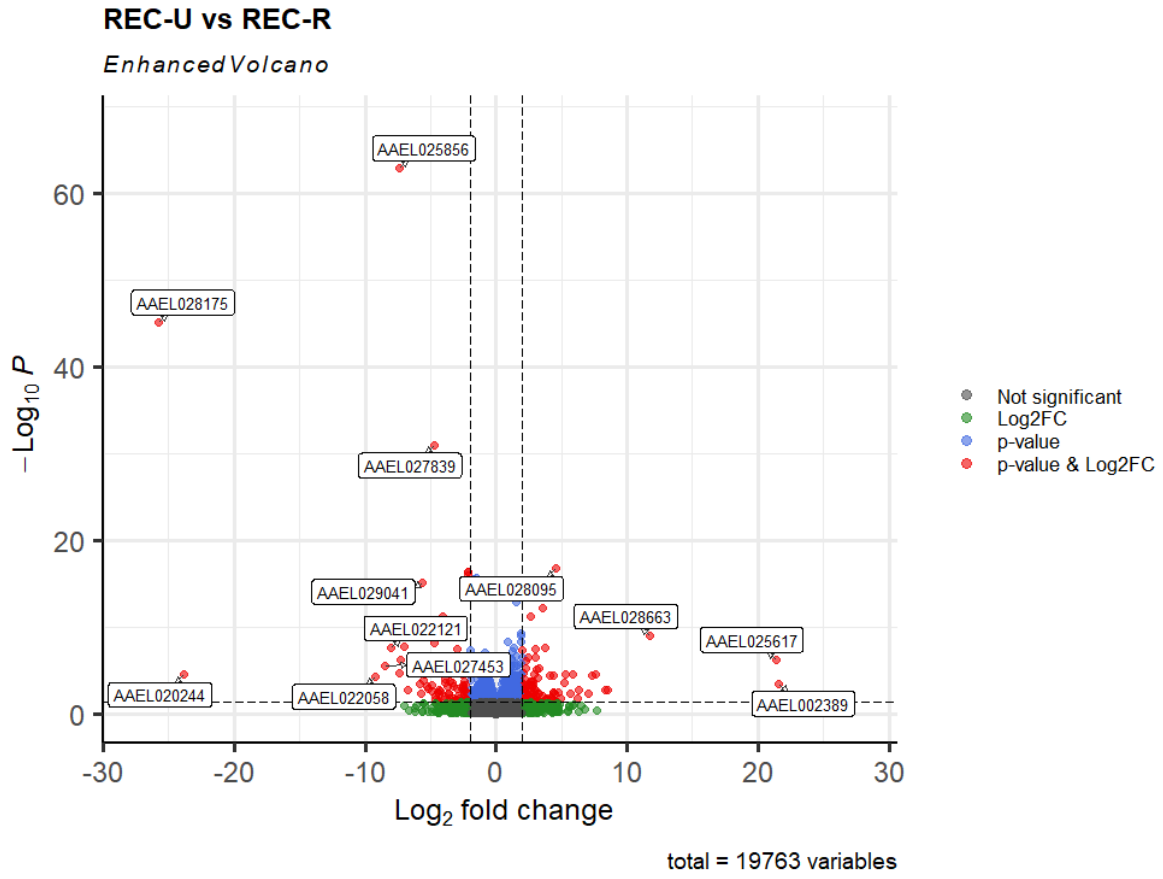


Figure 6 – Volcano plot for differentially expressed genes in REC U vs REC R
Significantly differentially expressed genes in REC U relative to REC R are coloured red (log_2 fold change ≥2 and adjusted p value ≤0.05). A table of all differentially expressed genes and annotations for this comparison is available in **Supplementary Table 5**.

Gene ontology enrichment analysis revealed several terms relating to antibacterial immune responses (GO:0042742, GO:0009617, GO:0050829, GO:001973), and other aspects of innate immunity (GO:0006952, GO:0006955, GO:0002376, GO:0045087, GO:0006959, GO:0019730) were significantly enriched (Bonferroni p <0.05) relating to genes that were under-expressed in REC U compared to REC R (**Figure 7** & **Supplementary Table 8**). These terms were related to a total of 9 genes that were under-expressed in REC U compared to REC R. A single GO term relating to lyase activity was associated with 4 genes that were over-expressed in REC U compared to REC R. A number

of KEGG pathways linked to genes that were over-expressed in REC U compared to REC R, were significantly enriched. These related to a number of biosynthetic and metabolic pathways (**Figure 8**). There were no KEGG pathways relating to genes that were under-expressed in REC U compared to REC R.

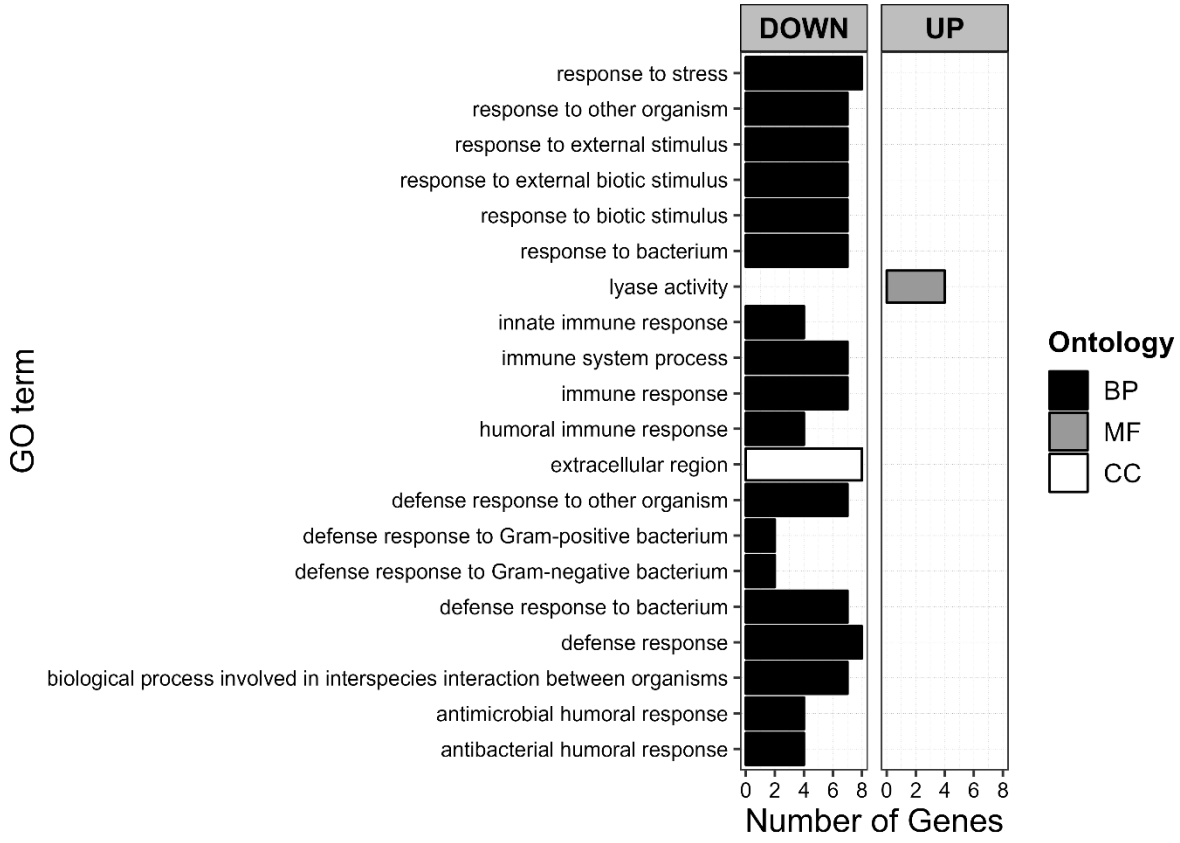


Figure 7 - Significantly enriched GO terms in REC U vs REC R
DOWN denotes the GO terms are related to genes that were under-expressed in REC U compared to REC R. UP denotes GO terms that were related to genes that were over-expressed in REC U compared to REC R. GO terms shown have a Bonferroni p value ≤0.05. GO terms shown are from all available ontologies (BP = biological processes, MF = molecular function, CC = cellular component).

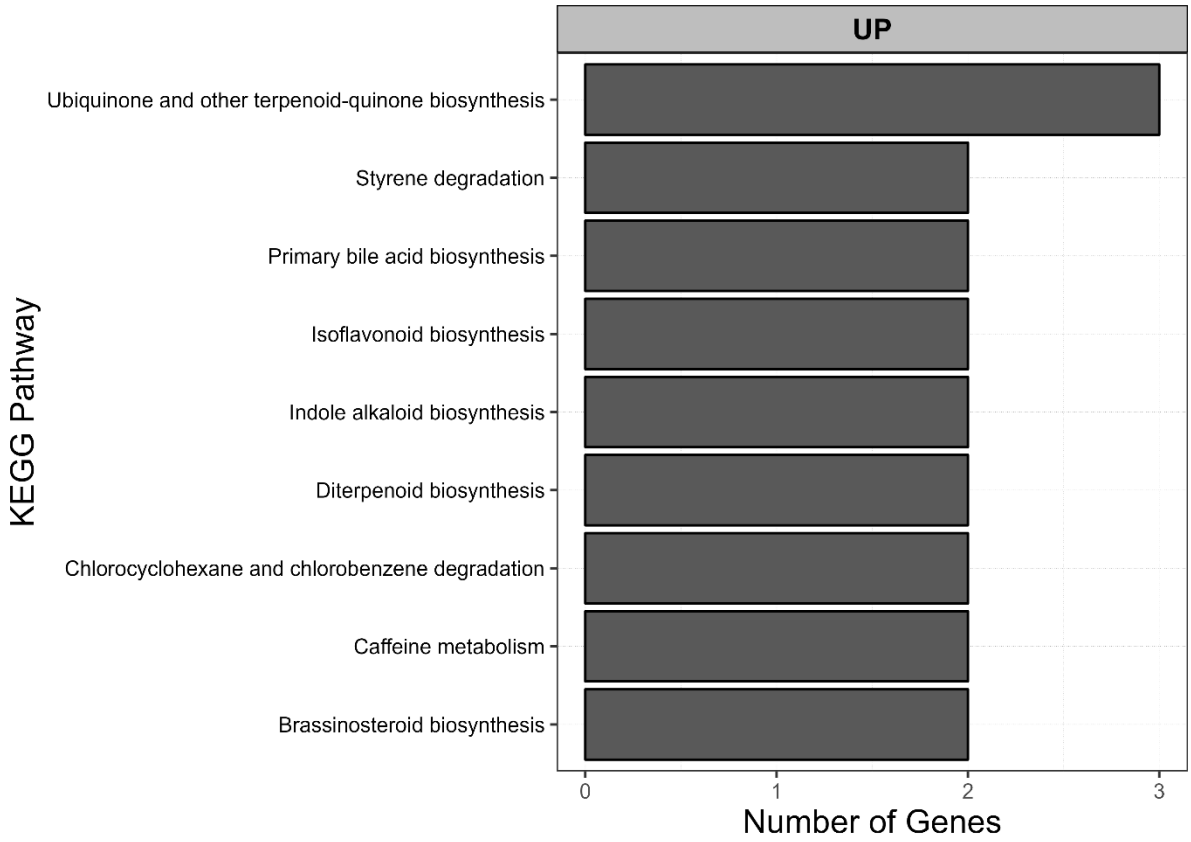


Figure 8 - Significantly enriched KEGG pathways - REC U compared to REC R
UP denotes KEGG terms that were related to genes that were over-expressed in REC U compared to REC R. KEGG pathways shown have a p value ≤0.05. There were no significantly enriched pathways associated with genes that were under-expressed in REC U compared to REC R.

3.4.2.2 The transcriptomic effects switching to malathion selection compared to maintaining temephos selection pressure – REC M versus REC R

The malathion selected REC M strain was compared to the temephos selected REC R strain to investigate the transcriptomic response to switching to an organophosphate larvicide (malathion) compared to maintaining selection with an adulticide (temephos). A total of 280 genes were differentially expressed in REC M compared to REC R (86 under-expressed, 194 over-expressed) (**Figure 9**). Annotations were available for 49 of the gene transcripts (**Supplementary Table 6**). As in other comparisons, a serine/threonine-protein phosphatase 2A inhibitor (AAEL020244) was highly under-expressed in REC M compared to REC R, and a SCP domain-containing protein (AAEL027045) was highly over-expressed.

Gene ontology analysis revealed a limited number of significantly enriched (Bonferroni p≤0.05) GO terms (**Supplementary Table 9**). Of note, six genes that were over-expressed in REC M compared to REC R were identified as being structural constituents of the cuticle (GO:0042302). A total of 14 KEGG pathways were significantly enriched (p≤0.05) relating to over-expressed genes, and 3 to under-expressed genes in REC M compared to REC R (**Figure 10**). Drug metabolism pathways were associated with both under and over expressed genes in this comparison.

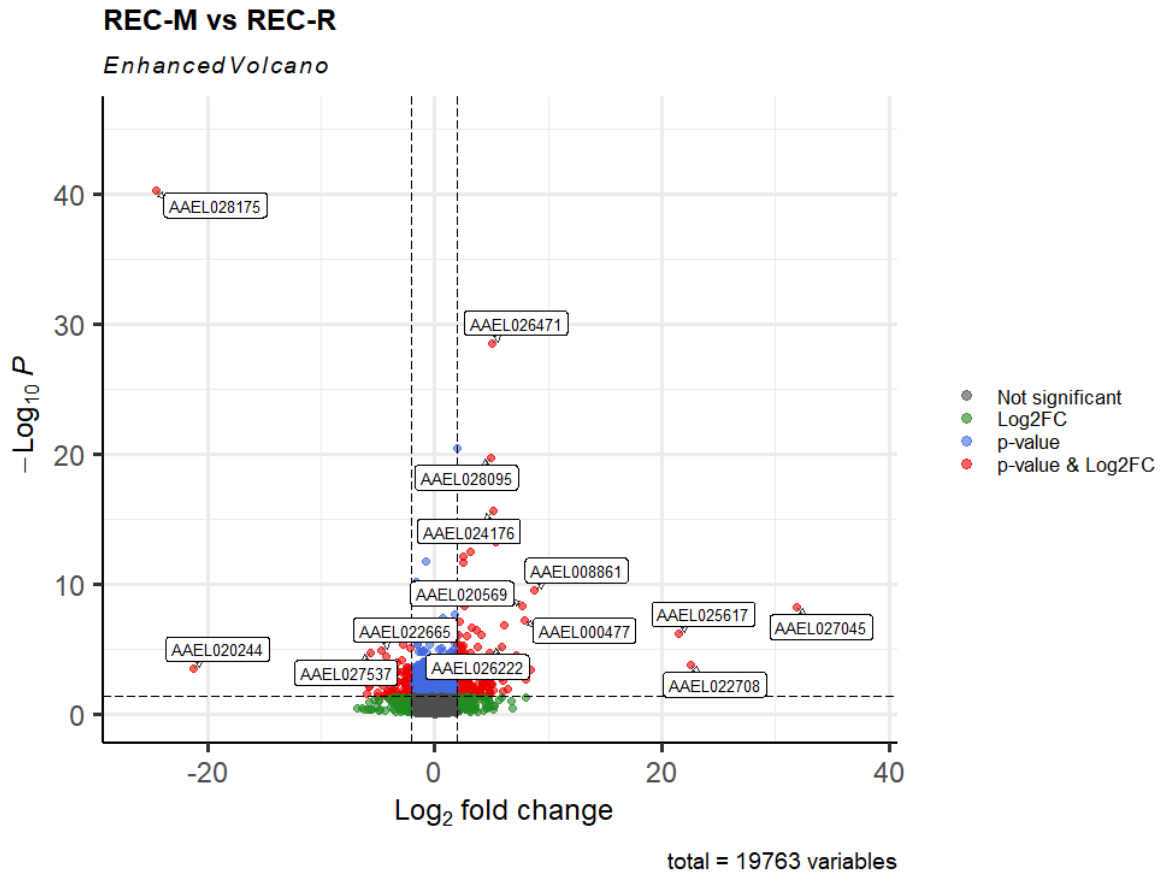


Figure 9 – Volcano plot for differentially expressed genes in REC M compared to REC R
Significantly differentially expressed genes are coloured red (log_2 fold change ≥2 and adjusted p value ≤0.05). A table of all differentially expressed genes and annotations for this comparison is available in **Supplementary Table 6**.

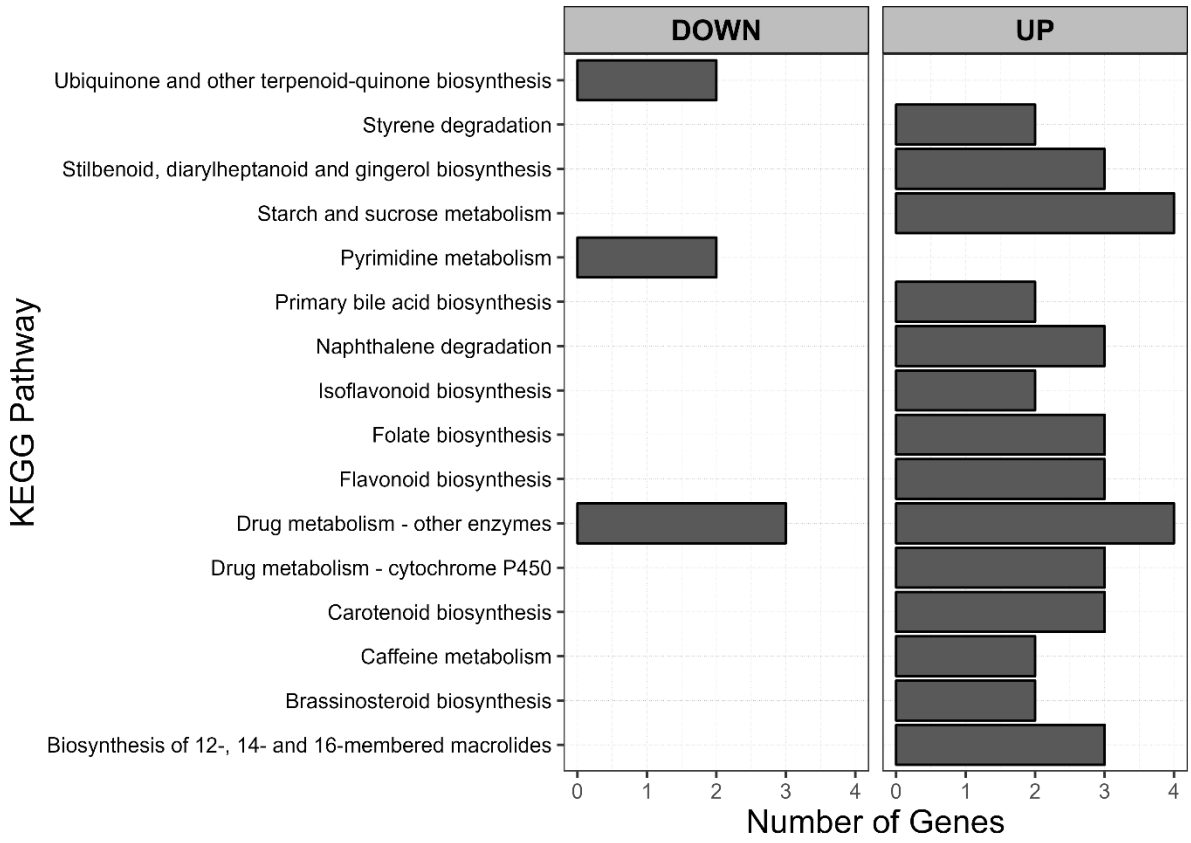


Figure 10 - Significantly enriched KEGG pathways REC M compared to REC R
DOWN denotes pathways that were associated with genes that were under-expressed in REC M compared to REC R. UP denotes pathways that were associated with genes that were over-expressed in REC M compared to REC R. KEGG pathways shown have a p value ≤0.05.

3.4.2.3 The transcriptomic effects switching to permethrin selection compared to maintaining temephos selection – REC P versus REC R

A total of 336 genes were differentially expressed in REC P relative to REC R (222 overexpressed, 114 under-expressed) (**Figure 11**). Annotations were available for 48 of the gene transcripts, and 288 were unspecified transcripts (**Supplementary Table 7**). As with other comparisons, the serine/threonine-protein phosphatase 2A activator (AAEL020244) was highly under-expressed, and an SCP domain-containing protein (AAEL027045) was highly over-expressed in REC P compared to REC R.

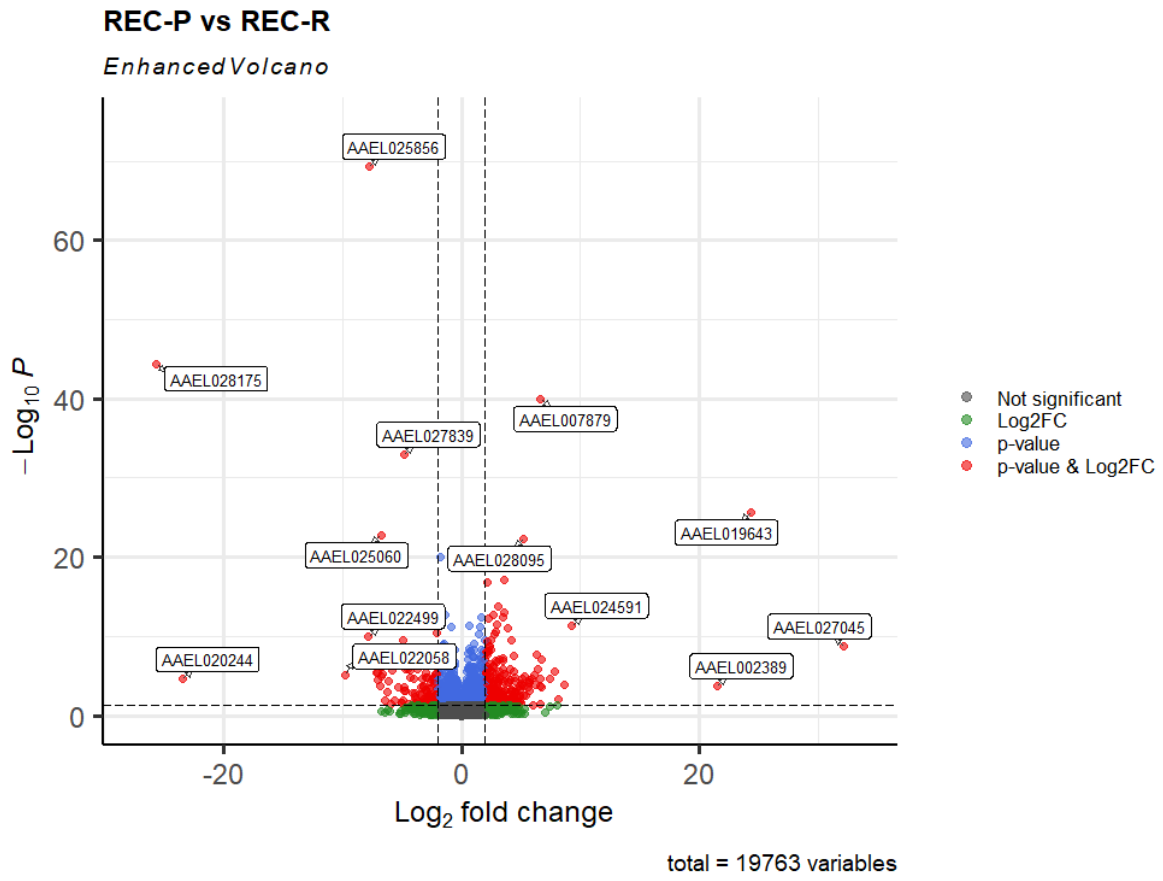


Figure 11 – Volcano plot for differentially expressed genes in REC P compared to REC R
Significantly differentially expressed genes are coloured red (log_2 fold change ≥2 and adjusted p value ≤0.05). A table of all differentially expressed genes and annotations for this comparison is available in **Supplementary Table 7**.

There were no significantly enriched GO terms relating to genes that were over-expressed in REC P compared to REC R. There were several terms enriched in genes that were under-expressed in REC P relative to REC R relating to antibacterial and other immune responses (**Figure 12** & **Supplementary Table 10**). Pathways analysis revealed significantly enriched KEGG pathways relating to metabolism of xenobiotics by cytochrome P450s and glutathione metabolism pathways (**Figure 13**). These were associated with genes that were over-expressed in REC P compared to R. There were no pathways that were significantly enriched relating to genes that were under-expressed in REC P versus REC R.

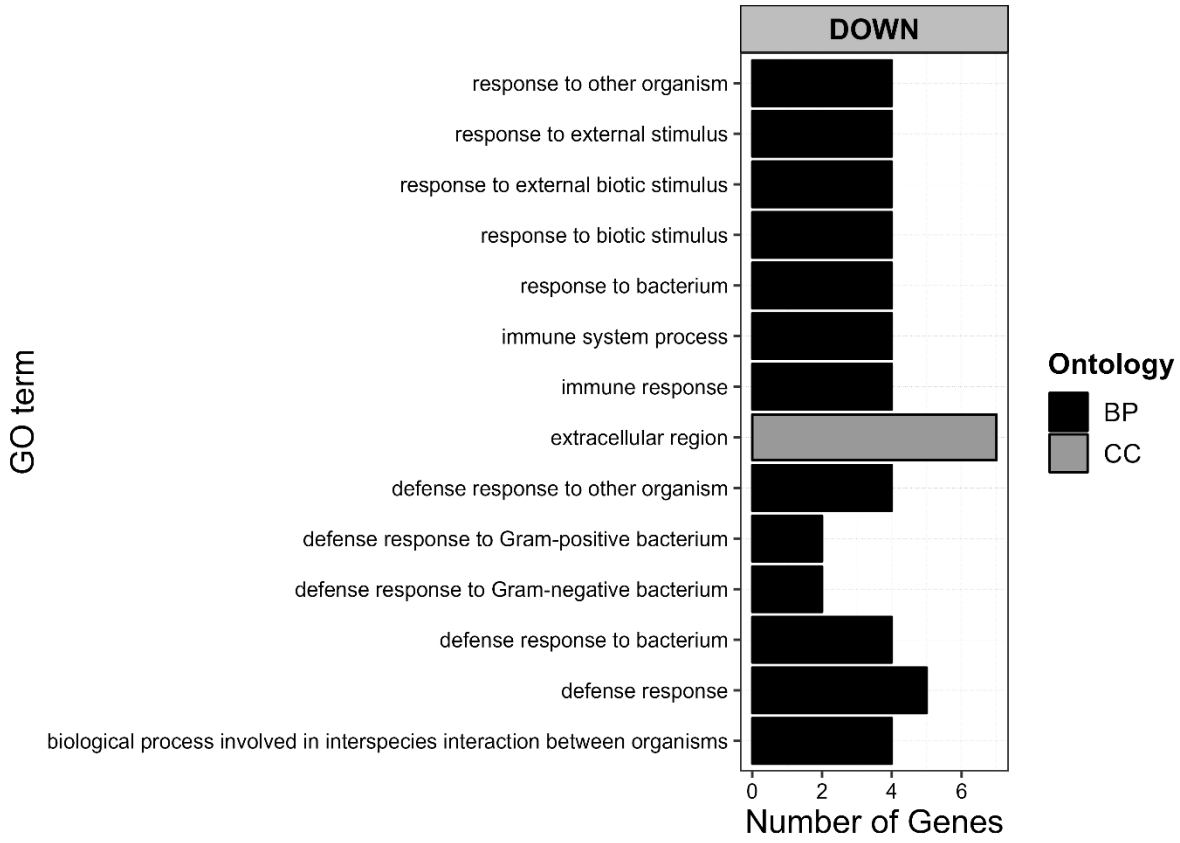


Figure 12- Significantly enriched GO terms in REC P compared to REC R
DOWN denotes GO terms that were related to genes that were under-expressed in REC P compared to REC R. GO terms shown have a Bonferroni p value ≤0.05. GO terms shown are from all available ontologies (BP = biological processes & CC = cellular component

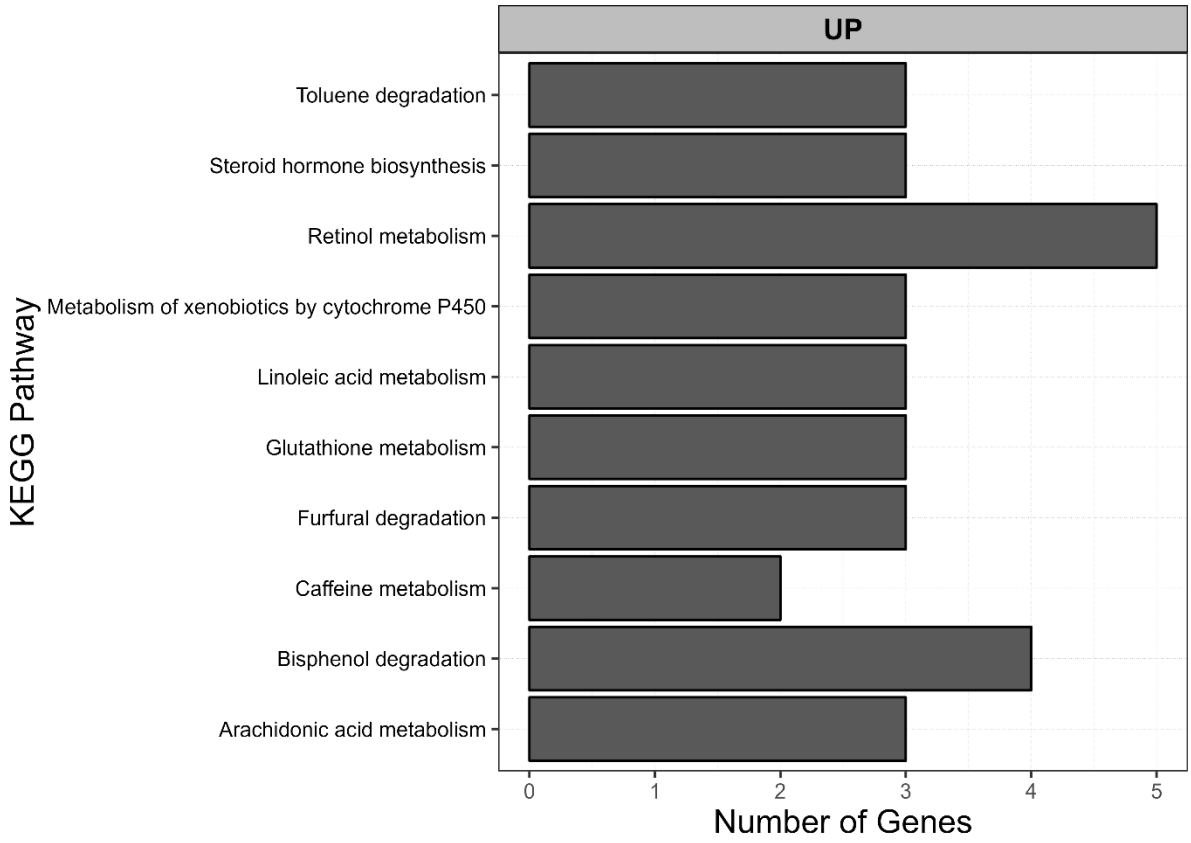


Figure 13 - Significantly enriched KEGG pathways in REC P compared to REC R
UP denotes that KEGG pathways are related to genes that were over-expressed in REC P compared to REC R. KEGG pathways shown have a Bonferroni p value ≤0.05. There were no significantly enriched KEGG pathways associated with genes that were under-expressed in REC P compared to REC R.

3.4.2.4 Genes potentially involved in metabolic insecticide resistance

To directly investigate genes that may be involved in the different insecticide resistance phenotypes of the Recife strains, genes coding for cytochromes, glutathione transferases, and carboxylesterase enzymes were selected (**Figure 14**). All four strains had increased transcription of CYP6F2 compared to REC R (REC U log_2FC 3.31; REC P 2.64; REC M 2.52; REC B 2.13). The cytochrome B561 gene (CYP9J245) was under-expressed in REC U compared to REC R (-2.32). There were several genes that were uniquely differentially expressed in one of the four Recife strains compared to REC R. The cytochrome p450 genes CYP6F3 and CYP325R1 were only significantly overexpressed in REC M versus REC R (log_2FC 3.64, and 2.51, respectively), and CYP9J23 was uniquely under-expressed (-2.31) in this comparison. Three enzymes from different families were only upregulated in REC P compared to REC R: the glutathione S-transferase GSTx2 (2.72); the carboxylesterase CCEae5B (2.38); and the cytochrome P450 CYP6Z6 (2.11). The cytochrome P450 gene CYP4H30 was only significantly differentially expressed in U compared to R (2.22). The expression of detoxification genes in the baseline REC B strain relative to REC R is shown in the **Supplementary Figure 4**.

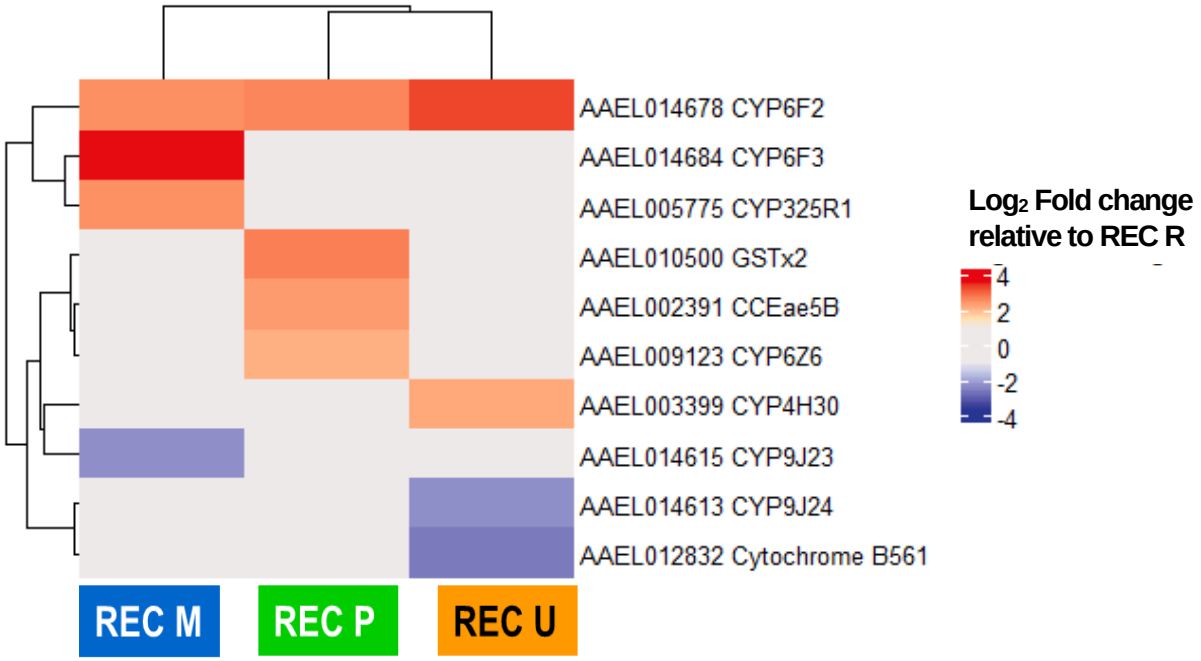


Figure 14 - Heatmap of expression for genes that are potentially involved in insecticide degradation
Scale shows log_2 fold change expression in each strain relative to REC R. Grey colouration indicates the gene was not differentially expressed (Log_2FC ≥2 and adjusted p-value ≤0.05) compared to REC R for that strain. Clustering was performed using hierarchical clustering method. A heatmap for all four strains (including REC B) is shown in **Supplementary Figure 4**.

3.4.2.5 Genes potentially involved in antiviral immunity

There were a number of differentially expressed genes between REC strains that are potentially involved in the antiviral immune response (**Figure 15**). All three REC strains had reduced expression of the cecropin-D genes AAEL029041 (REC U log_2FC -5.63; REC P -3.24; REC M -2.85) and AAEL29046 (REC U log_2FC -3.87; REC M -2.26; REC P -2.19), relative to REC R. REC U had several additional cecropin (AAEL029044 (CECE) log_2FC -2.45; AAEL029047 (CECN) -2.65) and defensin (AAEL003832 (DEFC) -2.53, AAEL003857 (DEFD) -2.94, AAEL003841 (DEFA) -3.01; AAEL027792 (DEFA) -3.01) genes under-expressed relative to REC R. Two of these defensin-A genes were also under-expressed in REC P (AAEL003841 -2.07; AAEL027792 -2.07). The Clip domain serine protease B28 (AAEL013245) which is potentially involved in activation of the Toll pathway, were uniquely under-expressed in REC U (-2.41) compared to REC R. The expression of innate immune genes in the REC B strain compared to REC R is shown in the **Supplementary Figure 5**.

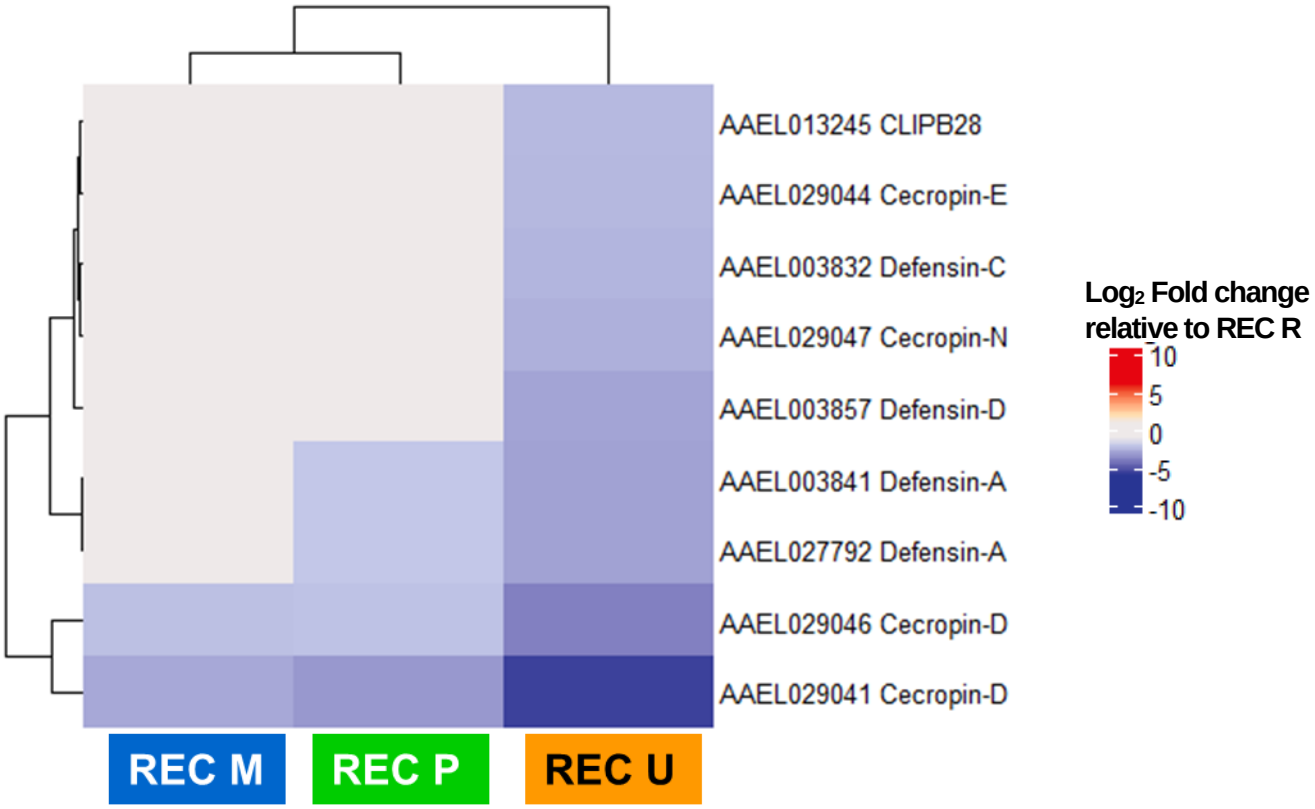


Figure 15 – Heatmap of expression for genes that are potentially involved in antiviral immunity
Scale shows log_2 fold change expression in each strain relative to REC R. Grey colouration indicates the gene was not differentially expressed (Log_2FC ≥2 and adjusted p-value ≤0.05) compared to REC R for that strain. Clustering was performed using hierarchical clustering method. A heatmap for all four strains (including REC B) is shown in **Supplementary Figure 5**.

3.5 Discussion

Bi-annual rotation with insecticides of different classes and modes of action is recommended by the World Health Organization for managing insecticide resistance in *Aedes* vectors [238]. This technique is increasingly being used for the control of insecticide resistant vector populations across the globe [273, 306, 307]. It is clear that changes in insecticide selection regimens can have profound phenotypic effects including survival, and reproductive success [190]. We hypothesised that alterations in insecticide selection pressures would have widespread effects on the transcriptome of *Ae. aegypti* in multiple biological domains, including genes involved in metabolic resistance to insecticides, and innate antiviral immune pathways. Compared to the continuation of temephos selection, removal of temephos, switching to malathion, and switching to permethrin, resulted in the differential expression of 211, 280, and 336 transcripts, respectively, within 10 generations. Whilst there were a number of differentially expressed transcripts that were common in these comparisons (189), the majority (68.6%) were unique to each pairing. Our data, and similar work by others [280, 286, 287, 295], clearly show that alterations in insecticide selection pressure can rapidly lead to profound transcriptomic changes within a small number of generations.

3.5.1 Metabolic insecticide resistance

Altering insecticide selection pressures in the Recife strains led to numerous changes in the expression of detoxification enzymes, many of which have been linked to metabolic insecticide resistance. Whilst there can be significant overlap in detoxification pathways for different insecticides, and previous studies [294] have highlighted marked variability in the suite of detoxification enzymes associated with the observed insecticide resistance phenotype of a vector population, a number of general trends are apparent from the data [286, 287].

3.5.1.1 The effects of the removal of temephos – REC U versus REC R

The cytochrome CYP9J24 was overexpressed in REC R compared to REC U. It is a validated metaboliser of deltamethrin and permethrin [308], and seen in some, but not all, permethrin resistant populations of *Ae. aegypti* [309, 310]. However, as it was overexpressed in the temephos selected strain compared to the susceptible baseline strain in the investigation by Strode et al [280], and similarly seen here to be overexpressed in the temephos selected REC R compared to REC U, it may have a broader role than exclusively pyrethroid metabolism.

The expression of the cytochrome P450 CYP6N12, and the carboxylesterase CCEae3A, have been previously reported by Strode et al [280] to decline within thirteen generations of temephos withdrawal, however, these genes were not differentially expressed between REC R and REC U in our study after nine generations. PCR-based gene expression analysis of these mosquito strains performed by Thornton et al [283], at the same generation as the current study, did not report significant differences in the expression of these genes. The temephos resistant strain used by Strode et al [280] had a higher

LC50 for temephos (1.23mg/L) than the REC R strain used here (0.40 mg/L), and the decline in temephos resistance in their reverting strain was more profound (0.03) than seen in our REC U strain (0.15). Therefore, it is possible that differences in the transcription of CYP6N12 and CCEae3A may only occur in cases of more extreme temephos resistance than achieved here. One cytochrome P450 (CYP4H30), that has previously been observed in permethrin resistant strains of *Ae. aegypti*, was uniquely overexpressed in REC U compared to REC R [311]. It is unclear why this transcript would be over-transcribed in response to the removal of temephos, and suggests it may have important metabolic roles beyond insecticide degradation.

3.5.1.2 The effects of switching to malathion compared to maintaining temephos selection – REC M versus REC R

Despite being from the same class of insecticides, and having the same mechanism of action, temephos and malathion are used to target different life stages, and appear to affect the expression of detoxification genes differently. Comparison of the temephos selected REC R and malathion selected REC M revealed a number of differentially expressed transcripts that may be implicated in organophosphate resistance at these different mosquito lifecycle stages. The cytochrome CYP9J23 was overexpressed in REC R compared to REC M, and its overexpression in organophosphate and pyrethroid resistant mosquitoes has been observed in several studies [287, 311-313]. In addition, CYP325R1, which has been reported to be overexpressed in pyrethroid resistant strains [314], and CYP6F3, which has been implicated in both pyrethroid [315] and temephos resistance [316], were both overexpressed in REC M compared to REC R. The differential expression of these genes in strains selected with different organophosphate insecticides, targeting different lifecycle stages, implies a degree of insecticide specificity in the cytochrome P450 detoxification pathways. However, given the molecular relatedness of temephos and malathion, and the similarity of the temephos and malathion resistance phenotypes in these two strains, it is likely there is significant overlap in the organophosphate detoxification pathways. Furthermore, as temephos is a larvicide, the transcriptomic response to organophosphate exposure may not fully persist into the adult life stage, and therefore would not be detected in our analysis.

3.5.1.3 The effects of switching to permethrin compared to maintaining temephos selection – REC P versus REC R

Comparison of the temephos selected REC R and pyrethroid selected REC P revealed three transcripts that may be involved in permethrin resistance. A carboxylesterase (CCEae5B) and glutathione transferase (GSTX2) were both overexpressed in REC P compared to REC R, and have both been associated with pyrethroid resistance [313, 317, 318]. The cytochrome P450 CYP6Z6 was also overexpressed in REC P (log_2FC 2.11) compared to REC R. This enzyme has been reported to be over-expressed in several populations of permethrin resistant *Aedes* [287, 313, 319, 320]. Furthermore,

cytochrome P450 enzymes of the CYP6 family are considered important markers of permethrin resistance in *Anopheles* vectors [321]. As such, this enzyme may be implicated in the ~5-fold increase in permethrin LC50 seen in REC P compared to REC R. However, as previously reported, permethrin resistance is associated with a diverse and variable suite of detoxification enzymes, which makes it unlikely that the small number of over-expressed genes described here are solely responsible for the dramatic change in resistance phenotype for permethrin [287].

3.5.2 Expression of genes potentially involved in antiviral immunity

Alteration of insecticide selection pressure was associated with several differences in the expression of genes potentially involved in innate antiviral immune responses. Changes in the expression of immune genes can be transient in nature and occur in response to a wide range of stimuli including bacteria, fungi, and insect specific viruses [322, 323]. As such, further investigation will be required to determine whether the observed changes are transitory in nature or represent persistent, intergenerational differences in gene expression. Nevertheless, several interesting trends are present.

Two genes encoding cecropin-D (AAEL029046 & AAEL029041) were over-expressed in REC R compared to all the other strains. Cecropins constitute an important family of AMPs and are known to be involved in the antiviral immune response [114, 324-327]. Both these cecropins were also over-expressed in REC R compared to the baseline REC B strain (see **Supplementary data** section 3.7.1.2). This shows that there was a unique upregulation of the innate immune response in REC R during the selection process, that was not present in the baseline strain prior to selection, nor any of the other strains following the selection regimens. It is difficult to retrospectively determine the reason for this immune upregulation, as these components of the innate immune system respond similarly to a wide range of stimuli [322]. As REC R was continued on the same selection regimen as the original REC B strain, it is unclear why there is a difference in immune gene expression.

However, the removal of temephos appears to be associated with decreased immune gene expression. For example, the permethrin selected REC P showed a unique under-expression of two genes encoding defensin-A (AAEL03841 and AAEL027792). In comparison, switching to malathion from temephos, both organophosphate insecticides, was associated with the fewest number of differentially expressed immune genes. Our data show a unique under-expression of several genes encoding cecropins (CECE and CECN), defensins (DEFC and DEFD) and the Clip-domain serine proteinase B28 in the unselected REC U compared to the temephos selected REC R. Clip-domain serine proteases may be involved in the initiation of the Toll pathway through activation of the Spätzle ligand, which ultimately leads to the production of antimicrobial peptides including defensins and cecropins [328]. Taken together, these data suggest that there could be an effect of organophosphate insecticide resistance on innate immune gene expression. This was not observed in a previous evaluation of the transcriptomic effects of removing temephos in the Recife strain [280].

A potential explanation for the differences in immune gene expression between strains could relate to changes in the composition and/or diversity of the microbiome.The microbiome is known to be influenced by insecticide resistance status and is also a modulator of the innate immune system [202, 329, 330]. It is possible that uncharacterised differences in the microbiome of the Recife strains, resulting from differing insecticide selection pressures, may be involved in the observed differences in immune gene expression. Further investigation investigating the microbial composition and diversity of these strains in the presence and absence of insecticide exposure, will be required to explore this hypothesis.

An additional hypothesis for the reduced expression of a larger number of immune genes in REC U could be due to a reduction in cytochrome P450 activity in the absence of insecticide pressure in REC U. Cytochrome P450s are known to increase basal levels of ROS, which can trigger AMP production via activation of the Toll pathway [331, 332]. A decline in P450 activity following the withdrawal of temephos in REC U may have resulted in reduced activation of the Toll pathway, and led to decreased expression of genes encoding AMPs.

There has been limited investigation on the effects of insecticide resistance on immune gene expression. David et al [295] reported that in *Ae. aegypti* selected with permethrin, imidacloprid or propoxur, several genes encoding cecropin, defensins and Clip-domain serine proteases were under-expressed. However, investigation of insecticide resistant *Anopheles* and *Culex* reported higher constitutive expression of cecropins and defensins than susceptible populations [298, 299]. It is clear that the immune responses associated with insecticide exposure and resistance are complex and require further investigation. Importantly, it has been shown that a DENV-refractory *Ae. aegypti* had higher long-term basal expression of cecropins and other AMPs [333]. Therefore, if the observed differences in immune gene transcription are not a transient phenomenon, and persist intergenerationally, this could have consequences for the vector competence of these insecticide selected *Aedes* strains for arboviruses. As such, it would be interesting to see whether vector competence for arboviruses is altered in these Recife strains. This is investigated in **Chapter 4**.

Future work should also further explore the effects of temephos selection on immune gene expression by additional insecticide rotations in the Recife strains, particularly reintroducing temephos to REC U. Furthermore, the insecticide selection pressures could be removed from REC M and P, and the transcriptomic response monitored, to see if similar effects on immune gene transcription occur with the removal of other insecticides. Vector competence experiments could be conducted with accompanying gene expression analyses to identify any changes in innate immune responses. Finally, other critical antiviral immune pathways, especially RNA interference, should be investigated in these strains, to fully understand the breadth of the innate immune response.

3.5.3 Limitations

The data presented here are derived from only one replicate of each insecticide rotation scenario. Obtaining further replicates of each insecticide, by deriving multiple sub-colonies for each scenario, would improve confidence in the observed results and allow an assessment of the replicability of the findings. Another important limitation that may have influenced our results relates to population bottlenecks and the potential for genetic drift. The insecticide selection regimens used may have led to the stochastic over-representation of alleles in subsequent generations that would not be observed in larger populations in the field. Performing replicates of each rotation scenario would help highlight this effect. Further timepoints during the selection process would help produce a more complete picture of the effects of insecticide rotation on the transcriptome.

3.5.4 Conclusion

Alteration of insecticide selection regimens has widespread effects on the transcriptome of *Ae. aegypti* within a relatively small number of generations. Differential expression of cytochrome P450s, glutathione transferases, and carboxylesterases, provides an insight into the detoxification pathways involved in the insecticide resistance phenotypes seen in the Recife strains. Our data suggest that changes in insecticide selection pressures could be linked with alterations in the expression of genes encoding AMPs, and activators of the Toll pathway. This could have consequences for vector competence and warrants further investigation.

3.6 Acknowledgements

I would like to thank Sanjay Nagi for his help with the initial analysis using the RNASeqPop pipeline. I would also like to thank Laura Brettell for her help with differential expression and gene ontology analyses. The original experimental design, mosquito selection regimens, RNA sample collection, and sequencing were performed by Lisa Reimer, Bruno Gomes, Jonathan Thornton, and other members of the team.

3.7 Supplementary data

3.7.1 Recife Baseline (REC B) compared to temephos selected Recife (REC R)

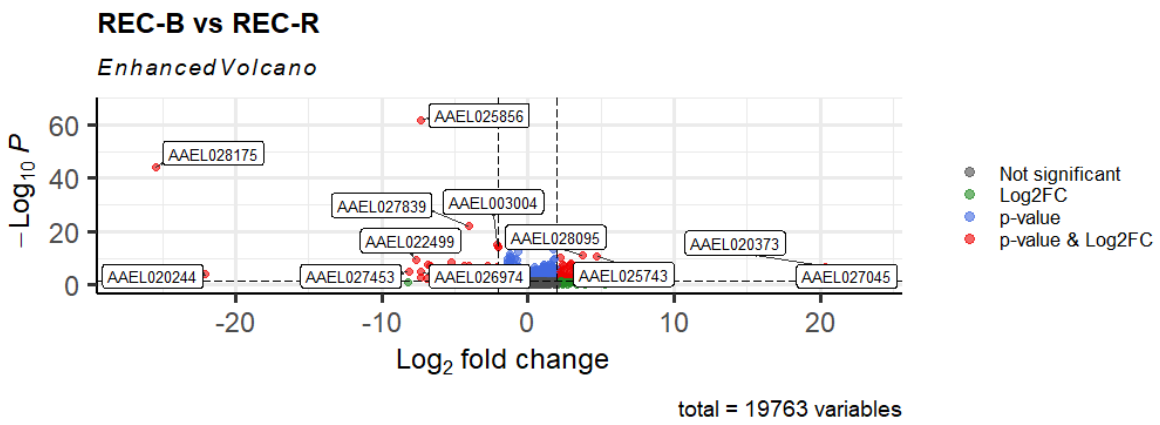
To investigate what effect laboratory colonisation had on the transcriptome of the Recife strain, the baseline REC B strain was compared to the REC R strain which was maintained under the same temephos selection regimen. The sequencing and alignment quality data for REC B are shown in **Supplementary Table 1**. A total of 202 genes were differentially expressed in REC B compared to REC R (**Supplementary Table 2**). One hundred of these were underexpressed, and 102 were over-expressed in REC B compared to REC R (**Supplementary Figure 2**). These genes had 249 associated transcripts, 53 of which had annotations available and 196 were unknown transcripts. The most highly overexpressed (log_2FC 25.5 gene in REC R compared to REC B (AAEL028175) has no known function. Another highly overexpressed gene (AAEL020244) coding for a serine/threonine-protein phosphatase 2A activator was observed in REC R compared to REC B. A gene coding for an SCP domain-containing protein (AAEL027045) was highly overexpressed in REC B versus REC R.

Gene ontology enrichment analysis revealed a number of metabolic and immunity associated differences between REC R and REC B. Significantly enriched GO terms (Bonferroni $p \leq 0.05$) for serine hydrolase and peptidase activity, and other catabolic processes were associated with under-expressed genes in REC B compared to REC R (**Supplementary Figure 3**, **Supplementary Tables 3 & 4**). All observed GO terms relating to innate immunity were associated with 3 genes that were significantly under-expressed in REC B (AAEL003389, AAEL029041, AAEL029046) compared to REC R. Pathways analysis revealed that there were no significantly enriched pathways relating to genes over-expressed in REC B versus REC R, and a single KEGG pathway (ec00120) relating to primary bile acid biosynthesis was enriched relating to genes that were under-expressed in REC B compared to REC R.

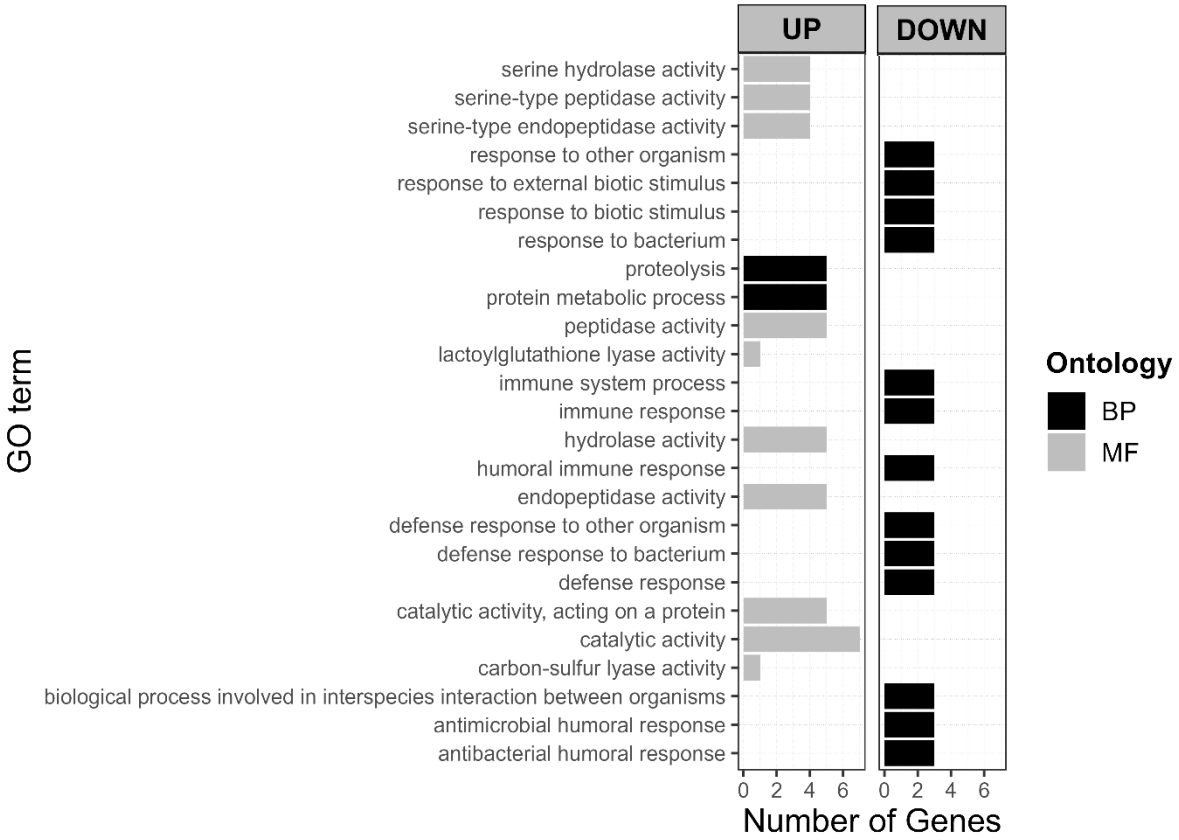
Supplementary Table 1 – Illumina sequencing and alignment quality

Strain	Replicates	Untrimmed	Trimmed	Pair R1/R2	Total aligned	Aligned (%)
REC-B	5	268,638,244	267,309,403	132,996,598	121309298	91.21

Supplementary Table 1 – Illumina sequencing and quality for REC B
Five biological replicates were collected for the Recife baseline strain at generation 37. A total of approx. 268 thousand reads were mapped to the reference *Aedes aegypti* Liverpool AWGW genome. >91% paired R1/R2 reads were aligned.



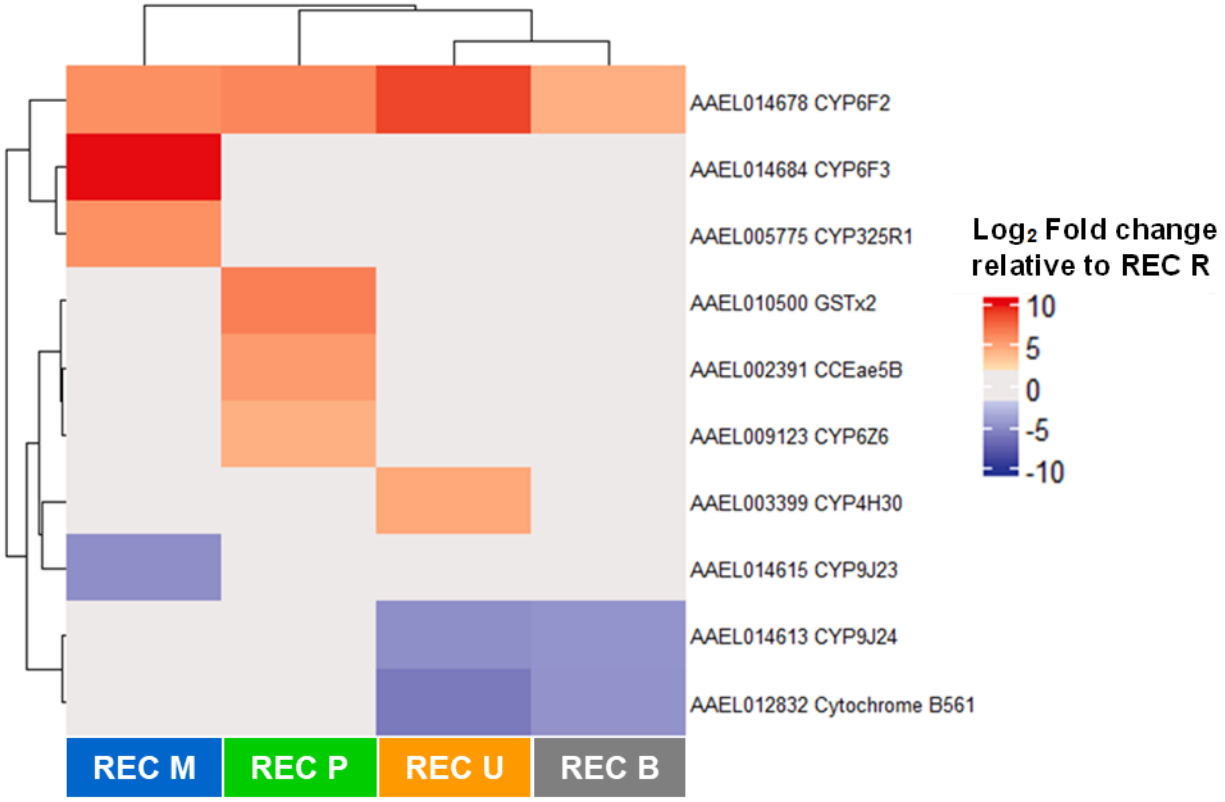
Supplementary Figure 2 – Volcano plot for differentially expressed gene in REC B vs REC R
Significantly differentially expressed genes are coloured red (log_2 fold change ≥2 and adjusted p value ≤0.05). A table of all differentially expressed genes and annotations for this comparison is available in **Supplementary Table 2**.



Supplementary Figure 3 - Significantly enriched GO terms in REC B vs REC R
DOWN denotes the GO terms are related to genes that were under-expressed in REC B compared to REC R. UP denotes GO terms that were related to genes that were over-expressed in REC B compared to REC R. GO terms shown have a Bonferroni p value ≤0.05. GO terms shown are from all available ontologies (BP = biological processes, MF = molecular function). No GO terms significantly enriched from cellular component ontology.

3.7.1.1 Detoxification genes

REC B had lower expression of a cytochrome B561 gene (-2.25), and cytochrome P450 CYP9J24 (-2.23) than REC R. Like the other strains, the expression of CYP6F2 was higher than REC R.

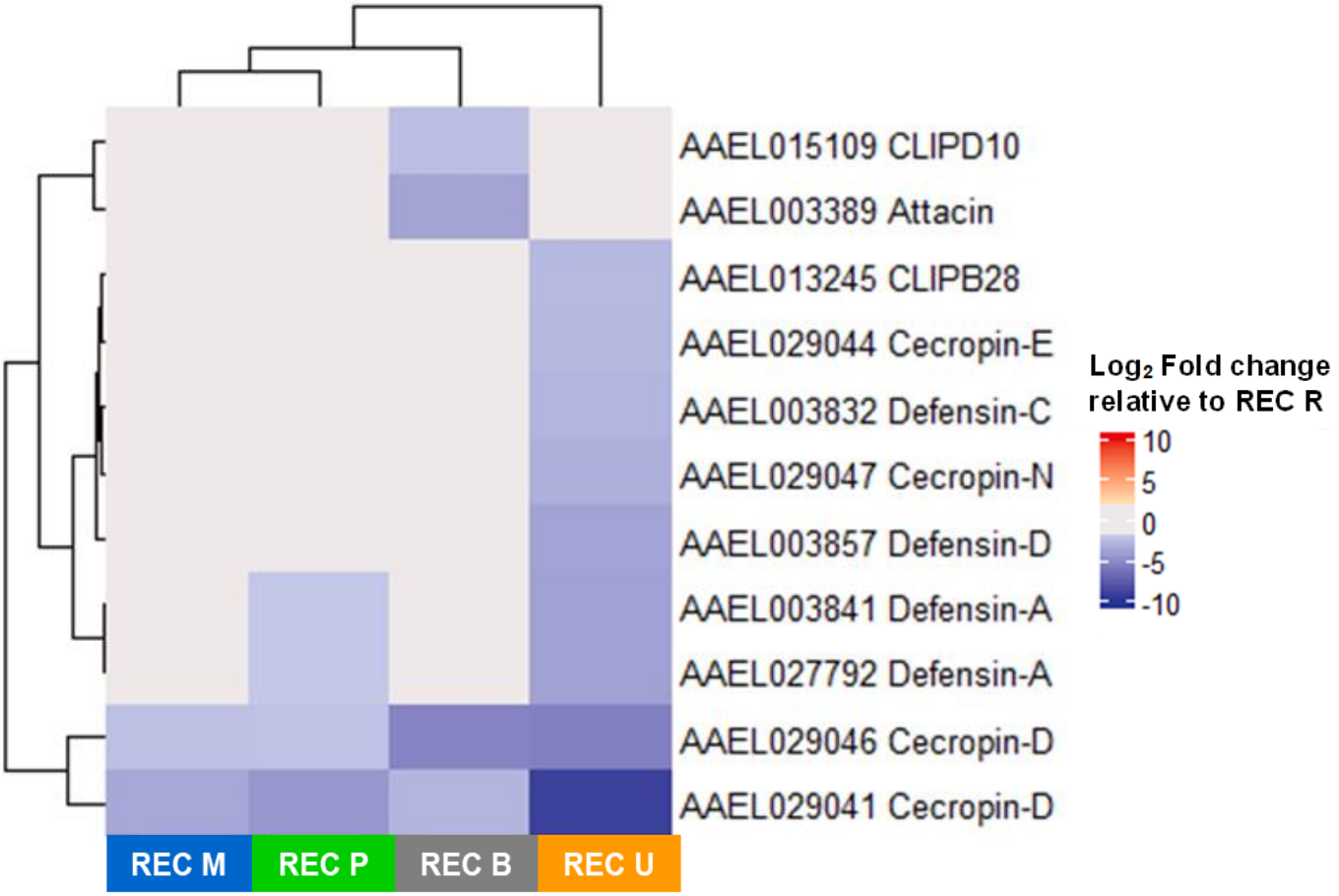


Supplementary Figure 4 – Detoxification gene expression in all Recife strains
Scale shows log_2 fold change expression in each strain relative to REC R. Grey colouration indicates the gene was not differentially expressed ($Log_2FC \geq 2$ and adjusted p-value ≤ 0.05) compared to REC R for that strain. Clustering was performed using hierarchical clustering method.

3.7.1.2 Innate immunity genes

REC B had two genes encoding cecropin-D (AAEL029041 & AAEL29046) that were under-expressed (Log_2FC -2.51 and -3.75, respectively) compared to REC R. These genes were also under-expressed in the other Recife strains relative to REC R, suggesting that they were uniquely upregulated in REC R during the selection process. Genes encoding attacin were uniquely under-expressed in REC B compared to REC R (AAEL003389, Log_2FC -2.51) Attacins have been implicated in the antiviral response of *Drosophila melanogaster* for Sindbis virus [334], and are overexpressed in *Ae. aegypti* infected with dengue virus (DENV) [135]. In addition, the Clip domain serine protease D10 was uniquely under-expressed in REC B compared to REC R (-2.26). Clip domain serine proteases can be involved in activation of the Toll pathway, and therefore may potentially be involved in antiviral immune pathways [328]. Neither of these genes were differentially expressed in REC R compared to REC U, REC P, or REC M, suggesting these strains have similar expression of attacin and CLIPD10 to REC R. Therefore,

it seems that all strains have upregulated CLIPD10 and attacin to a broadly similar level during the selection regimens. Taken together, these changes suggest that the selection regimens produced upregulation of CLIPD10 and attacin in all strains relative to REC B.



Supplementary Figure 5 – Innate immunity gene expression in all Recife strains
Scale shows log_2 fold change expression in each strain relative to REC R. Grey colouration indicates the gene was not differentially expressed ($Log_2FC \geq 2$ and adjusted p-value ≤ 0.05) compared to REC R for that strain. Clustering was performed using hierarchical clustering method.

3.7.1.3 Differentially expressed genes in REC B compared to REC R

Supplementary Table 2 - Differentially expressed genes in REC B compared to REC R			
Gene ID	**Log_2 Fold Change**	**Adjusted p-value**	**Annotation**
AAEL028175	-25.49	8.40E-45	
AAEL020244	-22.11	8.96E-05	Serine/threonine-protein phosphatase 2A activator
AAEL027453	-8.15	6.39E-06	
AAEL022499	-7.67	3.41E-10	
AAEL022058	-7.35	1.41E-03	
AAEL025856	-7.31	1.63E-62	
AAEL026974	-7.29	1.52E-05	
AAEL024804	-6.92	4.42E-03	
AAEL027537	-6.86	2.19E-08	
AAEL021627	-6.67	8.74E-08	
AAEL012867	-6.34	6.52E-07	
AAEL024645	-6.27	8.02E-04	
AAEL019388	-6.12	9.40E-05	
AAEL022121	-5.75	1.56E-04	
AAEL021292	-5.74	1.51E-04	
AAEL013998	-5.74	7.41E-05	General transcription factor IIF subunit 2
AAEL012866	-5.55	2.21E-04	
AAEL020040	-5.53	3.61E-02	
AAEL025887	-5.50	8.88E-03	Zinc finger CCCH-type with G patch domain-containing protein
AAEL028245	-5.36	1.24E-03	
AAEL024622	-5.23	3.54E-02	
AAEL014763	-5.23	4.77E-05	tak1 binding protein-1
AAEL027009	-5.22	5.14E-09	
AAEL012860	-5.09	1.51E-03	
AAEL020882	-4.97	5.64E-04	
AAEL020699	-4.71	5.73E-04	
AAEL001650	-4.71	7.43E-06	ML domain-containing protein
AAEL025491	-4.65	3.06E-06	
AAEL027629	-4.64	1.05E-03	
AAEL027983	-4.47	4.18E-02	
AAEL025362	-4.47	6.49E-04	60S ribosomal protein L10
AAEL023113	-4.45	7.95E-04	
AAEL003467	-4.43	2.29E-06	
AAEL020850	-4.38	8.72E-08	

AAEL012864	-4.33	1.98E-06	
AAEL025812	-4.12	1.25E-03	
AAEL009149	-4.06	2.45E-05	kinectin, putative
AAEL027839	-4.05	8.86E-23	Putative translocon-associated protein
AAEL023018	-4.03	1.02E-07	
AAEL010048	-4.01	4.09E-02	26S proteasome non-ATPase regulatory subunit
AAEL027924	-4.00	7.22E-05	
AAEL007641	-3.87	3.78E-06	
AAEL027922	-3.85	4.86E-04	
AAEL020619	-3.81	2.32E-04	
AAEL026117	-3.81	1.79E-02	
AAEL029046	-3.75	1.72E-05	Cecropin-D
AAEL027895	-3.68	3.16E-02	
AAEL021346	-3.64	1.51E-02	
AAEL021528	-3.48	3.71E-04	
AAEL010045	-3.45	1.77E-02	Protein FAM50 homolog
AAEL022033	-3.28	1.21E-03	
AAEL022306	-3.28	2.26E-04	
AAEL021637	-3.18	1.49E-02	
AAEL000012	-3.18	9.64E-03	gustatory receptor Gr6
AAEL010393	-3.06	3.87E-02	ferritin subunit, putative
AAEL026734	-3.06	8.87E-03	
AAEL028076	-2.96	1.67E-02	
AAEL003389	-2.95	2.93E-03	attacin anti-microbial peptide
AAEL023587	-2.93	7.71E-05	
AAEL023901	-2.85	3.61E-05	
AAEL028653	-2.85	4.44E-02	
AAEL009181	-2.84	7.62E-06	Uncharacterized protein AEGI23
AAEL024161	-2.74	8.43E-08	
AAEL026026	-2.73	4.70E-03	
AAEL025278	-2.72	1.84E-03	
AAEL010894	-2.61	1.34E-04	carbonic anhydrase II, putative
AAEL005777	-2.60	1.92E-04	
AAEL009484	-2.60	4.08E-02	
AAEL022056	-2.54	8.27E-03	
AAEL008012	-2.53	3.60E-02	
AAEL029041	-2.51	1.96E-03	
AAEL001118	-2.42	5.17E-04	
AAEL028157	-2.39	1.68E-04	

AAEL022559	-2.36	4.91E-04	
AAEL002612	-2.35	3.33E-05	
AAEL017538	-2.35	3.33E-05	
AAEL023370	-2.31	5.60E-03	
AAEL021632	-2.31	1.36E-03	
AAEL028240	-2.30	2.68E-03	
AAEL011014	-2.29	6.21E-03	
AAEL015109	-2.26	6.37E-04	CLIP D10. Clip-Domain Serine Protease family D.
AAEL012832	-2.25	3.14E-04	cytochrome B561
AAEL007814	-2.24	1.24E-02	n-twist
AAEL014613	-2.23	2.14E-02	CYP9J24 cytochrome P450
AAEL010434	-2.21	2.73E-02	vitellogenin-A1 precursor
AAEL023034	-2.17	7.95E-03	
AAEL003773	-2.16	1.83E-06	
AAEL026801	-2.15	4.69E-02	
AAEL010677	-2.15	1.25E-07	Oxidoreductase
AAEL006953	-2.15	5.09E-04	
AAEL003004	-2.09	7.86E-16	
AAEL023260	-2.08	4.53E-02	
AAEL023683	-2.07	3.72E-02	
AAEL015313	-2.05	6.25E-03	odorant binding protein OBP59
AAEL025231	-2.04	1.13E-03	
AAEL010140	-2.03	9.50E-03	sodium/potassium-dependent ATPase beta-2 subunit
AAEL023819	-2.03	3.19E-03	
AAEL022633	-2.01	1.29E-02	
AAEL002993	-2.00	8.12E-15	mitochondrial ribosomal protein L43
AAEL021617	.00	8.12E-15	
AAEL025568	2.01	1.25E-03	
AAEL020874	2.03	2.63E-04	
AAEL021964	2.04	7.49E-03	
AAEL016998	2.06	1.03E-06	
AAEL022817	2.06	4.72E-03	
AAEL025940	2.08	2.70E-04	
AAEL006421	2.09	2.92E-02	trypsin, putative
AAEL025652	2.10	4.65E-04	
AAEL022186	2.11	1.29E-03	
AAEL017252	2.12	1.06E-02	
AAEL014678	2.13	2.27E-03	cytochrome P450
AAEL022414	2.13	3.45E-02	

AAEL013415	2.13	2.81E-03	alpha-tropomyosin 5a, putative
AAEL021393	2.14	2.40E-02	
AAEL025770	2.14	1.85E-04	
AAEL025354	2.14	1.38E-02	
AAEL021463	2.15	3.04E-05	
AAEL011591	2.19	3.19E-02	
AAEL023170	2.21	1.80E-03	
AAEL019524	2.23	7.67E-06	
AAEL019508	2.24	3.58E-11	
AAEL006365	2.28	9.77E-03	trypsin-alpha, putative
AAEL021469	2.29	2.56E-02	
AAEL027695	2.29	1.79E-04	
AAEL026050	2.31	3.08E-03	
AAEL023599	2.31	2.70E-02	
AAEL022102	2.38	2.75E-02	
AAEL020836	2.39	8.63E-03	
AAEL013449	2.41	2.18E-02	metalloproteinase, putative
AAEL006627	2.41	1.18E-02	serine-type endopeptidase,
AAEL022185	2.41	1.69E-08	
AAEL024271	2.42	1.06E-07	
AAEL021207	2.42	1.23E-06	
AAEL027763	2.43	5.22E-03	
AAEL027592	2.44	1.61E-02	
AAEL020404	2.49	3.62E-03	
AAEL024453	2.49	2.04E-02	
AAEL015092	2.49	4.38E-06	
AAEL024275	2.55	6.49E-04	
AAEL027758	2.55	2.91E-02	
AAEL008467	2.55	2.40E-03	cysteine synthase
AAEL008752	2.58	3.87E-04	
AAEL023766	2.58	1.74E-02	
AAEL020188	2.59	1.64E-02	
AAEL028122	2.63	8.19E-03	
AAEL006430	2.63	2.53E-03	Trypsin
AAEL021854	2.64	1.01E-02	
AAEL026541	2.65	2.23E-02	
AAEL005108	2.71	5.67E-03	manganese-iron (Mn-Fe) superoxide dismutase
AAEL021656	2.74	6.18E-03	
AAEL025866	2.84	4.02E-02	

AAEL001196	2.85	3.96E-08	Cadherin
AAEL022664	2.90	1.62E-05	
AAEL025604	2.92	3.17E-08	
AAEL027653	2.93	1.53E-03	
AAEL022921	2.94	1.13E-05	
AAEL004398	2.95	1.21E-08	GPCR Octopamine/Tyramine Family
AAEL022380	2.98	7.82E-05	
AAEL025598	2.99	2.20E-02	
AAEL020226	3.01	1.42E-02	
AAEL025894	3.06	5.66E-03	
AAEL009796	3.11	1.24E-03	cuticle protein, putative
AAEL021842	3.11	3.43E-02	
AAEL029066	3.11	2.60E-03	
AAEL021890	3.21	7.52E-08	
AAEL026781	3.25	3.73E-02	
AAEL026953	3.45	1.70E-03	
AAEL007870	3.46	1.83E-07	
AAEL006429	3.48	3.48E-04	Trypsin
AAEL026362	3.52	1.38E-02	
AAEL027752	3.52	1.38E-02	
AAEL014020	3.63	4.82E-03	
AAEL017488	3.68	4.65E-04	gustatory receptor Gr49
AAEL028095	3.75	9.63E-12	
AAEL022554	3.77	1.76E-05	
AAEL020163	3.79	5.73E-04	
AAEL027911	3.79	1.29E-02	
AAEL004762	3.88	2.61E-04	pupal cuticle protein, putative
AAEL021553	4.00	5.17E-04	
AAEL026228	4.05	2.08E-06	
AAEL020569	4.17	1.64E-03	
AAEL025068	4.44	4.11E-03	
AAEL023686	4.55	4.72E-03	
AAEL026198	4.58	3.27E-07	
AAEL025144	4.60	3.83E-03	
AAEL025743	4.68	1.40E-11	
AAEL021683	4.81	4.12E-03	
AAEL026222	4.88	5.70E-05	
AAEL026268	4.91	7.58E-06	
AAEL021914	5.28	2.59E-02	

AAEL027016	5.43	3.32E-05	
AAEL028077	5.48	3.44E-05	
AAEL027747	5.67	1.96E-06	
AAEL025877	5.71	2.77E-03	
AAEL020633	6.23	4.87E-04	
AAEL023533	6.61	7.69E-04	
AAEL021122	6.71	2.19E-05	
AAEL020726	6.74	2.74E-02	
AAEL020298	7.26	1.66E-03	
AAEL023226	8.21	3.46E-02	
AAEL020373	20.32	1.17E-07	
AAEL027045	21.63	1.74E-04	SCP domain-containing protein

3.7.1.4 Enriched gene ontology terms in REC R versus REC B

Supplementary Table 3
GO terms relating to significantly over-expressed genes in REC B versus REC R

GO ID	Ontology	GO term	Associated genes	Bonferroni p value
0004175	MF	Endopeptidase activity	AAEL006365,AAEL006421, AAEL006627, AAEL013449, AAEL021656,	6.47E-05
0008233	MF	Peptidase activity		3.03E-04
0004252	MF	Serine-type endopeptidase activity	AAEL006365, AAEL006421, AAEL006627, AAEL021656,	7.24E-04
0006508	BP	Proteolysis	AAEL006365, AAEL006421, AAEL006627, AAEL013449, AAEL021656	7.42E-04
0008236	MF	Serine-type peptidase activity	AAEL006365, AAEL006421, AAEL006627, AAEL021656	9.63E-04
0017171	MF	Serine hydrolase activity		9.63E-04
0140096	MF	Catalytic activity, acting on a protein	AAEL006365, AAEL006421, AAEL006627, AAEL013449, AAEL021656,	5.29E-03
0003824	MF	Catalytic activity	AAEL005108, AAEL006365, AAEL006421, AAEL006627, AAEL013449, AAEL021656, AAEL027763	9.47E-03
0016787	MF	Hydrolase activity	AAEL006365, AAEL006421, AAEL006627, AAEL013449, AAEL021656	1.94E-02
0004462	MF	Lactoylglutathione lyase activity	AAEL027763	3.03E-02
0019538	BP	Protein metabolic process	AAEL006365, AAEL006421, AAEL006627, AAEL013449, AAEL021656	3.74E-02
0016846	MF	Carbon-sulfur lyase activity	AAEL027763	4.54E-02

Supplementary Table 4 - GO terms relating to significantly under-expressed genes in REC B versus REC R				
GO ID	**Ontology**	**GO term**	**Associated genes**	**Bonferroni p value**
0019720	BP	Antimicrobial humoral response	AAEL003389, AAEL029041, AAEL029046	2.21E-03
0006959	BP	Humoral immune response		2.21E-03
0019731	BP	Antibacterial humoral response		2.21E-03
009617	BP	Response to bacterium		8.26E-03
0042742	BP	Defense response to bacterium		8.26E-03
0006955	BP	Immune response		3.58E-02
0044419	BP	Biological process involved in interspecies interaction between organisms		3.58E-02
0098542	BP	Defence response to other organism		3.58E-02
0009607	BP	Response to biotic stimulus		3.58E-02
0051707	BP	Response to other organism		3.58E-02
0043207	BP	Response to external biotic stimulus		3.58E-02
0006952	BP	Defense response		4.57E-02
0002376	BP	Immune system process		4.57E-02

3.7.2 Lists of differentially expressed genes for all other comparisons

3.7.2.1 Differentially expressed genes in REC U compared to REC R

Supplementary Table 5 - Differentially expressed genes in REC U compared to REC R			
Gene ID	**Log_2 Fold Change**	**Adjusted p-value**	**Annotation**
AAEL028175	-25.78	8.0E-46	
AAEL020244	-23.84	3.4E-05	Serine/threonine-protein phosphatase 2A activator
AAEL022058	-9.20	5.9E-05	
AAEL027453	-8.53	3.1E-06	
AAEL022121	-8.00	3.0E-08	
AAEL025856	-7.38	1.4E-63	
AAEL026974	-7.37	2.2E-05	
AAEL021292	-7.29	7.5E-07	
AAEL021627	-7.00	2.1E-08	
AAEL025887	-6.75	2.0E-03	Zinc finger CCCH-type with G patch domain-containing protein
AAEL012867	-6.48	4.8E-07	
AAEL022499	-6.48	4.9E-07	
AAEL012860	-5.83	4.2E-04	
AAEL027924	-5.77	1.2E-09	
AAEL024645	-5.71	5.4E-03	
AAEL027537	-5.65	1.8E-05	
AAEL029041	-5.63	8.2E-16	
AAEL020882	-5.57	1.4E-04	
AAEL014763	-5.48	3.0E-05	tak1 binding protein-1
AAEL013998	-5.27	7.3E-04	General transcription factor IIF subunit 2
AAEL012866	-5.17	1.4E-03	
AAEL026117	-4.94	2.7E-03	
AAEL010393	-4.87	6.0E-04	ferritin subunit, putative
AAEL023018	-4.79	6.1E-11	
AAEL027839	-4.72	1.3E-31	Putative translocon-associated protein
AAEL007641	-4.71	6.7E-09	
AAEL012864	-4.64	3.6E-07	
AAEL025746	-4.63	8.6E-03	
AAEL026300	-4.62	2.5E-02	
AAEL027629	-4.48	3.4E-03	
AAEL002929	-4.46	3.9E-02	AMP dependent ligase
AAEL007762	-4.41	4.2E-02	mitochondrial ribosomal protein, L40, putative

AAEL025362	-4.36	1.8E-03	60S ribosomal protein L10
AAEL010202	-4.34	4.4E-03	trypsin
AAEL023113	-4.28	2.7E-03	
AAEL009181	-4.10	6.5E-12	Uncharacterized protein AEGI23
AAEL015147	-3.96	2.3E-02	odorant receptor (Or4)
AAEL020619	-3.93	2.6E-04	
AAEL009149	-3.93	9.5E-05	kinectin, putative
AAEL020699	-3.91	1.2E-02	
AAEL029046	-3.87	1.4E-05	cecropin
AAEL025491	-3.71	6.5E-04	
AAEL020850	-3.70	2.2E-05	
AAEL000012	-3.66	4.9E-03	gustatory receptor Gr6
AAEL028076	-3.58	5.4E-03	
AAEL022056	-3.56	2.0E-04	
AAEL025812	-3.52	1.6E-02	
AAEL021346	-3.50	4.4E-02	
AAEL022378	-3.37	2.9E-04	
AAEL023260	-3.29	8.4E-04	
AAEL013406	-3.28	3.6E-02	venom allergen
AAEL028653	-3.26	3.8E-02	
AAEL000021	-3.25	2.1E-02	
AAEL010894	-3.17	2.6E-06	carbonic anhydrase II, putative
AAEL009108	-3.07	2.4E-02	protease m1 zinc metalloprotease
AAEL005777	-3.06	1.0E-05	
AAEL027984	-3.05	2.6E-02	
AAEL003841	-3.01	1.8E-06	Defensin-A
AAEL027792	-3.01	1.8E-06	Defensin-A
AAEL008050	-2.98	4.2E-02	
AAEL003857	-2.94	1.2E-03	INVERT_DEFENSINS domain-containing protein
AAEL009484	-2.93	3.8E-02	
AAEL027377	-2.93	3.9E-08	
AAEL003467	-2.87	8.8E-03	
AAEL026734	-2.84	3.6E-02	
AAEL027922	-2.82	3.1E-02	
AAEL026985	-2.80	2.1E-02	
AAEL002467	-2.70	1.3E-05	
AAEL029047	-2.65	1.4E-03	cecropin
AAEL025231	-2.62	1.7E-05	
AAEL012832	-2.60	3.5E-05	cytochrome B561

AAEL024391	-2.54	7.3E-04	
AAEL003832	-2.53	2.6E-06	defensin anti-microbial peptide
AAEL010140	-2.49	2.0E-03	sodium/potassium-dependent ATPase beta-2 subunit
AAEL022509	-2.47	1.1E-05	
AAEL027518	-2.47	5.7E-04	
AAEL006109	-2.46	1.8E-03	odorant binding protein OBP23
AAEL029044	-2.45	6.8E-03	cecropin
AAEL021528	-2.43	4.1E-02	
AAEL013245	-2.41	2.0E-04	Clip-Domain Serine Protease family B.
AAEL006381	-2.41	1.8E-02	sphingomyelin phosphodiesterase
AAEL021360	-2.40	1.9E-02	
AAEL024777	-2.37	5.3E-04	
AAEL024985	-2.37	2.3E-03	
AAEL026265	-2.36	2.2E-02	
AAEL014613	-2.32	3.5E-02	CYP9J24 cytochrome P450
AAEL025738	-2.21	2.6E-02	
AAEL027734	-2.21	2.6E-02	
AAEL025278	-2.20	3.4E-02	
AAEL023901	-2.18	5.7E-03	
AAEL003773	-2.16	2.8E-06	
AAEL004730	-2.14	3.1E-02	
AAEL002993	-2.13	5.0E-17	mitochondrial ribosomal protein L43
AAEL021617	-2.13	5.0E-17	
AAEL003004	-2.13	1.1E-16	
AAEL005374	-2.06	4.6E-02	Class B Scavenger Receptor (CD36 domain)
AAEL005952	-2.02	3.4E-05	GPCR Dopamine Family
AAEL005330	2.00	5.0E-08	atrial natriuretic peptide receptor
AAEL022120	2.01	7.2E-04	
AAEL005842	2.02	7.6E-04	
AAEL025354	2.06	3.8E-02	
AAEL027143	2.07	4.0E-03	
AAEL023079	2.07	5.0E-03	
AAEL010624	2.08	7.6E-03	Osiris, putative
AAEL006365	2.08	3.8E-02	trypsin-alpha, putative
AAEL004398	2.09	2.5E-05	GPCR Octopamine/Tyramine Family
AAEL028209	2.11	2.5E-02	
AAEL020053	2.11	7.8E-03	
AAEL012447	2.11	2.3E-03	elastase, putative
AAEL009434	2.12	2.0E-02	

AAEL023492	2.13	1.4E-03	
AAEL024603	2.13	4.5E-04	
AAEL013910	2.14	7.5E-03	Fatty acyl-CoA reductase
AAEL020777	2.16	5.1E-04	
AAEL026455	2.17	2.5E-03	
AAEL026198	2.19	1.9E-02	
AAEL015092	2.20	1.1E-04	
AAEL021808	2.21	2.1E-02	
AAEL006382	2.21	6.3E-03	trypsin-eta, putative
AAEL022942	2.21	2.7E-03	
AAEL022924	2.22	5.4E-03	
AAEL003399	2.22	1.7E-03	CYP4H30 cytochrome P450
AAEL022908	2.22	2.6E-02	
AAEL020163	2.23	3.4E-02	
AAEL023463	2.26	3.6E-03	
AAEL024123	2.26	5.6E-06	
AAEL027727	2.27	4.5E-02	
AAEL008467	2.27	1.4E-02	cysteine synthase
AAEL028222	2.28	4.8E-03	
AAEL023283	2.29	3.1E-02	
AAEL024271	2.29	9.5E-07	
AAEL027763	2.29	1.7E-02	
AAEL021355	2.30	4.0E-02	
AAEL013598	2.31	5.0E-03	
AAEL018005	2.32	4.0E-02	DDE Tnp4 domain-containing protein
AAEL014731	2.34	7.9E-03	
AAEL024389	2.37	7.8E-03	
AAEL028196	2.40	3.9E-02	
AAEL011591	2.43	4.2E-02	
AAEL021266	2.47	4.3E-02	
AAEL022532	2.48	4.0E-02	
AAEL021111	2.49	3.4E-07	
AAEL022288	2.49	4.0E-02	
AAEL027051	2.58	5.3E-04	
AAEL022817	2.60	5.5E-04	
AAEL026349	2.61	1.3E-02	
AAEL024638	2.62	2.7E-03	
AAEL027968	2.62	2.3E-04	
AAEL017488	2.63	1.6E-02	gustatory receptor Gr49

AAEL024868	2.67	1.7E-02	
AAEL025175	2.68	2.7E-03	
AAEL028243	2.68	7.1E-12	
AAEL024892	2.73	2.2E-04	
AAEL008798	2.75	7.5E-04	
AAEL021104	2.76	4.7E-02	
AAEL027714	2.79	4.5E-02	
AAEL000502	2.80	4.7E-04	NADPH FAD oxidoreductase
AAEL025743	2.81	2.3E-04	
AAEL026679	2.88	1.2E-03	
AAEL027747	2.90	3.6E-03	
AAEL022921	2.92	2.6E-05	
AAEL026268	2.93	1.7E-02	
AAEL021369	2.94	4.3E-02	
AAEL007434	2.98	2.9E-02	testis-specific serine/threonine kinase 22c
AAEL027327	2.99	3.7E-08	
AAEL021890	3.04	3.5E-07	
AAEL025583	3.04	2.2E-03	
AAEL023669	3.04	6.3E-03	
AAEL007870	3.05	7.7E-06	
AAEL020569	3.13	4.9E-02	
AAEL025328	3.16	3.0E-02	
AAEL026228	3.17	8.7E-05	
AAEL002677	3.17	5.7E-03	matrix metalloproteinase
AAEL002955	3.22	3.1E-02	
AAEL026953	3.28	5.8E-03	
AAEL014678	3.31	6.6E-06	CYP6F2 cytochrome P450
AAEL024082	3.40	4.1E-02	
AAEL021779	3.46	4.1E-02	
AAEL021598	3.58	1.1E-02	
AAEL021088	3.59	6.1E-13	
AAEL008464	3.76	2.3E-08	
AAEL026285	3.82	2.7E-02	
AAEL026996	3.82	2.7E-02	
AAEL022862	3.82	1.6E-02	
AAEL005108	4.08	3.6E-05	manganese-iron (Mn-Fe) superoxide dismutase
AAEL027911	4.19	1.4E-02	
AAEL025243	4.22	1.9E-02	
AAEL022004	4.34	1.6E-02	

AAEL003983	4.37	2.9E-02	adenylate cyclase
AAEL024029	4.39	3.5E-03	
AAEL014136	4.40	4.1E-05	
AAEL020226	4.41	3.4E-03	
AAEL022302	4.51	4.6E-03	
AAEL028095	4.58	2.0E-17	
AAEL027704	4.67	4.7E-02	
AAEL024278	4.71	4.1E-03	
AAEL027474	4.94	1.7E-02	
AAEL021122	5.23	2.8E-04	
AAEL026222	5.27	2.9E-05	
AAEL025877	5.69	5.8E-03	
AAEL028077	5.83	2.8E-05	
AAEL025838	6.22	1.9E-02	
AAEL021571	6.28	2.0E-03	
AAEL020298	7.03	4.5E-03	
AAEL020633	7.35	4.3E-05	
AAEL025068	7.58	3.5E-05	
AAEL025333	8.31	2.1E-03	
AAEL002622	8.55	1.9E-03	vitamin k-dependent gamma-carboxylase
AAEL028663	11.77	1.2E-09	
AAEL025617	21.39	6.3E-07	
AAEL002389	21.59	3.6E-04	gamma-secretase subunit aph-1

3.7.2.2 Differentially expressed genes in REC M compared to REC R

Supplementary Table 6 - Differentially expressed genes in REC M compared to REC R			
Gene ID	**Log_2 Fold Change**	**Adjusted p-value**	**Annotation**
AAEL028175	-24.53	0.0000	
AAEL020244	-21.24	0.0004	Serine/threonine-protein phosphatase 2A activator
AAEL027453	-7.31	0.0001	
AAEL024645	-6.89	0.0003	
AAEL027602	-5.91	0.0276	
AAEL020296	-5.74	0.0084	
AAEL022818	-5.74	0.0084	
AAEL027537	-5.64	0.0000	
AAEL028212	-5.51	0.0005	
AAEL015286	-5.06	0.0426	Odorant receptor
AAEL025887	-5.04	0.0256	Zinc finger CCCH-type with G patch domain-containing protein
AAEL023113	-4.92	0.0003	
AAEL022665	-4.74	0.0000	
AAEL004292	-4.58	0.0002	
AAEL001704	-4.55	0.0066	
AAEL017347	-4.33	0.0347	odorant receptor
AAEL006159	-4.31	0.0301	
AAEL025491	-4.31	0.0000	
AAEL028076	-4.29	0.0004	
AAEL013339	-4.18	0.0457	alphaA-crystallin, putative
AAEL022253	-4.17	0.0182	
AAEL012866	-3.96	0.0178	
AAEL002654	-3.84	0.0077	
AAEL013284	-3.81	0.0010	late trypsin 1, serine-type enodpeptidase
AAEL017402	-3.75	0.0002	
AAEL013998	-3.72	0.0323	General transcription factor IIF subunit 2
AAEL015163	-3.58	0.0175	cuticle protein, putative
AAEL025812	-3.53	0.0112	
AAEL003467	-3.41	0.0009	
AAEL012867	-3.41	0.0315	
AAEL028653	-3.41	0.0189	
AAEL025278	-3.36	0.0001	
AAEL027439	-3.34	0.0005	
AAEL024421	-3.28	0.0007	

AAEL021178	-3.21	0.0037	
AAEL019912	-3.19	0.0489	
AAEL012575	-3.18	0.0036	serine protease, putative
AAEL021632	-2.88	0.0001	
AAEL029041	-2.85	0.0006	
AAEL023843	-2.81	0.0161	
AAEL026304	-2.80	0.0038	
AAEL013348	-2.79	0.0271	lethal(2)essential for life protein, l2efl
AAEL025231	-2.78	0.0000	
AAEL022684	-2.74	0.0341	
AAEL002022	-2.71	0.0227	protein serine/threonine kinase, putative
AAEL008012	-2.69	0.0349	
AAEL023456	-2.67	0.0042	
AAEL025738	-2.67	0.0029	
AAEL027734	-2.67	0.0029	
AAEL026306	-2.56	0.0064	
AAEL013511	-2.55	0.0009	
AAEL023018	-2.55	0.0040	
AAEL005374	-2.54	0.0054	Class B Scavenger Receptor (CD36 domain)
AAEL001319	-2.51	0.0052	
AAEL022261	-2.49	0.0010	
AAEL026801	-2.48	0.0271	
AAEL008360	-2.48	0.0005	GPCR Serotonin Family
AAEL012864	-2.47	0.0228	
AAEL002908	-2.46	0.0193	
AAEL027989	-2.41	0.0003	
AAEL022033	-2.39	0.0337	
AAEL027291	-2.33	0.0207	
AAEL006953	-2.33	0.0002	
AAEL014615	-2.31	0.0489	CYP9J23 cytochrome P450
AAEL024985	-2.30	0.0030	
AAEL009178	-2.29	0.0390	Gram-Negative Binding Protein (GNBP) or Beta-1 3-Glucan Binding Protein (BGBP).
AAEL017500	-2.28	0.0213	
AAEL029046	-2.26	0.0260	cecropin
AAEL024509	-2.25	0.0022	
AAEL020007	-2.24	0.0478	
AAEL021785	-2.24	0.0008	
AAEL025608	-2.22	0.0250	Putative cuticle protein

AAEL021360	-2.22	0.0260	
AAEL011714	-2.19	0.0004	
AAEL021842	-2.15	0.0366	
AAEL023587	-2.14	0.0093	
AAEL022509	-2.13	0.0003	
AAEL022863	-2.11	0.0008	
AAEL022378	-2.11	0.0393	
AAEL026166	-2.11	0.0000	
AAEL002467	-2.10	0.0013	
AAEL013018	-2.09	0.0455	odorant binding protein OBP56
AAEL010858	-2.09	0.0497	AMP dependent ligase
AAEL019870	-2.05	0.0243	
AAEL021995	-2.01	0.0049	
AAEL013766	-2.00	0.0159	
AAEL021808	2.01	0.0300	
AAEL022664	2.01	0.0054	
AAEL022710	2.01	0.0008	
AAEL024929	2.02	0.0006	
AAEL027012	2.02	0.0000	
AAEL024890	2.03	0.0000	
AAEL026163	2.03	0.0042	
AAEL015683	2.04	0.0025	Histone H2B
AAEL019636	2.04	0.0025	Histone H2B
AAEL023942	2.07	0.0000	
AAEL027800	2.08	0.0022	
AAEL021393	2.09	0.0441	
AAEL018025	2.11	0.0408	
AAEL023492	2.11	0.0017	
AAEL004398	2.11	0.0000	GPCR Octopamine/Tyramine Family
AAEL025652	2.13	0.0007	
AAEL023742	2.13	0.0316	
AAEL023233	2.13	0.0124	
AAEL024203	2.14	0.0412	
AAEL024260	2.15	0.0061	
AAEL023272	2.16	0.0372	
AAEL026050	2.16	0.0096	
AAEL022414	2.17	0.0464	
AAEL024111	2.17	0.0034	
AAEL022929	2.19	0.0305	

AAEL022564	2.20	0.0000	
AAEL000066	2.21	0.0078	
AAEL011174	2.22	0.0025	gustatory receptor Gr11
AAEL022119	2.22	0.0244	
AAEL014731	2.22	0.0114	
AAEL023803	2.23	0.0001	
AAEL008884	2.23	0.0005	
AAEL025282	2.24	0.0214	
AAEL022554	2.25	0.0151	
AAEL022466	2.26	0.0000	
AAEL021207	2.26	0.0000	
AAEL022120	2.27	0.0002	
AAEL003653	2.27	0.0099	Serine Protease Inhibitor (serpin) homologue - unlikely to be inhibitory.
AAEL010367	2.31	0.0333	aldehyde oxidase
AAEL023549	2.32	0.0000	
AAEL027638	2.41	0.0173	
AAEL026349	2.41	0.0182	
AAEL011591	2.42	0.0356	
AAEL009463	2.43	0.0000	
AAEL025470	2.43	0.0001	
AAEL024275	2.43	0.0022	
AAEL021656	2.45	0.0226	
AAEL025896	2.45	0.0320	
AAEL020424	2.47	0.0000	
AAEL022093	2.48	0.0074	
AAEL027747	2.49	0.0116	
AAEL027449	2.50	0.0008	
AAEL006424	2.50	0.0384	37 kDa salivary gland allergen Aed a 2 Precursor (Protein D7)(Allergen Aed a 2)
AAEL005775	2.51	0.0023	CYP325R1 cytochrome P450
AAEL024240	2.52	0.0000	
AAEL014678	2.52	0.0007	CYP6F2 cytochrome P450
AAEL010682	2.52	0.0000	armc4
AAEL026605	2.56	0.0000	
AAEL010628	2.56	0.0100	
AAEL026953	2.59	0.0303	
AAEL026198	2.59	0.0034	
AAEL025260	2.61	0.0188	
AAEL022293	2.62	0.0000	

AAEL025354	2.63	0.0060	
AAEL009683	2.64	0.0004	
AAEL021469	2.65	0.0194	
AAEL024271	2.67	0.0000	
AAEL022575	2.72	0.0356	
AAEL023690	2.72	0.0266	
AAEL023336	2.74	0.0295	
AAEL022652	2.74	0.0139	
AAEL008199	2.79	0.0489	serine collagenase 1 precursor, putative
AAEL025083	2.84	0.0000	
AAEL009434	2.84	0.0020	
AAEL006627	2.85	0.0047	serine-type enodpeptidase,
AAEL021341	2.87	0.0439	
AAEL023956	2.88	0.0292	
AAEL014757	2.89	0.0036	
AAEL021527	2.90	0.0433	
AAEL023429	2.90	0.0271	
AAEL024638	2.90	0.0022	
AAEL024609	2.92	0.0025	
AAEL013598	2.93	0.0004	
AAEL026298	2.94	0.0025	
AAEL020902	2.96	0.0042	
AAEL025244	2.99	0.0024	
AAEL002677	2.99	0.0082	matrix metalloproteinase
AAEL015472	3.02	0.0017	catrin, putative
AAEL028196	3.04	0.0057	
AAEL020641	3.05	0.0223	
AAEL022714	3.06	0.0006	
AAEL024605	3.06	0.0248	
AAEL006003	3.07	0.0131	odorant receptor
AAEL020531	3.08	0.0088	
AAEL010631	3.09	0.0474	
AAEL014993	3.10	0.0012	
AAEL022759	3.11	0.0181	
AAEL023689	3.12	0.0000	
AAEL025912	3.13	0.0101	
AAEL023988	3.15	0.0110	
AAEL026735	3.16	0.0498	
AAEL022185	3.16	0.0000	

AAEL025345	3.22	0.0366	
AAEL026228	3.22	0.0001	
AAEL020428	3.25	0.0000	
AAEL024617	3.26	0.0063	
AAEL010752	3.28	0.0106	
AAEL020549	3.29	0.0246	
AAEL021749	3.30	0.0433	
AAEL021487	3.40	0.0410	
AAEL021594	3.42	0.0008	
AAEL022555	3.42	0.0201	
AAEL025185	3.45	0.0265	
AAEL028036	3.47	0.0007	
AAEL021191	3.48	0.0000	
AAEL003289	3.49	0.0107	alkaline phosphatase
AAEL004343	3.52	0.0188	odorant binding protein OBP19
AAEL020498	3.56	0.0327	
AAEL026674	3.60	0.0435	
AAEL027293	3.63	0.0122	
AAEL025612	3.63	0.0004	
AAEL014684	3.64	0.0485	CYP6F3 cytochrome P450
AAEL024603	3.65	0.0000	
AAEL025743	3.70	0.0000	
AAEL024868	3.72	0.0006	
AAEL013621	3.72	0.0002	
AAEL024082	3.72	0.0213	
AAEL025426	3.74	0.0274	
AAEL000502	3.75	0.0000	NADPH FAD oxidoreductase
AAEL020085	3.79	0.0457	
AAEL026860	3.84	0.0270	
AAEL011543	3.84	0.0455	metalloproteinase, putative
AAEL023669	3.88	0.0008	
AAEL028663	3.89	0.0495	
AAEL009543	3.90	0.0046	
AAEL026268	3.93	0.0006	
AAEL026398	3.97	0.0225	
AAEL027763	3.99	0.0004	
AAEL025243	4.01	0.0207	
AAEL020325	4.01	0.0006	
AAEL026679	4.02	0.0010	

AAEL022862	4.02	0.0131	
AAEL023605	4.06	0.0011	
AAEL022591	4.06	0.0000	
AAEL028118	4.14	0.0396	
AAEL021910	4.17	0.0063	
AAEL025860	4.19	0.0029	
AAEL026029	4.19	0.0097	
AAEL008873	4.21	0.0054	pupal cuticle protein 78E, putative
AAEL028085	4.25	0.0341	
AAEL021779	4.29	0.0070	
AAEL023406	4.40	0.0015	
AAEL017488	4.49	0.0004	gustatory receptor Gr49
AAEL020870	4.50	0.0495	
AAEL024068	4.53	0.0067	
AAEL025942	4.53	0.0042	
AAEL008708	4.63	0.0416	lysosomal pro-X carboxypeptidase, putative
AAEL025593	4.71	0.0103	
AAEL024029	4.71	0.0012	
AAEL024875	4.73	0.0288	
AAEL025068	4.83	0.0039	
AAEL006975	4.84	0.0349	leucine aminopeptidase
AAEL026943	4.89	0.0000	
AAEL022745	4.90	0.0182	Histone H2A
AAEL026212	4.90	0.0182	Histone H2A
AAEL021122	4.95	0.0006	
AAEL028095	4.98	0.0000	
AAEL020209	5.00	0.0054	
AAEL026471	5.02	0.0000	
AAEL021683	5.10	0.0055	
AAEL028077	5.12	0.0002	
AAEL024369	5.12	0.0163	
AAEL024176	5.16	0.0000	
AAEL008798	5.39	0.0000	
AAEL021598	5.42	0.0002	
AAEL026222	5.91	0.0000	
AAEL020633	5.94	0.0016	
AAEL025838	5.97	0.0189	
AAEL021571	6.03	0.0027	
AAEL024278	6.13	0.0001	

AAEL021553	6.15	0.0000	
AAEL020131	6.46	0.0116	
AAEL022004	6.75	0.0003	
AAEL023409	6.78	0.0000	
AAEL027911	7.07	0.0001	
AAEL020968	7.13	0.0000	
AAEL020569	7.67	0.0000	
AAEL000477	7.97	0.0000	NADPH FAD oxidoreductase
AAEL025333	8.06	0.0025	
AAEL020298	8.50	0.0004	
AAEL008861	8.82	0.0000	deoxyribonuclease I, putative
AAEL025617	21.50	0.0000	
AAEL022708	22.51	0.0002	Peptidyl-prolyl cis-trans isomerase
AAEL027045	31.89	0.0000	SCP domain-containing protein

3.7.2.3 Differentially expressed genes in REC P compared to REC R

Supplementary Table 7 - Differentially expressed genes in REC P compared to REC R

Gene ID	Log2 Fold Change	Adjusted p-value	Annotation
AAEL028175	-25.59	3.9E-45	
AAEL020244	-23.38	2.6E-05	Serine/threonine-protein phosphatase 2A activator
AAEL022058	-9.78	6.9E-06	
AAEL022499	-7.79	9.8E-11	
AAEL025856	-7.73	4.8E-70	
AAEL028245	-7.12	4.4E-06	
AAEL026974	-7.05	2.8E-05	
AAEL019388	-7.02	3.4E-06	
AAEL021627	-6.94	1.2E-08	
AAEL024645	-6.87	1.7E-04	
AAEL025060	-6.77	1.8E-23	
AAEL027895	-6.73	9.6E-06	
AAEL020882	-6.69	6.3E-07	
AAEL022121	-6.62	6.1E-06	
AAEL027602	-6.43	1.4E-02	
AAEL027453	-6.22	1.0E-03	
AAEL021292	-6.11	3.9E-05	

AAEL020040	-5.90	3.4E-02	
AAEL014763	-5.85	2.3E-06	tak1 binding protein-1
AAEL027983	-5.59	1.1E-02	
AAEL013998	-5.34	2.9E-04	General transcription factor IIF subunit 2
AAEL020296	-4.97	2.8E-02	
AAEL022818	-4.97	2.8E-02	
AAEL007641	-4.95	3.5E-10	
AAEL002929	-4.92	1.1E-02	AMP dependent ligase
AAEL027009	-4.91	3.5E-08	
AAEL027839	-4.85	1.1E-33	Putative translocon-associated protein
AAEL027537	-4.84	2.9E-04	
AAEL027629	-4.77	7.4E-04	
AAEL027924	-4.69	1.3E-06	
AAEL012867	-4.66	6.5E-04	
AAEL021875	-4.50	3.5E-02	
AAEL009149	-4.48	1.6E-06	kinectin, putative
AAEL020850	-4.48	2.5E-08	
AAEL026026	-4.43	4.1E-07	
AAEL025491	-4.34	1.4E-05	
AAEL012864	-4.30	1.8E-06	
AAEL025812	-4.07	1.7E-03	
AAEL003467	-4.02	2.1E-05	
AAEL012860	-4.02	2.1E-02	
AAEL025362	-3.93	3.9E-03	60S ribosomal protein L10
AAEL020100	-3.90	3.6E-02	
AAEL029058	-3.88	1.4E-03	
AAEL012866	-3.84	2.1E-02	
AAEL023018	-3.67	1.6E-06	
AAEL015147	-3.66	3.1E-02	odorant receptor (Or4)
AAEL021346	-3.65	2.1E-02	
AAEL003297	-3.59	4.3E-02	alkaline phosphatase
AAEL026734	-3.49	2.6E-03	
AAEL010045	-3.48	2.4E-02	Protein FAM50 homolog
AAEL020537	-3.45	3.9E-02	
AAEL027922	-3.37	3.3E-03	
AAEL022056	-3.34	3.0E-04	
AAEL028653	-3.31	2.2E-02	
AAEL029041	-3.24	2.6E-05	
AAEL027518	-3.24	4.4E-07	

AAEL010393	-3.20	4.1E-02	ferritin subunit, putative
AAEL021690	-3.20	1.6E-02	
AAEL028973	-3.18	9.1E-03	
AAEL011685	-3.16	3.1E-04	ARL2_Bind_BART domain-containing protein
AAEL010166	-3.16	2.0E-04	
AAEL021637	-3.12	2.5E-02	
AAEL025334	-3.06	3.5E-08	
AAEL022863	-3.03	5.4E-08	
AAEL019978	-3.02	4.7E-02	
AAEL009566	-3.02	3.8E-06	apolipoprotein D, putative
AAEL025278	-2.96	5.8E-04	
AAEL005777	-2.94	1.3E-05	
AAEL001118	-2.86	1.9E-05	
AAEL023260	-2.86	3.7E-03	
AAEL020334	-2.85	1.0E-03	
AAEL020649	-2.85	1.0E-03	
AAEL006138	-2.78	5.6E-03	
AAEL020068	-2.72	2.1E-02	
AAEL020215	-2.70	4.3E-03	
AAEL025608	-2.66	4.3E-03	Putative cuticle protein
AAEL005293	-2.66	3.2E-04	Galectin
AAEL019842	-2.65	1.8E-03	
AAEL026261	-2.63	1.5E-02	
AAEL009166	-2.62	5.0E-03	
AAEL013127	-2.62	5.0E-03	
AAEL023456	-2.61	4.0E-03	
AAEL021461	-2.59	5.7E-05	
AAEL024421	-2.54	1.1E-02	
AAEL022306	-2.50	9.7E-03	
AAEL024509	-2.50	2.3E-04	
AAEL021915	-2.49	2.9E-05	
AAEL005374	-2.46	6.0E-03	Class B Scavenger Receptor (CD36 domain)
AAEL002612	-2.40	2.0E-05	
AAEL017538	-2.40	2.0E-05	
AAEL005952	-2.38	1.4E-07	GPCR Dopamine Family
AAEL014067	-2.36	4.1E-02	
AAEL022509	-2.35	1.8E-05	
AAEL026801	-2.32	4.3E-02	
AAEL021648	-2.30	1.6E-02	

AAEL020923	-2.30	3.4E-03	
AAEL001552	-2.28	1.8E-02	
AAEL011714	-2.27	9.1E-05	
AAEL024046	-2.22	4.3E-02	
AAEL026289	-2.19	4.7E-02	
AAEL010894	-2.19	2.2E-03	carbonic anhydrase II, putative
AAEL029046	-2.19	3.2E-02	cecropin
AAEL007381	-2.18	2.3E-02	
AAEL025874	-2.17	5.3E-06	
AAEL010580	-2.14	2.7E-02	3-hydroxyisobutyrate dehydrogenase, putative
AAEL024985	-2.12	5.5E-03	
AAEL012628	-2.11	5.8E-06	
AAEL014515	-2.07	2.0E-07	metalloproteinase, putative
AAEL003841	-2.07	2.0E-03	Defensin-A
AAEL027792	-2.07	2.0E-03	Defensin-A
AAEL022087	-2.07	3.5E-04	
AAEL009181	-2.06	2.3E-03	Uncharacterized protein AEGI23
AAEL026570	-2.06	3.5E-11	
AAEL021686	-2.02	3.8E-02	
AAEL003358	2.01	3.3E-02	
AAEL026639	2.01	5.8E-03	
AAEL022656	2.02	9.4E-08	
AAEL002361	2.02	4.3E-02	
AAEL023050	2.03	6.9E-06	
AAEL022714	2.03	2.7E-02	
AAEL007669	2.03	4.7E-06	RpL9 60S ribosomal protein L9
AAEL024892	2.04	4.5E-03	
AAEL026476	2.04	9.7E-09	
AAEL024755	2.05	3.8E-03	
AAEL025469	2.06	1.0E-02	
AAEL025031	2.08	3.1E-04	
AAEL006812	2.08	3.6E-03	
AAEL023039	2.09	5.3E-04	
AAEL024300	2.09	1.3E-05	
AAEL026612	2.09	4.9E-05	
AAEL021417	2.10	3.3E-02	
AAEL024128	2.11	6.6E-06	
AAEL009123	2.11	1.4E-06	CYP6Z6 cytochrome P450
AAEL023492	2.14	7.8E-04	

AAEL010154	2.16	1.2E-08	cytochrome P450
AAEL025354	2.16	2.0E-02	
AAEL026818	2.16	3.7E-03	
AAEL021212	2.16	1.6E-06	
AAEL020321	2.18	4.4E-02	
AAEL020454	2.18	5.8E-07	
AAEL020424	2.19	4.2E-05	
AAEL026466	2.20	1.6E-17	
AAEL009448	2.20	2.2E-08	
AAEL021207	2.21	9.4E-06	
AAEL007147	2.21	4.8E-10	
AAEL022924	2.22	3.4E-03	
AAEL025936	2.22	1.8E-02	
AAEL007667	2.23	1.6E-06	
AAEL003772	2.24	7.2E-09	
AAEL024260	2.24	3.0E-03	
AAEL013598	2.24	4.2E-03	
AAEL021283	2.25	7.4E-05	
AAEL025157	2.27	2.6E-02	
AAEL025743	2.27	3.0E-03	
AAEL027362	2.28	1.3E-02	
AAEL022664	2.30	7.8E-04	
AAEL026198	2.30	7.8E-03	
AAEL007941	2.30	4.3E-02	triacylglycerol lipase, putative
AAEL020575	2.31	5.2E-13	
AAEL001513	2.33	1.9E-19	WD-repeat protein
AAEL027279	2.33	9.8E-04	
AAEL026455	2.34	7.8E-04	
AAEL009434	2.35	6.2E-03	
AAEL022119	2.35	1.5E-02	
AAEL010721	2.36	4.8E-04	reticulon/nogo receptor
AAEL005759	2.37	3.9E-07	Rhodanese domain-containing protein
AAEL002391	2.38	3.3E-05	CCEae5B Carboxylic ester hydrolase
AAEL024883	2.38	1.6E-09	
AAEL021901	2.39	2.9E-05	
AAEL000043	2.39	4.8E-02	gustatory receptor 64e, putative
AAEL021469	2.42	2.9E-02	
AAEL025140	2.42	1.5E-09	
AAEL015092	2.42	7.1E-06	

AAEL021919	2.43	3.0E-10	
AAEL024684	2.44	5.7E-04	
AAEL001196	2.48	2.0E-06	cadherin
AAEL022458	2.48	2.2E-04	
AAEL020937	2.49	4.3E-02	
AAEL027763	2.51	5.9E-03	
AAEL012350	2.51	2.9E-02	lipase 1 precursor
AAEL026924	2.53	4.6E-09	
AAEL012774	2.53	5.5E-04	protease m1 zinc metalloprotease
AAEL005287	2.54	5.7E-05	
AAEL021775	2.55	4.9E-04	
AAEL027139	2.55	4.4E-06	
AAEL024275	2.59	5.8E-04	
AAEL021266	2.61	2.8E-02	
AAEL025156	2.61	8.8E-04	
AAEL028173	2.61	2.5E-02	
AAEL026725	2.61	1.6E-02	
AAEL014731	2.61	2.6E-03	
AAEL020350	2.63	6.3E-07	
AAEL014678	2.64	1.7E-04	CYP6F2 cytochrome P450
AAEL024240	2.67	4.4E-07	
AAEL022102	2.67	2.2E-02	
AAEL025323	2.71	2.1E-13	
AAEL027829	2.71	1.4E-03	
AAEL020325	2.71	4.9E-03	
AAEL010500	2.72	1.5E-21	GSTx2 glutathione transferase
AAEL014018	2.72	1.6E-02	
AAEL023906	2.72	4.2E-05	
AAEL020531	2.74	1.2E-02	
AAEL022185	2.75	5.9E-11	
AAEL022728	2.76	4.7E-02	
AAEL000459	2.76	5.4E-05	
AAEL023049	2.79	1.2E-02	
AAEL022130	2.83	1.1E-07	
AAEL003337	2.85	4.8E-03	
AAEL026239	2.85	1.8E-02	
AAEL026324	2.85	1.9E-05	
AAEL017252	2.85	1.1E-03	
AAEL021906	2.89	1.6E-02	

AAEL025940	2.89	5.6E-06	
AAEL024271	2.90	2.8E-11	
AAEL027994	2.91	1.0E-02	
AAEL006762	2.91	4.5E-04	
AAEL024389	2.92	5.8E-04	
AAEL025655	2.94	2.1E-04	
AAEL027012	2.98	3.7E-12	
AAEL022074	3.01	1.8E-03	
AAEL020097	3.02	1.5E-07	
AAEL025445	3.03	3.6E-02	
AAEL024355	3.03	5.5E-04	
AAEL023820	3.06	5.3E-05	
AAEL008752	3.08	9.0E-05	
AAEL027939	3.09	1.6E-14	
AAEL026544	3.10	1.2E-03	
AAEL027457	3.10	3.4E-03	
AAEL021854	3.12	4.5E-03	
AAEL023463	3.13	2.6E-05	
AAEL020273	3.14	4.0E-02	
AAEL022742	3.15	9.7E-03	
AAEL023088	3.16	4.2E-20	
AAEL021890	3.19	8.8E-08	
AAEL008798	3.21	1.9E-05	
AAEL012129	3.23	2.8E-06	cationic amino acid transporter
AAEL011029	3.27	2.8E-02	
AAEL024501	3.31	4.0E-02	
AAEL026228	3.32	2.9E-05	
AAEL020711	3.33	4.3E-02	
AAEL026509	3.34	2.3E-02	
AAEL024405	3.35	6.2E-03	
AAEL023201	3.39	1.0E-02	
AAEL025554	3.44	1.0E-03	
AAEL002677	3.44	2.1E-03	matrix metalloproteinase
AAEL027880	3.48	1.0E-07	
AAEL026225	3.49	1.6E-06	
AAEL025759	3.49	4.4E-13	
AAEL023980	3.51	2.2E-03	
AAEL027968	3.51	5.6E-08	
AAEL022205	3.53	2.5E-02	

AAEL021463	3.56	8.5E-14	
AAEL024868	3.57	4.9E-04	
AAEL008467	3.60	4.7E-05	cysteine synthase
AAEL023483	3.61	7.3E-18	
AAEL022018	3.62	5.4E-03	
AAEL026151	3.62	5.4E-03	
AAEL021204	3.63	6.1E-06	
AAEL023686	3.65	3.7E-02	
AAEL026953	3.72	7.1E-04	
AAEL001801	3.73	4.0E-02	
AAEL013415	3.75	9.2E-06	alpha-tropomyosin 5a, putative
AAEL021640	3.77	1.4E-02	
AAEL025328	3.80	9.0E-03	
AAEL025852	3.82	9.1E-03	
AAEL023608	3.84	1.0E-02	
AAEL005108	3.85	5.7E-05	manganese-iron (Mn-Fe) superoxide dismutase
AAEL022777	3.91	3.0E-02	
AAEL024603	3.92	9.3E-12	
AAEL025612	4.00	7.4E-05	
AAEL017128	4.02	2.9E-02	
AAEL026349	4.05	2.8E-05	
AAEL022444	4.09	1.6E-06	
AAEL025860	4.09	2.0E-03	
AAEL027911	4.14	1.0E-02	
AAEL025175	4.17	4.5E-04	
AAEL009796	4.18	7.8E-04	cuticle protein, putative
AAEL025243	4.20	1.4E-02	
AAEL023997	4.22	9.8E-03	
AAEL007870	4.22	3.4E-10	
AAEL025912	4.23	1.2E-03	
AAEL021783	4.33	4.6E-02	
AAEL026965	4.35	4.4E-03	
AAEL023740	4.36	1.6E-02	
AAEL027489	4.36	3.2E-06	
AAEL028068	4.37	2.8E-08	
AAEL024462	4.37	3.6E-03	
AAEL026268	4.46	3.9E-05	
AAEL025282	4.47	1.5E-05	
AAEL025466	4.49	1.7E-03	

AAEL026362	4.54	1.2E-03	
AAEL027752	4.54	1.2E-03	
AAEL020443	4.57	5.3E-04	
AAEL002291	4.58	1.4E-02	
AAEL017799	4.69	5.1E-03	U1 spliceosomal RNA
AAEL027831	4.74	1.9E-02	
AAEL028151	4.80	2.4E-02	
AAEL028077	4.85	2.0E-04	
AAEL022233	4.89	1.5E-02	
AAEL022112	4.89	4.0E-03	
AAEL020892	4.90	4.9E-04	
AAEL022852	4.91	2.9E-03	
AAEL006975	4.96	3.0E-02	leucine aminopeptidase
AAEL027638	5.05	9.0E-05	
AAEL026222	5.09	2.7E-05	
AAEL027283	5.10	1.4E-04	
AAEL021656	5.21	1.8E-04	
AAEL028095	5.26	4.6E-23	
AAEL024851	5.26	3.3E-03	
AAEL021715	5.27	9.9E-06	
AAEL021760	5.41	3.5E-03	
AAEL020556	5.57	2.4E-04	
AAEL024752	5.67	2.9E-05	
AAEL021190	5.69	6.2E-05	
AAEL020209	5.70	7.3E-04	
AAEL021122	5.76	6.1E-05	
AAEL022032	5.96	2.0E-05	
AAEL018948	6.03	4.7E-02	
AAEL014136	6.07	6.9E-06	
AAEL025622	6.24	1.3E-04	
AAEL021553	6.30	2.2E-08	
AAEL027747	6.46	1.6E-06	
AAEL028768	6.58	3.4E-02	
AAEL014020	6.64	2.2E-05	
AAEL020633	6.64	1.7E-04	
AAEL007879	6.66	1.1E-40	
AAEL020569	6.68	9.8E-08	
AAEL021023	6.74	3.1E-04	
AAEL025068	7.45	2.5E-05	

AAEL022430	7.80	3.1E-06	
AAEL003310	8.13	7.9E-03	
AAEL020298	8.62	1.5E-04	
AAEL024591	9.30	4.4E-12	
AAEL002389	21.44	1.9E-04	gamma-secretase subunit aph-1
AAEL019643	24.31	2.3E-26	
AAEL027045	32.11	1.8E-09	SCP domain-containing protein

3.7.3 Enriched gene ontology terms

Ontology denotes which GO term vocabulary the term arises from (BP = biological processes, MF = molecular function, CC = cellular component).

3.7.3.1 REC U versus REC R

There was only a single GO term associated with genes that were over-expressed in REC U compared to REC R:

- GO:0016829 Lyase activity
- Associated genes: AAEL002622, AAEL005330, AAEL008467, AAEL027763

Bonferroni p-value 1.43E-02

Supplementary Table 8 - GO terms relating to significantly under-expressed genes in REC U versus REC R

GO ID	Ontology	GO term	Associated genes	Bonferroni p value
0042742	BP	Defense response to bacterium	AAEL003832, AAEL003841, AAEL027792, AAEL029041, AAEL029044, AAEL029046, AAEL029047	8.33E-11
0009617	BP	Response to bacterium		8.33E-11
0006952	BP	Defense response	AAEL003832, AAEL003841, AAEL003857, AAEL027792, AAEL029041, AAEL029044, AAEL029046, AAEL029047	8.43E-11
0051707	BP	Response to other organism	AAEL003832, AAEL003841, AAEL027792, AAEL029041, AAEL029044, AAEL029046, AAEL029047	4.33E-09
0044419	BP	Biological process involved in interspecies interaction between organisms		4.33E-09
0098542	BP	Defense response to other organism	AAEL003832, AAEL003841, AAEL027792, AAEL029041, AAEL029044, AAEL029046, AAEL029047	4.33E-09
0006955	BP	Immune response		4.33E-09
0043207	BP	Response to external biotic stimulus		4.33E-09
0009607	BP	Response to biotic stimulus		4.33E-09
0002376	BP	Immune system process		8.17E-09
0009605	BP	Response to external stimulus		2.92E-06
0019731	BP	Antibacterial humoral response	AAEL029041, AAEL029044, AAEL029046, AAEL029047	3.00E-05
0006959	BP	Humoral immune response		3.00E-05
0019730	BP	Antimicrobial humoral response		3.00E-05
0045087	BP	Innate immune response		1.91E-04
0006950	BP	Response to stress	AAEL003832, AAEL003841, AAEL003857, AAEL027792, AAEL029041, AAEL029044, AAEL029046, AAEL029047	1.23E-03
0050829	BP	Defense response to Gram-negative bacterium	AAEL003841, AAEL027792	6.51E-03
0050830	BP	Defense response to Gram-positive bacterium		6.51E-03
0005576	CC	Extracellular region	AAEL002467, AAEL003832, AAEL003841, AAEL027792, AAEL029041, AAEL029044, AAEL029046, AAEL029047	1.18E-02

3.7.3.2 REC M versus REC R

There were no significantly GO terms (Bonferroni $p \leq 0.05$) associated with genes that were over-expressed in REC M compared to REC R.

Supplementary Table 9 - GO terms relating to significantly under-expressed genes in REC M versus REC R

GO ID	Ontology	GO term	Associated genes	Bonferroni p value
0042302	BP MF	Structural constituent of cuticle	AAEL004292, AAEL013511, AAEL015163, AAEL022261, AAEL026306, AAEL028212	1.55E-02
0005576	CC	Extracellular region	AAEL002467, AAEL006159, AAEL006953, AAEL013766, AAEL029041, AAEL029046	3.99E-02

3.7.3.3 REC P versus REC R

Supplementary Table 10 GO terms relating to significantly under-expressed genes in REC P versus REC R				
GO ID	**Ontology**	**GO term**	**Associated genes**	**Bonferroni p value**
0006952	BP	Defense response	AAEL003841, AAEL003857, AAEL027792, AAEL029041, AAEL029046	2.84E-05
0009617	BP	Response to bacterium	AAEL003841, AAEL027792, AAEL029041, AAEL029046	1.41E-04
0042742	BP	Defense response to bacterium	AAEL003841, AAEL027792, AAEL029041, AAEL029046	1.41E-04
0051707	BP	Response to other organism		1.07E-03
0043207	BP	Response to external biotic stimulus		1.07E-03
0098542	BP	Defense response to other organism		1.07E-03
0044419	BP	Biological process involved in interspecies interaction between organisms		1.07E-03
0006955	BP	Immune response		1.07E-03
0009607	BP	Response to biotic stimulus		1.07E-03
0002376	BP	Immune system process		1.49E-03
0050830	BP	Defense response to Gram-positive bacterium	AAEL003841, AAEL027792	5.41E-03
0050829	BP	Defense response to Gram-negative bacterium		5.41E-03
0005576	CC	Extracellular region	AAEL002612, AAEL003841, AAEL017538, AAEL025334, AAEL027792, AAEL029041, AAEL029046	3.16E-02
0009605	BP	Response to external stimulus	AAEL003841, AAEL027792, AAEL029041, AAEL029046	3.54E-02

There were no significantly enriched GO terms (Bonferroni $p \leq 0.05$) associated with genes over-expressed in REC P compared to REC R.

Chapter 4 – Insecticide selection of *Aedes aegypti* is associated with alterations in vector competence for Zika virus

4.1 Abstract

Insecticide selection pressures and insecticide resistance are associated with multiple fitness costs in mosquito vectors. Four strains of *Aedes aegypti*, derived from the same parent strain, were subjected to different insecticide selection regimens over multiple generations. The vector competence of these strains for Zika virus (ZIKV) was compared using oral infection and intrathoracic microinjections. The dissemination prevalence of ZIKV was 25% lower at 5 days post oral infection in the temephos selected strain compared to the unselected strain. This was associated with lower body titres in the temephos selected strain at all timepoints compared to the unselected strain following oral infection. This could reflect differences in midgut infection and or/escape barriers between these strains resulting from insecticide selection. Following infection by intrathoracic injection, the salivary infection prevalence was markedly lower in the temephos selected strain compared to the unselected strain at all timepoints. This may represent the presence of a salivary gland infection or escape barrier. The differences may reflect previously established differences between these strains in the constitutive expression of genes relating to innate immunity. These results suggest that temephos resistance may be associated with decreased vector competence for ZIKV virus. Further investigation is required to determine the exact mechanisms responsible for the observed decreases in vector competence.

4.2 Introduction

Due to a lack of effective vaccines and specific medical treatments for arboviral diseases spread by *Aedes aegypti*, current control efforts are heavily dependent on successful vector control [41]. The use of neurotoxic insecticides via indoor residual spraying, treatment of larval sites, and fogging, forms the mainstay of control efforts [48]. Concerningly, insecticide resistance is now highly prevalent in *Ae. aegypti* populations worldwide and presents a major challenge to the control of these important vectors [54-57]. Target-site mutations to insecticide binding sites on voltage-gated sodium channels and acetylcholinesterases have been widely reported in *Ae. aegypti* populations [56]. The enhanced degradation of insecticides by detoxification enzymes, known as metabolic insecticide resistance, is also highly prevalent in *Aedes* vectors [55-57, 335]. Enzymes from the cytochrome P450 monooxygenase (CYP), carboxylesterase (CCE), and glutathione-s-transferase (GST) families, have been repeatedly implicated in insecticide resistance to pyrethroid and organophosphate insecticides [56]. The chance of an insecticide resistant mosquito surviving through the extrinsic incubation period of an arbovirus, in areas of insecticide use, is likely to be higher than for an insecticide susceptible individual. Therefore,

insecticide resistance could have important implications for the epidemiology of arboviral diseases, reflected in its recent association with hotspots of dengue transmission [60]. Metabolic insecticide resistance has also been associated with numerous pleiotropic effects on mosquito physiology including longevity, host-seeking behaviour, energy reserves, and reproductive success [190-194]. Given the scale of the physiological changes associated with metabolic insecticide resistance, there are concerns that it may also have pleiotropic effects on the vector competence of mosquitoes [58, 242].

There is evidence that metabolic insecticide resistance could increase vector competence for arboviruses. A field strain of *Ae. aegypti*, that was selected for high permethrin resistance over multiple generations, had significantly higher dissemination of dengue virus (DENV) to the heads of mosquitoes compared to the unselected parent strain [175]. However, the presence of target site mutations, in addition to differences in the expression of CYPs and GSTs, made it difficult to determine the mechanism for the observed differences in vector competence. Duplication of the esterase^2 SA2 locus, which has been associated with organophosphate resistance [336], was associated with higher dissemination of West Nile virus (WNV) from the midguts of *Culex pipiens*, and higher transmission efficiency than wildtype insecticide susceptible controls [183]. Interestingly, the *ace1* target site mutation produced similar effects, suggesting that the increased permissiveness to WNV may be due to other uncharacterised differences between insecticide resistant and susceptible strains.

In contrast, vector competence can be reduced in mosquitoes with metabolic insecticide resistance. Isofemale lines of *Ae. aegypti* with mixed metabolic and target site insecticide resistance to deltamethrin exhibited lower chikungunya virus (CHIKV) dissemination from the midgut than strains that were more susceptible to insecticide [181]. Similarly, deltamethrin selected *Ae. albopictus* were reported to have lower dissemination of DENV to the heads and salivary glands, with lower viral titres, than the insecticide susceptible parent strain [195]. Whilst insecticide resistant mosquitoes were able to transmit DENV to susceptible mice during blood feeding, the infected mice took longer to develop viraemia than those bitten by insecticide susceptible mosquitoes. This suggests that the inoculum delivered, and/or the viral fitness, was lower in the insecticide resistant strain [195]. The generalisability of these findings to other insecticides and *Aedes* species is unclear however, as selection of *Ae. aegypti* larvae with an organochlorine insecticide, dichlorodiphenyltrichloroethane (DDT), was not associated with any alterations in vector competence for DENV [337]. It is clear that further investigation is required to determine what effects metabolic insecticide resistance may have on vector competence for arboviruses. The mechanisms for an interaction remain poorly characterised, however, there are a number of possibilities.

Increased activity of insecticide detoxification enzymes leads to changes in the levels of reactive oxygen species (ROS). These are known to be activators of antiviral immune pathways, therefore there is potential for this to alter vector competence [197]. Metabolic insecticide resistance requires high levels of

energy and resource investment by mosquitoes. It is thought that this may divert resources away from those required by immune responses, and potentially increase vector competence [58, 191]. Conversely, energy diversion to insecticide detoxification could reduce the availability of resources for viral replication, potentially lowering vector competence [58]. The microbiome of insecticide resistant mosquitoes can vary from insecticide susceptible strains. As the microbiome is considered to be an important determinant of vector competence, this could provide another mechanism for an interaction with insecticide resistance [203, 204].

The aim of this study was to determine whether insecticide selection regimens, resulting in different insecticide resistance phenotypes, led to alterations in the vector competence of four closely related strains of *Ae. aegypti*. Previous RNA sequencing analysis of the Recife *Ae. aegypti* strains used here (see **Chapter 3**), revealed constitutive differences in the expression of genes encoding antimicrobial peptides, and potential activators of Toll innate immune pathways (Clip domain serine proteases). This study sought to determine whether these transcriptomic differences may be associated with differences in vector competence relating to different insecticide selection regimens and their resulting insecticide resistance phenotypes.

4.3 Methods

4.3.1 Mosquito strains

The Recife strain of *Ae. aegypti* is a temephos resistant colony which was originally collected in Araripina, Brazil by Melos Santos et al [301] (72˚ 32'S and 40˚ 34' W). This colony was split by Thornton et al [283] and subjected to different insecticide selection regimens to create four strains with differing insecticide resistance phenotypes. Following the initial insecticide selection regimens described by Thornton et al [283] (see **Chapter 3**), we maintained these colonies under the following insecticide selection regimens prior to undergoing vector competence analyses. The insecticide susceptible REC U was not exposed to any insecticides for a further 9 generations (G47-G55). The temephos resistant REC R was exposed to 1.5mg/L temephos for 24 hours, every 3 generations, for a further 10 generations (G47-G56). The malathion resistant REC M was exposed to 1.5% malathion in WHO tube assays for 1 hour, every 3 generations, for a further 7 generations (G46-G53). The permethrin resistant REC P was exposed to 0.75% permethrin, every 3 generations, for a further 9 generations (G46-G55). All strains were maintained at the Liverpool School of Tropical Medicine (LSTM) under standard insectary conditions (27˚C +/- 1, relative humidity 80% +/- 5, and 12:12 hours light:dark).

4.3.2 Insecticide resistance phenotype testing

To determine the relative insecticide resistance phenotypes of the Recife strains, REC U was exposed to temephos, malathion and permethrin. In addition, REC R was exposed to temephos, REC M was exposed to malathion, and REC P was exposed to permethrin. For temephos selections, temephos powder (Chem Service Inc) was dissolved in acetone to produce 1000x stock solutions. One millilitre of

temephos stock solution was diluted in 1L of a 1 in 5 mixture of larval tray water and distilled water. Groups of 25 L3 instar larvae were exposed in plastic pots to 200mL of 1.5% temephos [283]. The mortality was assessed at 24 hours. Controls were exposed to 1mL/L acetone. REC M and REC U were exposed to 1.5% malathion using WHO tube assays. A 1.5% malathion solution was prepared by dissolving malathion powder (Chem Service Inc) in acetone. A 2mL mixture (1:1:1 v/v) of 1.5% malathion solution, acetone, and corning oil was pipetted onto a filter paper. These papers were dried overnight and stored in a refrigerator before use. Control papers were treated with acetone and corning oil only. Both strains were exposed for 30 mins before being transferred back into holding tubes with access to 10% sucrose on cotton pads. Mortality was assessed at 24 hours post-exposure. The susceptibility of REC U and REC P to 0.75% permethrin was assessed using WHO tube assays according to standard protocols [302]. Both strains were exposed for 30 mins and survival was assessed at 24 hours. A 30 minute time period was chosen as previous attempts with 1 hour exposure to the doses used for selections resulted in high mortality, and the surviving individuals were required to maintain the colonies. Controls were exposed to non-insecticide treated filter papers.

4.3.3 Per os (PO) infections

Female mosquitoes were sugar starved with access to water for 48 hours prior to being offered a blood meal. Stocks of Zika virus (ZIKV) DAK AR 41524 (BEI Resources, NR-50338) were obtained from colleagues at LSTM, passaged once in Vero cells (titre $2x10^7$ PFU/mL), and frozen at -80˚C. To prepare the blood meals, the virus stocks were defrosted and mixed 1:1 with whole human blood containing the phagostimulant adenosine triphosphate (900µM). In arthropod containment level 2 (ArCL2) facilities, 5-7 day old female mosquitoes were offered a blood meal via a Hemotek membrane feeding system for 1 hour. Samples of the blood meal were taken for back titration via plaque assay. Following feeding, mosquitoes were briefly cold anaesthetised to allow removal of partially fed and unfed mosquitoes before being transferred to an incubator (27˚C +/- 1, relative humidity 80% +/- 5, and 12:12 hours light:dark) with access to 10% sucrose solution via cotton wool pads.

4.3.4 Intrathoracic (IT) microinjections

An inoculum titre of 200 PFU/injection was prepared by diluting frozen stocks of ZIKV DAK AR 41524 which had been passaged once in Vero cells. This inoculum titre was based on published experimental data [266, 338, 339]. The virus was diluted in Dulbecco's Modified Eagle Media (DMEM) containing 2% v/v foetal bovine serum (FBS) and 1:1000 v/v 50mg/mL gentamicin. Non-blood fed females aged 3-5 days old were cold anaesthetised at -20˚C for 30 seconds and transferred to a cold plate. In ArCL2 facilities each mosquito was intrathoracically injected with 69nL ZIKV at a rate of 50nL/second using a Nanoject III (Drummond Scientific). Samples of the injection media were taken for back titre via plaque assay and confirmed the average inoculum to be $2.7x10^6$ PFU/mL (187 PFU/injection). Following injection, mosquitoes were housed in cardboard cups in an incubator with access to 10% sucrose until further processing.

4.3.5 Dissections and salivations

Following PO infections, cohorts of mosquitoes were sampled at 5-, 7-, 14- and 21 days post-infection (dpi), and head, body (thorax & abdomen), and saliva were collected. Following IT injections, mosquitoes were dissected into head, body (thorax & abdomen), and legs, and salivated at 5-, 7-, and 10 dpi. Briefly, mosquitoes were cold anaesthetised at -20˚C for 2 minutes and placed on a cold plate. Wings and legs were removed, and the tip of the proboscis was placed in a 20µL pipette tip containing mineral oil. After 15 minutes of forced salivation, saliva was ejected into a tube containing 100µL infection media (DMEM + 2% FBS v/v + 1:1000 50mg/mL gentamicin + 1:200 v/v 10mg/mL Fungin). Body parts were placed into separate Safelock tubes containing infection media and a stainless steel ball bearing (5/32" diameter). All samples were immediately frozen at -80˚C until further processing.

4.3.6 Virological assays

All mosquito samples were analysed using plaque assays. 24-well cell culture plates were seeded with 500µL Vero cells at a density of $2.5x10^5$ cell/mL in growth media (DMEM + 10% FBS + 1:1000 v/v 50mg/mL gentamicin), and incubated overnight (37˚C, 5% CO_2). Body part samples were homogenised using a TissueLyser (Qiagen) at 26Hz for 5mins, and centrifuged at 10000rpm for 5 minutes to pellet debris. Saliva samples were vortexed for 15 seconds. Each sample was serially diluted in infection media and 100µL inoculum was added to the confluent monolayer of Vero cells in 24-well tissue culture plates. Following a 1 hour incubation (37˚C, 5% CO_2), a 0.4% agarose overlay was applied. The plates were incubated for a further 72 hours before being fixed with formaldehyde. The plaque assays were stained with 0.25% v/v crystal violet solution and allowed to dry before the plaques were counted.

4.3.7 Statistical analysis

All statistical analyses were performed in the R-studio environment (version 2.2.2) (**Appendix 4**). For insecticide susceptibility data, and infection, dissemination, and transmission prevalence data, Fisher's exact tests were used to determine differences between strains. The fisher.test function was used for these calculations. Wilson binomial confidence intervals for proportion data were calculated using the binconf function. The normality of ZIKV titre data of each sample type, at each timepoint, was assessed using a Shapiro-Wilks test. If these data were normally distributed, differences in the mean ZIKV of different body parts, at different timepoints, between the strains were assessed using ANOVA. Strain and sample type were used as grouping variables at each timepoint. If the data were non-normally distributed, a Kruskall-Wallis test was used.

4.3.8 Sample size

Sample sizes were considered for the primary outcomes relating to comparing the prevalence of infection, dissemination, and transmission between treatment groups. These data are binomial in nature, as mosquitoes can either be positive or negative for ZIKV. Exact sample size calculations for these comparisons require prior knowledge of the expected proportions for these outcomes in each

experimental group, which were not known prior to this experiment. Furthermore, mortality and variable blood feeding rates, makes attainment of precise sample sizes difficult. A sample size of 30 mosquitoes per treatment, per timepoint, has been reported to be sufficiently powered to detect differences between experimental groups, and this was used as a target in this study [339]. As mosquitoes from all treatment groups must be sampled at the same fixed timepoints, this figure is near the limit of what can be logistically achieved. The statistical power to detect differences between strains associated with this sample size was simulated for a range of proportions, and their combinations between treatment groups (**Supplementary Figure 1**). For each combination, data were simulated using the rbinom function in R and a beta value was calculated using the prop.test function. This was bootstrapped 10,000 times per combination, and beta values with an associated alpha threshold of 0.05 were averaged to produce a mean beta value for each comparison of proportions. This analysis revealed that the minimum statistically significant difference ($\beta \geq 0.8$) between treatment groups across all combinations of proportions, with a sample size of 30, is approximately 30-40%.

4.4 Results

4.4.1 Insecticide resistance phenotypes

The unselected REC U strain was almost completely susceptible to 24-hours exposure to 0.5mg/L temephos (99.4% mortality [95% CI 98.4-99.9]) (**Figure 1**). This was significantly higher than the temephos selected REC R strain (77.6% [74.2-80.8]) (Fisher's exact p<2.20e-16). REC U was also highly susceptible to 30mins exposure to 1.5% malathion (97.5% mortality [94.3-99.2]) compared to the malathion selected REC M (61.1% mortality [50.5-70.9]) (Fisher's exact p=4.70e-16). The permethrin selected REC P exhibited 56.6% mortality [48.3-64.6] to 30 mins exposure to 0.75% permethrin. This was significantly lower (p<2.20e-16) than the unselected REC U strain, which was completely susceptible to the same exposure (100% mortality [96.2-100.0]). There was no mortality in negative control groups.

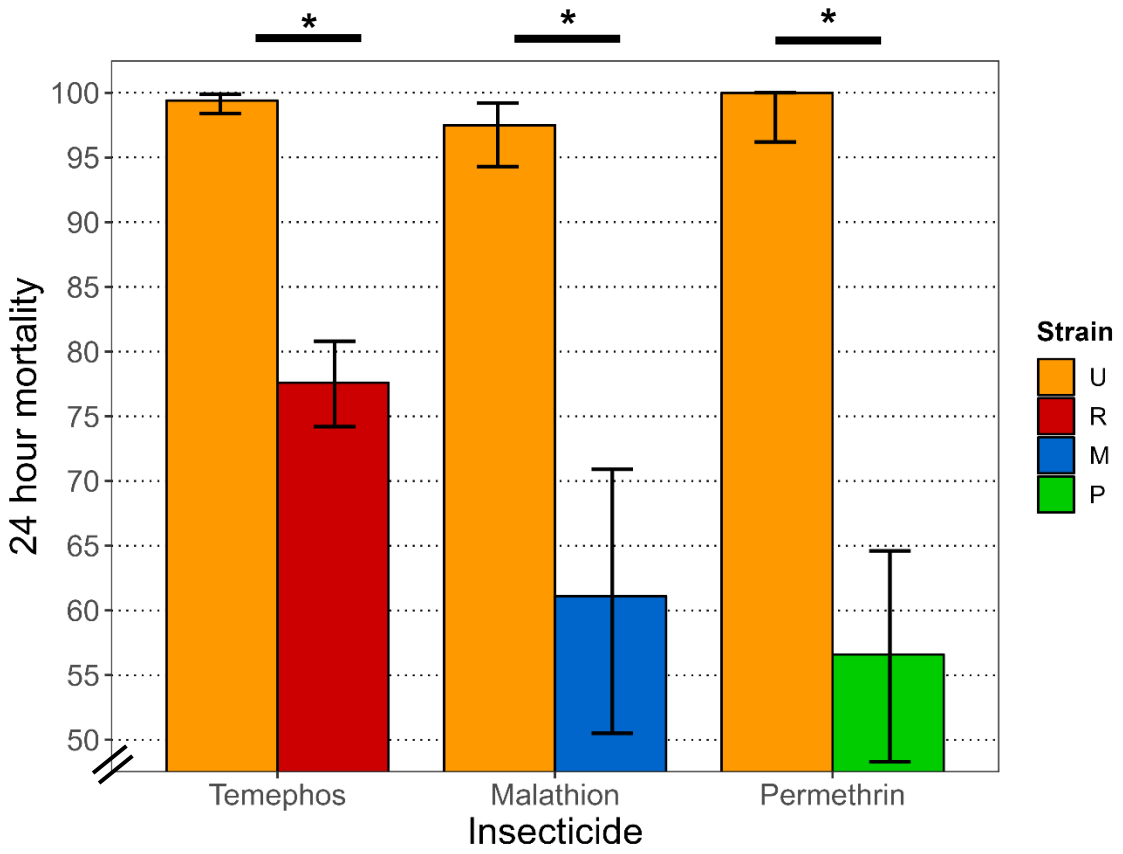


Figure 1 – Insecticide resistance phenotypes of the Recife strains
REC R (n=634) and REC U (n=529) were exposed as larvae to 1.5mg/L temephos for 24 hours. This resulted in 77.6% [95% CI 74.2-80.8] mortality in REC R, and 99.4% [98.4-99.9] mortality in REC U, which was significantly different (p<2.2e-16). REC M (n=95) and REC U (n=200) were exposed to 1.5% malathion in WHO tube assays for 30 minutes. This resulted in 61.1% [50.5-70.9] mortality in REC M, and 97.5% [94.3-99.2] in REC U (p=4.7e-16). REC P (n=152) and REC U (n=97) were exposed to 0.75% permethrin in WHO tube assays for 30 minutes. This resulted in 56.6% [48.3-64.6] mortality in REC P, and 100% mortality in REC U [96.2-100.0] (p=<2.2e-16). Negative controls were used in all three comparisons, and no mortality was recorded.
NB Y-axis scale starts at 50%.

4.4.2 PO infections

Despite extensive optimisation efforts, feeding rates remained low in REC M and REC P. This prevented the attainment of a sufficient sample size for these strains to make statistically meaningful comparisons. As such, oral infection data are presented for REC R and REC U only. In addition, there were insufficient numbers to allow for a 21dpi timepoint to be collected for REC R. Sample sizes are shown in **Supplementary Table 1**. Plaque assay of blood meal samples from feeders used for both strains confirmed a starting ZIKV titre of $1x10^7$ pfu/mL. Samples of infected blood taken at the beginning, and following the 1 hour feeding period, showed there was no significant drop in viral titre in all feeders. The prevalence of infection and dissemination were high in both strains at all timepoints following a blood meal containing ZIKV, except at 5dpi where there was a 25% lower prevalence of disseminated infection in REC R (75.0% [95% CI 46.8-91.1]) than REC U (100% [88.6-100.0]) (Fisher's exact p=0.01) (**Figure**

2). The prevalence of salivary infection was <20% in both strains at all timepoints. Virus was present in the saliva of REC U by 5dpi, but it was not seen in the saliva of REC R until 14dpi. The small number of samples make interpretation difficult, however, this may represent an elongation of the extrinsic incubation period as a consequence of slower initial dissemination from the midgut (**Figure 2**). At 14dpi REC R had a higher prevalence (16.8% [4.7-44.8] of salivary infection than REC U (11.1% [3.9-28.1], but this was not statistically significant (Fisher's exact $p>0.05$).

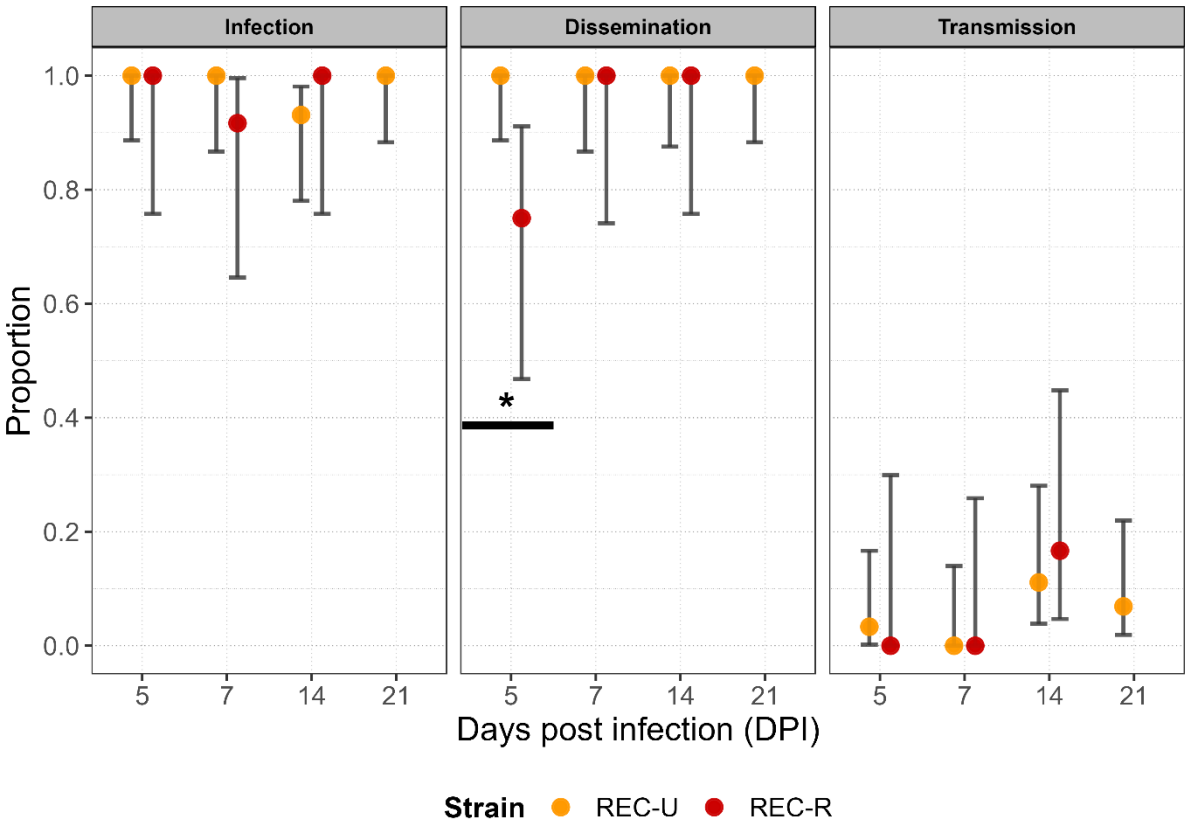


Figure 2 – Infection, dissemination and transmission prevalence following PO infections
Infection, dissemination and transmission prevalence for REC U and REC R following PO infection with ZIKV. There was a 25% lower prevalence of disseminated infection in REC R (75.0% [46.8-91.1]) compared to REC U (100% [88.6-100.0]) (p=0.01) at 5dpi. No other comparisons between strains were significant. Sample sizes for each strain x timepoint combination are shown in **Supplementary Table 1**. Error bars represent 95% binomial confidence intervals (Wilson). Due to low number, there are no data for 21dpi for REC R.

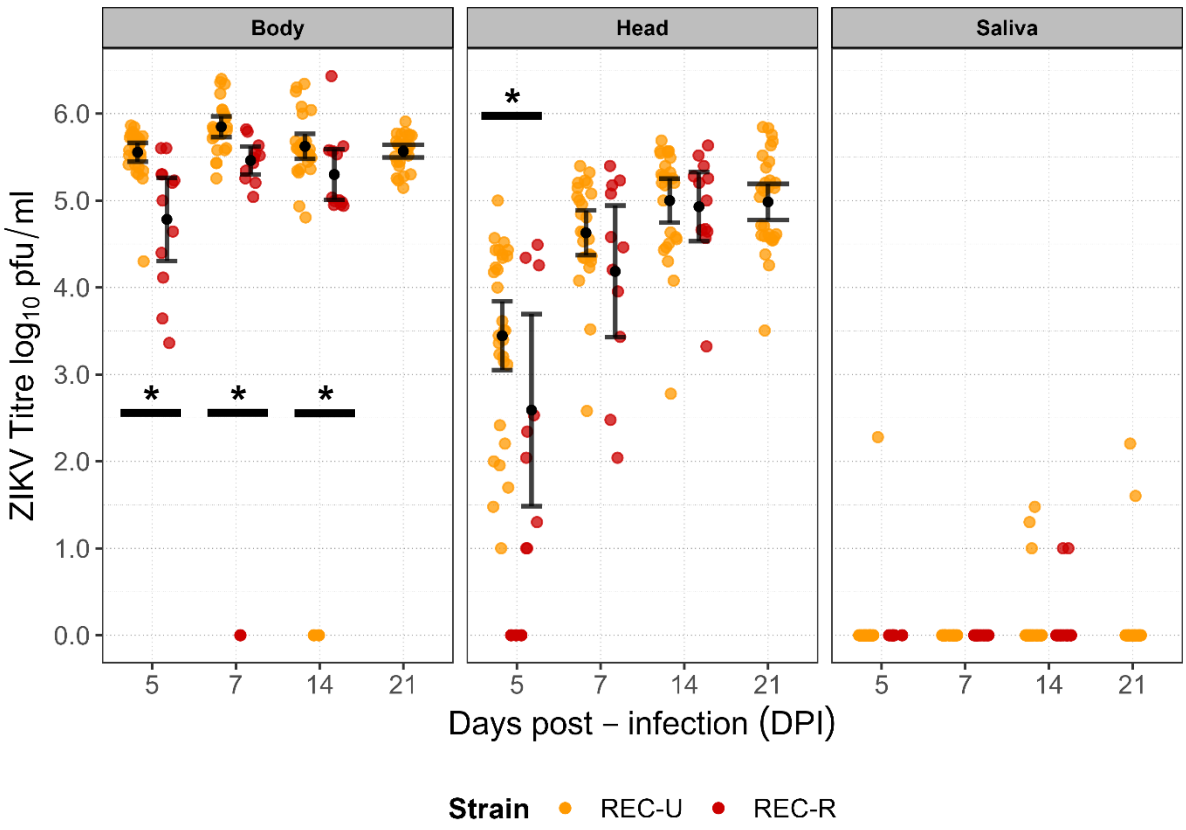


Figure 3 – ZIKV titres following PO infections
ZIKV titres of mosquito bodies, heads, and saliva at multiple timepoints post-infection were determined by plaque assay. The bodies of REC R had consistently lower titres across all timepoints compared to REC U. There were no significant differences in the head or saliva titres between strains. Mean titres are indicated in black. Error bars represent 95% confidence intervals of the mean. Means and confidence intervals were not calculated for saliva samples due to the low number of ZIKV-positive samples. Titres of zero were not included in calculations of means and confidence intervals.

Following oral infections, the mean ZIKV titres in the bodies of REC R mosquitoes (5dpi: log_{10} pfu/body 4.78 [4.31-5.26]; 7dpi: 5.46 [5.30-5.62]; 14dpi: 5.30 [5.01-5.59]) were lower at all timepoints than REC U (5dpi: 5.56 [5.45-5.66] Kruskal-Wallis *p*=6.05e-5; 7dpi: 5.85 [5.73-5.97] *p*=7.08e-4; 14dpi: 5.57 [5.50-5.64] *p*=0.04). Despite these differences, ZIKV titres in the heads of both strains increased from approximately 3.0 log_{10} pfu/head to approximately 5.0 by 14dpi, and there were no significant differences between strains. The small number of ZIKV positive saliva samples from REC R precludes statistical analysis. However, at the 14dpi timepoint, the only timepoint when ZIKV was detected in the saliva of both strains, the mean ZIKV titres were similar between strains (REC U 1.26 [0.66-1.86], REC R 1.00) (**Figure 3** & **Supplementary Table 1**).

4.4.3 IT microinjections

The prevalence of infection in the bodies and heads of mosquitoes was high (>96%) in all four strains at all timepoints post-IT injection (**Figure 4**). There were no significant differences in infection prevalence between strains at any timepoint. The prevalence of infection in the legs of mosquitoes was slightly lower (92.0-96.2%), but there were no significant differences between strains (Fisher's exact p>0.05). There were notable differences in the prevalence of ZIKV in mosquito saliva between strains. At 7dpi, the prevalence of ZIKV in saliva was significantly higher in REC U (39.3% [23.6-57.6] Fisher's exact p = 2.38e-3) and REC M (48.0% [30.0-66.5] p=3.17e-4) than REC R (3.8% [0.0-18.9]). REC P had an intermediate salivary ZIKV prevalence of 18.5% [8.2-36.7], which was not significantly different from other strains. At 10dpi, REC U (41.9% [26.4-59.2]) and REC M (52.0% [33.5-70.0]) had a significantly higher prevalence (p=3.01e-4 and p=3.13e-05, respectively) of salivary infection than REC R (5.3% [1.5-17.3]). In addition, REC P had a significantly lower proportion of salivary infections (16.7% [7.3-33.6]) at 10dpi than both REC M (p=8.89e-3) and REC U (p=0.04). All other comparisons at 10dpi were not significantly different.

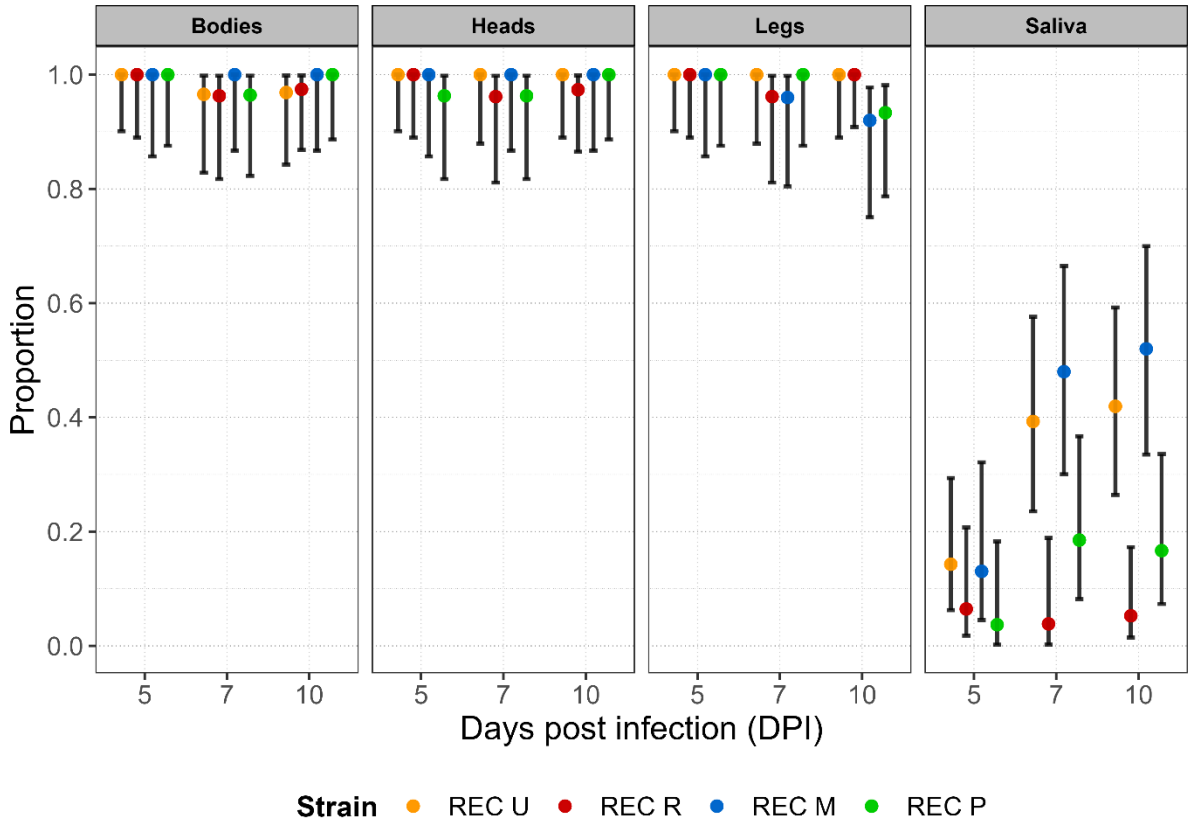


Figure 4 – Infection prevalence in body parts and saliva following IT injections
Infection and transmission prevalence for REC U and REC R following IT infection with ZIKV.
There were no significant differences in infection prevalence in the bodies, heads or legs between strains following IT injection. The prevalence of ZIKV in the saliva of REC R was low at all timepoints. REC U and REC M had the highest prevalence of salivary infection at 7- and 10dpi. Sample sizes for each strain x timepoint combination are shown in **Supplementary Table 2**. Error bars represent 95% binomial confidence intervals

Mosquitoes from the four Recife strains developed similar ZIKV titres in their heads and bodies at all timepoints following intrathoracic injection (**Figure 5**). There were differences in the ZIKV titres in the legs of mosquitoes at multiple timepoints post-injection. At 5dpi, REC P had a 0.37log_{10} PFU/sample higher leg titre than REC U (p = 6.65e-3), and a 0.36log_{10} PFU/sample higher titre than REC R (p=0.021). In addition, REC M had significantly higher leg titres (2.79 log_{10}PFU/sample [95% CI 2.92-3.26]) than REC P (2.43 [2.22-2.64] p= 6.08e-3) and REC U (2.48 [2.34-2.62] p=0.01) at 10dpi (see **Figure 5** & **Supplementary 2**). There were no significant differences in the salivary titres between strains at any timepoint following IT injections.

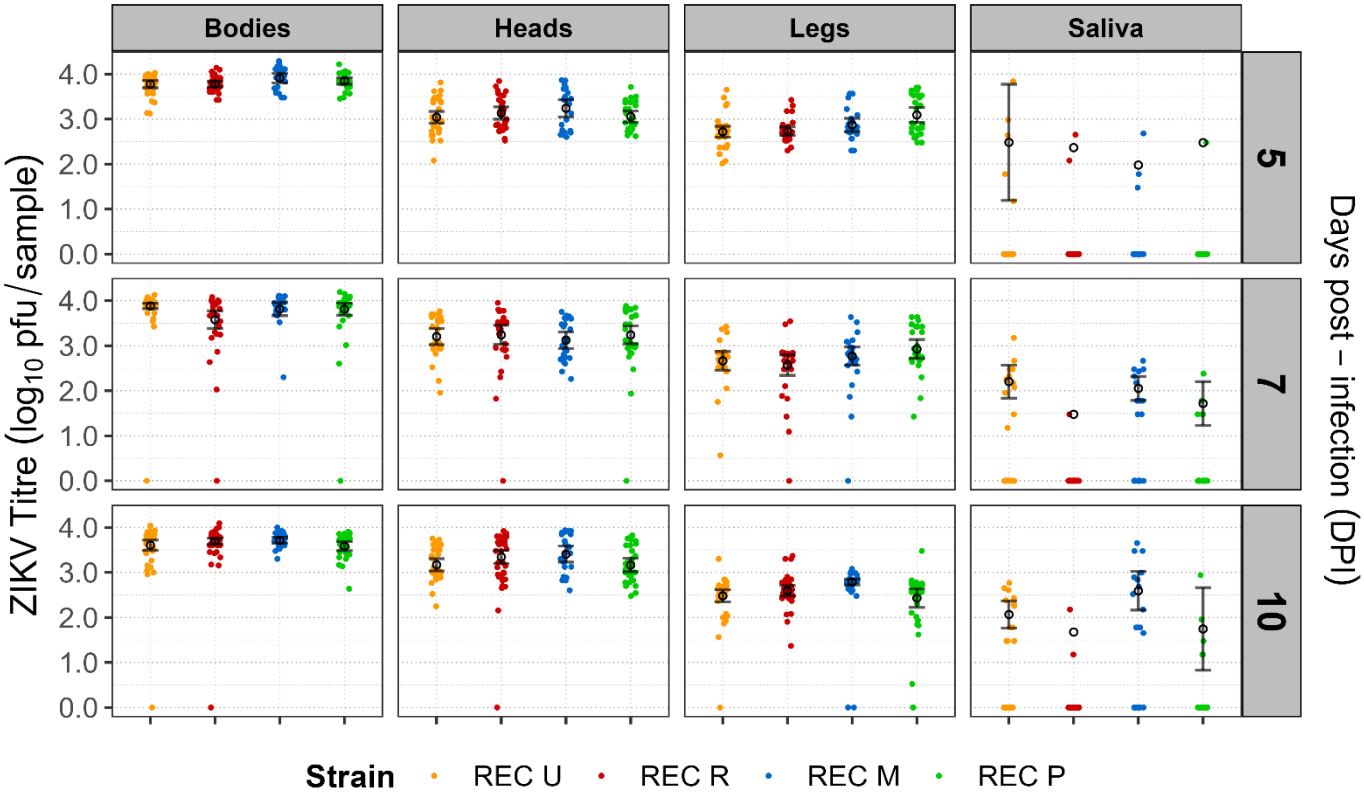


Figure 5– ZIKV titres following IT injections
ZIKV titres of mosquito bodies, heads, and saliva at multiple timepoints post-infection were determined by plaque assay. There were no significant differences in head or body titres at any timepoint between strains. At 5dpi RECP had a higher leg titre than REC U and REC R. At 10dpi REC M had higher leg titres than REC P and REC U. Titres of zero were not included in calculations of means and confidence intervals. Mean values and 95% confidence intervals (Wilson) are shown in black.

4.5 Discussion

Metabolic insecticide resistance is often associated with profound alterations in the physiology of vectors [190-193]. There is also a growing body of evidence that metabolic insecticide resistance may be capable of altering the competence of vectors for arboviruses [175, 181, 183, 195]. The aim of these experiments was to determine whether different metabolic insecticide resistance phenotypes are associated with alterations in the vector competence for ZIKV. As previous transcriptomic analysis of the Recife strains used here showed differences in the expression of genes relating to innate immunity (**Chapter 2**), we aimed to determine whether this was associated with alterations in vector competence. Our data suggest that metabolic insecticide resistance can influence vector competence for ZIKV . Findings from PO and IT infection routes suggest two main areas of difference in vector competence between the Recife strains: 1) systemic dissemination following oral infection, and 2) transmission prevalence.

4.5.1 Systemic ZIKV dissemination following oral infection

The data from PO infections show a 25% lower prevalence of disseminated infection at 5dpi in the temephos resistant REC R compared to the susceptible REC U. Similar results have been reported previously in deltamethrin resistant *Ae. aegypti* with mixed target site and metabolic resistance mechanisms where reduced dissemination of CHIKV from the midgut was observed [175]. Comparable results have also been observed in *Ae. aegypti* with DENV dissemination [195]. However, permethrin selected *Ae. aegypti*, with combined target site and metabolic resistance, had higher dissemination of DENV to the heads of mosquitoes than unselected controls [175]. A similar picture was also seen for *Cx. pipiens* with esterase^2 mediated organophosphate resistance and WNV dissemination from the midgut [183]. These data highlight that the relationship between insecticide resistance and dissemination from the midgut is complex, and is not well characterised to date.

An important implication of a slower rate of dissemination relating to temephos resistance would be an increase in the extrinsic incubation period (EIP) for ZIKV. The small number of ZIKV-positive saliva samples following PO infection make it difficult to make confident assertions about the length of the EIP in each strain. However, ZIKV was detected in the saliva of REC U by 5dpi and was not detected in REC R until 14 dpi. If this finding was replicated with larger sample sizes, this would suggest an elongation of the EIP in REC relating to temephos exposure. It may be that the slower initial rates of viral dissemination from the midgut result in subsequent delays in viral infection of the salivary glands. The length of the EIP is a major determinant of transmission potential, and therefore this could have important implications.

In addition to the reduced dissemination of ZIKV in REC R mosquitoes, body titres (thorax and abdomen) were significantly lower at all timepoints compared to REC U following oral infection. The similarity of body titres in all strains, at all timepoints following IT injection, shows that all the Recife strains permit similar replication of ZIKV when it is deposited directly into the haemocoele. Therefore, this suggests that the differences observed between REC R and REC U following oral infection, are due to midgut related

factors. Reduced dissemination and body titres could reflect fewer initial loci of infection or a reduced replication rate in midgut cells, reduced escape from the midgut into the haemocoele, differences in innate immune responses, or reductions in viral fitness relating to different population bottlenecks in the midgut [82, 93, 340]. Further work is required to determine the exact mechanisms.

Initial viral dose in an ingested bloodmeal is an important factor in determining the prevalence of infection and dissemination in mosquitoes, and is also important in influencing viral titres [83, 341, 342]. Back-titration of blood meals by plaque assay confirmed there were no differences in the ZIKV titre of the bloodmeal offered to REC R and REC U in our study, but it is possible that uncharacterised differences in blood meal volume ingested by these strains could have produced the observed results. Previous evaluation of the Recife strains found no significant difference in blood meal volume between REC U and REC R [190]. However, due to limited numbers of blood fed mosquitoes in this study, it was not possible to include this analysis.

Another possible contributor to the results seen from oral infections are potential differences in the microbiome of REC U and REC R. The constitution of the microbiome in these strains has not yet been investigated, and the microbiome is known to be an important contributor to vector competence [140, 201]. The microbiome of insecticide resistant and susceptible vector populations can differ, therefore it is possible that the insecticide selection regimens used here may have altered the microbiome [202]. The microbiome can both facilitate [343], and limit [344] viral infection of the midgut, and is also known to be important in priming the immune system through activation of innate immunity pathways [140]. Therefore, differences in microbiome between the strains could contribute to the results seen following PO infections. Further investigation will be required to explore the potential role of the microbiome in the vector competence of these mosquito strains.

4.5.2 Transmission prevalence

The presence of ZIKV in saliva was low following PO infection in our study, and there were no significant differences in the prevalence of salivary infection or titres between REC R and REC U. However, infection of all four Recife strains via IT injection revealed marked differences in the prevalence of salivary infection. The low prevalence of ZIKV in the saliva of REC R following IT injections may suggest the presence of salivary gland infection and/or escape barriers [345]. The mechanisms for these barriers are very poorly characterised, but changes to these barriers relating to insecticide resistance have not been previously reported [345]. Future work should determine the nature of this barrier using salivary gland dissection and viral titration, or immunofluorescent techniques, to determine whether these findings represent the presence of a salivary gland infection or escape barrier [346, 347]. Comparison of these barriers between the temephos resistant REC R and susceptible REC U may reveal differences relating to insecticide resistance status.

At 7- and 10dpi, REC U and REC M had significantly higher prevalence of ZIKV in saliva than REC R. The permethrin selected REC P had an intermediate prevalence of salivary infection at 7- and 10dpi, and REC R remained low throughout all tested timepoints. To our knowledge, IT injections have not been used in previous investigations of the effects of insecticide resistance on vector competence, and the findings relating to salivary infection from PO infections are mixed [242]. Atyame et al [183] reported higher WNV saliva positivity in organophosphate resistant *Cx. pipiens* compared to susceptible strains. This contrasts with findings of Deng et al [195] in *Ae. albopictus* where salivary DENV prevalence was lower in deltamethrin selected strains. As the use of IT injections bypasses the midgut, it is difficult to make robust comparisons with data from PO infections.

In this study, whilst different insecticide resistance phenotypes are associated with differences in transmission prevalence following IT injections, there is no clear direction of effect of insecticide resistance phenotype on transmission. REC M and REC U had similar salivary infection prevalence throughout, despite REC U being reared for multiple generations in the absence of insecticide selection pressure. Furthermore, REC R and REC M were both selected with organophosphate insecticides, but had marked differences in the prevalence of salivary infections. Due to poor feeding rates in REC M and REC P, there are no data for systemic ZIKV dissemination following oral infection in these strains. Therefore, it is not possible from IT injection data only, to exclude midgut factors that could alter transmission prevalence in these strains.

4.5.3 Correlation with immune gene expression

RNA sequencing analysis of previous generations of the Recife strains identified a number of differences in the expression of immune genes (**Chapter 3**). REC U had lower expression of a number of cecropins, defensins and a Clip domain serine protease compared to REC R. Cecropins and defensins are known to be involved in antiviral immune responses [114, 324-327]. Moreover, Clip domain serine proteases can be involved with activation of the Toll pathway, which is key to the innate immune response to viral infection in mosquitoes [328]. We hypothesised that this may be associated with alterations in measures of vector competence in REC U compared to REC R.

In this study there was higher dissemination prevalence at 5dpi and body viral titres in REC U compared to REC R from PO infections, and a significantly higher prevalence of salivary infection in REC U following IT injections. It has been reported that *Ae. aegypti* strains with higher constitutive expression of cecropins and clip domain serine proteases had lower infection prevalence and limited dissemination of DENV to salivary glands [132]. Furthermore, variations in the basal activation level of the Toll pathway have also been associated with differences in dissemination rates for DENV [348]. As immune gene expression was not examined contemporaneously in this experiment, it is not possible to determine that these disparities in immune gene expression resulted in the observed differences in vector competence. However, this provides an interesting avenue for future research, and existing mosquito samples

collected for the vector competence analyses could be used to determine the expression of this panel of immune genes at different timepoints post-infection.

REC M had a broadly similar expression of innate immune genes to REC R, except for the under-expression of two genes encoding cecropin D (**Chapter 3**). Given the established antiviral capabilities of cecropins, it is possible that these differences in expression could be linked to the marked differences in salivary infection prevalence observed between these strains [139].

4.5.4 Limitations

Achieving adequate sample sizes for statistically powered comparisons presents a challenge in vector competence experiments, due to blood feeding rates, mosquito survival, and small numbers of mosquitoes developing salivary infections [349]. The target of 30 mosquitoes per strain per timepoint was achieved for some, but not all, combinations in this study. Therefore, we may not have been able to detect smaller differences in the proportion of samples that were positive with ZIKV between strains.

Where resources permit, multiple replicates of each experiment could be performed to enhance sample sizes. The use of mosquito lines derived from the same parent strain is a strength of our study, however, laboratory mosquito colonies that have undergone repeated rounds of insecticide selection are prone to the effects of genetic drift. It is possible that the phenotypes observed may be influenced by the loss of genetic diversity and stochastic overrepresentation of alleles due to population bottlenecks. Performing replicates of each insecticide selection regimen may help identify these effects but would be logistically demanding.

4.5.5 Conclusion

The data presented here show that metabolic insecticide resistance, resulting from different insecticide selection regimens, is associated with differences in viral dissemination and presence in saliva within a relatively small number of generations. Following oral infections, dissemination of ZIKV from the midgut, and the viral titres of mosquito bodies, were higher in the temephos susceptible strain than the temephos resistant strain. Data from intrathoracic injections identified the presence of salivary gland barriers in the temephos resistant strain, resulting in low levels of salivary infection. The insecticide susceptible and malathion resistant strains had high salivary infection prevalence compared to the temephos resistant strain. The underlying mechanisms remain unclear and require further investigation. However, differences in immune gene transcription of antimicrobial peptides and potential activators of the Toll immune pathway may contribute to the differences seen and warrant further investigation.

4.6 Supplementary data

Supplementary Table 1 –Mean ZIKV titres from oral infections

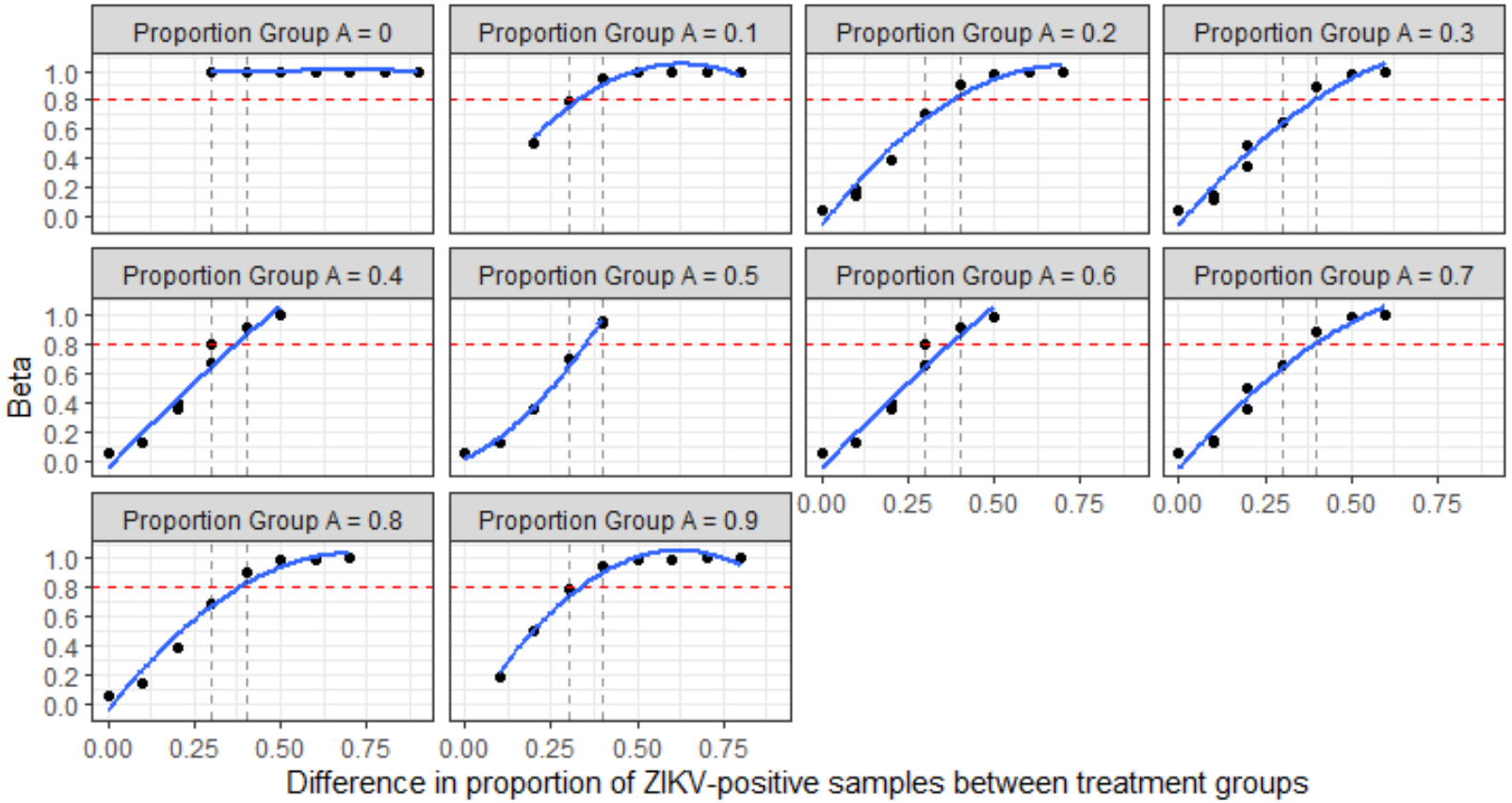
Strain	Bodies pfu/sample [95% CI]				Heads pfu/sample [95% CI]				Saliva pfu/sample [95% CI]			
	5dpi	**7dpi**	**14dpi**	**21dpi**	**5dpi**	**7dpi**	**14dpi**	**21dpi**	**5dpi**	**7dpi**	**14dpi**	**21dpi**
REC U	**5.56** [5.45-5.66] n=30	**5.85** [5.73-5.97] n=25	**5.62** [5.48-5.77] n=27	**5.57** [5.50-5.64] n=29	**3.44** [3.05-3.84] n=30	**4.63** [4.37-4.89] n=25	**5.00** [4.75-5.25] n=27	**4.98** [4.78-5.19] n=29	**2.28** n=1	0	**1.26** [0.66-1.86] n=3	**1.90** [0.00-5.73] n=2
REC R	**4.78** [4.31-5.26] n=12	**5.46** [5.30-5.62] n=11	**5.30** [5.01-5.59] n=12	NA	**2.59** [1.48-3.69] n=9	**4.18** [3.43-4.94] n=11	**4.93** [4.53-5.33] n=12	NA	0	0	**1.00** n=2	NA

Supplementary Table 1 – Mean ZIKV titres from PO infections
The mean ZIKV titres of bodies, heads, and saliva of REC U and REC R at multiple timepoint post-PO infection are shown in bold as plaque forming units/sample. Mosquitoes were fed ZIKV via an artificial blood meal (titre of blood meal $2x10^7$ pfu/mL). Viral titres were determined by plaque assay. The number of samples collected for each strain x timepoint are indicated. No data were collected for REC M or REC P following PO infection due to poor feeding rates. In addition, 21dpi samples were collected for REC R due to insufficient numbers.

Supplementary Table 2 – Mean ZIKV titres from intrathoracic microinjections

Strain	Bodies			Heads			Legs			Saliva		
	5dpi	**7dpi**	**10dpi**	**5dpi**	**7dpi**	**10dpi**	**5dpi**	**7dpi**	**10dpi**	**5dpi**	**7dpi**	**10dpi**
REC U	**3.77** [3.69-3.85] n=35	**3.88** [3.82-3.94] n=28	**3.61** [3.49-3.72] n=31	**3.04** [2.91-3.17] n=35	**3.20** [3.02-3.38] n=28	**3.17** [3.03-3.31] n=28	**2.72** [2.60-2.84] n=35	**2.66** [2.46-2.87] n=28	**2.48** [2.34-2.62] n=30	**2.48** [1.19-3.77] n=5	**2.20** [1.84-2.57] n=11	**2.07** [1.76-2.37] n=13
REC R	**3.78** [3.71-3.84] n=31	**3.58** [3.38-3.78] n=26	**3.69** [3.63-3.76] n=38	**3.13** [3.00-3.27] n=31	**3.24** [3.03-3.46] n=25	**3.35** [3.20-3.49] n=37	**2.73** [2.64-2.83] n=31	**2.57** [2.34-2.80] n=25	**2.59** [2.48-2.71] n=38	**2.37** [0.00-6.01] n=2	**1.48** n=1	**1.68** [0.00-8.03] n=2
REC M	**3.91** [3.80-4.02] n=23	**3.81** [3.67-3.96] n=25	**3.71** [3.65-3.78] n=25	**3.24** [3.05-3.43] n=23	**3.12** [2.94-3.31] n=25	**3.41** [3.23-3.59] n=25	**2.87** [2.72-3.02] n=23	**2.77** [2.57-2.97] n=24	**2.79** [2.72-2.85] n=23	**1.98** [0.42-3.54] n=3	**2.05** [1.79-2.32] n=12	**2.59** [2.16-3.02] n=13
REC P	**3.85** [3.77-3.92] n=27	**3.81** [3.68-3.95] n=27	**3.59** [3.48-3.69] n=30	**3.06** [2.93-3.18] n=26	**3.24** [3.04-3.44] n=26	**3.16** [3.02-3.31] n=30	**3.09** [2.92-3.26] n=27	**2.93** [2.72-3.14] n=27	**2.43** [2.22-2.64] n=28	**2.48** n=1	**1.72** [1.23-2.21] n=5	**1.74** [0.83-2.66] n=5

Supplementary Table 2 – Mean ZIKV titres from IT microinjections
The mean ZIKV titres of bodies, heads, and saliva of REC U and REC R at multiple timepoint post-IT infection are shown in bold as plaque forming units/sample. Mosquitoes were injected intrathoracically with 69nL of $2.7x10^6$ pfu/mL ZIKV. Viral titres were determined by plaque assay. The number of samples collected for each strain x timepoint are indicated.



Supplementary Figure 1 – Statistical power to detect differences in the proportion of ZIKV-positive samples between experimental groups for a sample size n=30 per treatment group, at each timepoint.

Each pane represents the proportion of ZIKV-positive samples observed in a theoretical treatment group A. All combinations of proportions between treatment group A and B were simulated. The x-axis represents the difference in the observed proportions of ZIKV-positive samples between two treatment groups of mosquitoes. The points indicate the mean beta values from 10,000 bootstraps for each proportion difference between groups. The red horizontal dashed line indicates a beta value of 0.8. The vertical black dashed lines indicate the range of points where the differences in proportions between treatment groups can be detected above the beta threshold (30-40%).

Chapter 5 – Can sublethal larval exposure to temephos alter the vector competence of *Aedes aegypti* for Zika virus?

5.1 Abstract

Mosquitoes may be exposed to doses of insecticides that are insufficient to cause rapid mortality. However, these sublethal doses of insecticide have been associated with numerous fitness costs and changes in life history traits. There is limited evidence that there may also be impacts of sublethal insecticide exposure on the vector competence of mosquitoes for arboviruses. Despite the widespread use of temephos as a larvicide for *Aedes* control, the potential effects of sublethal temephos exposure on vector competence have not been explored. Here, two strains of *Aedes aegypti* with differing susceptibilities to temephos, were sublethally exposed to temephos as larvae. The adults were infected with Zika virus via an artificial blood meal. Body parts and saliva from multiple timepoints post-infection were analysed to detect differences in vector competence. There were no significant differences in the prevalence of infection, dissemination, or transmission by temephos exposure status. Sublethal temephos exposure was associated with higher viral titres in the saliva of temephos susceptible mosquitoes. These data suggest that sublethal temephos exposure may be capable of altering aspects of vector competence, and further investigation using different insecticide doses, and bloodmeal viral titres is warranted.

5.2 Introduction

The efficacy of insecticides relies on their ability to interrupt transmission by delivering a lethal dose of insecticide to mosquitoes. However, there are situations where a mosquito may receive a sublethal exposure of insecticide that is insufficient to produce mortality. Insecticide resistance is widespread amongst arbovirus vectors and increasingly mosquitoes may survive a dose of insecticide that would produce high mortality in a susceptible population [55]. Adult mosquitoes are known to exhibit various avoidance behaviours which reduce time spent in contact with insecticide-treated surfaces, thereby reducing the dose of insecticide they receive [206-208]. Insecticide degradation on treated surfaces and in larval habitats over time can lead to sublethal exposure if regular dosing is not performed [209, 210]. Furthermore, agricultural run-off can lead to the contamination of larval habitats with diluted concentrations of insecticide [350].

Whilst these doses of insecticide may not result in mortality, they can still cause profound behavioural and physiological disturbances. Experimental sublethal insecticide exposure has been shown to affect a diverse range of life history traits in non-vector insects such as behaviour [211, 212], development [213, 214], sex ratios [351], longevity [215], and fecundity [216, 217]. The concern with vector species is that

sublethal exposure to insecticide may modulate their ability to transmit human pathogens. Sublethal insecticide exposure has been implicated in alterations in vectorial capacity, a metric of the overall transmission risk posed by a vector population [76]. In particular, the complex neurological systems controlling mosquito host-seeking behaviours appear vulnerable to the neurotoxic effects of commonly used insecticides, with sublethal exposure capable of disturbing host-seeking, and blood feeding behaviours [352-355]. For successful transmission to occur, a mosquito vector must survive long enough to allow completion of the extrinsic incubation period of a pathogen. As such, the observation that sublethal insecticide exposure can reduce vector longevity is important for disease transmission risk [353, 355, 356].

In addition to these effects, sublethal insecticide exposure may be capable of influencing vector competence, the intrinsic permissiveness of vectors to infection and transmission of pathogens. In multiple *Anopheles-Plasmodium* models, sublethal insecticide exposure as adults [250, 355, 357, 358], and larvae [359], has been associated with decreases in both the prevalence and density of parasites. Conversely, sublethal insecticide exposure of adult *Aedes* species does not appear to have an impact on infection prevalence for arboviruses. *Ae. aegypti* exposed to malathion prior to oral infection with dengue virus (DENV) did not exhibit significant differences in infection prevalence compared to unexposed controls [223]. Similarly, bifenthrin exposure of mosquitoes was not associated with differences in the infection prevalence for DENV [218] or Zika virus (ZIKV) [222].

Adult sublethal exposure has been associated with increased dissemination of ZIKV beyond the midgut to the peripheral tissues of mosquitoes [218, 222]. This could suggest interference with midgut escape barriers, allowing virus to enter the haemocoele more readily. It is also possible that replication dynamics of viruses could be affected by insecticide exposure. There is evidence that bifenthrin exposure may affect DENV replication, with bifenthrin exposed *Ae. aegypti* displaying lower body titres than unexposed [218]. However, as *Ae. albopictus* exposed to bifenthrin had lower body titres of ZIKV than unexposed, the direction of any effect remains unclear [222]. Furthermore, ZIKV titres in the legs of exposed mosquitoes were reported as being significantly higher than unexposed controls in one study, but this effect varied by the age at which the mosquitoes were infected [222]. To date there is no evidence to suggest sublethal insecticide exposure of adult mosquitoes modulates the prevalence of arboviruses in mosquito saliva, an important measure of vector competence, but few studies have directly investigated this outcome [218].

Larvicides are widely used to target mosquito vectors of arboviruses, but there has been little research into the potential impacts of larval sublethal exposure to insecticides on vector competence. Sublethal exposure to the *Bacillus thuringiensis* (Bti)-based larvicide was associated with significantly higher infection and dissemination prevalence for ZIKV in *Ae. aegypti* [220]. This effect has also been noted for infection and dissemination prevalence of certain strains of DENV, but there was no significant effect on

chikungunya virus (CHIKV) competence [221]. Few studies have investigated the effects of traditional neurotoxic larvicide exposure on vector competence, which have notably different mechanisms of action from Bti-insecticides [360]. Larval malathion exposure has been associated with higher prevalence of disseminated infection of Sindbis virus (SINV) to the heads of *Ae. aegypti*, but interestingly not *Ae. albopictus* [219]. However, as neither of these mosquito species are considered natural vectors of SINV, the generalisability of these findings to other vector-virus pairings, is unclear.

Here, the impact of larval sublethal temephos exposure on the vector competence for ZIKV is explored in two strains of *Aedes aegypti* with differing insecticide susceptibilities. The impacts of temephos exposure on vector competence have not previously been investigated, despite the fact temephos is widely used to target the larval stages of *Aedes* mosquitoes [361].

5.3 Methods

5.3.1 Mosquito strains

The Recife strain (REC) of *Ae. aegypti* was a temephos resistant field strain that was originally colonised in Brazil by Melos-Santos et al [294], and maintained at the Liverpool School of Tropical Medicine. We split this colony to maintain a temephos resistant REC R strain with larval temephos exposure every 3 generations, and an unselected REC U strain which was not exposed to insecticides [283]. The development and insecticide resistance phenotypes of these strains are described in detail here [283]. Briefly, to maintain the REC R line, larvae were selected every three generations for 21 generations with exposure to a LD_{50} dose (0.5mg/L temephos) for 24 hours. REC U were not exposed to any insecticide for 20 generations. The REC R mosquitoes used here were last selected 3 generations previously.

5.3.2 Sublethal temephos exposure

Two different concentrations of temephos were prepared, a 'low' dose (0.012mg/L) which is the WHO discriminating dose for temephos [362], and a 'high' dose (0.12mg/L) which is 10x the discriminating dose. Temephos powder (Chem Service Inc) was weighed and dissolved in acetone to produce 1000X stock solutions of each concentration. 200mL of water from larval trays was added to 800ml distilled water, and 1mL of stock temephos solution to give the required concentrations of temephos. 200mL of these dilutions was added to plastic dishes and larvae at the 3rd instar stage were added in batches of 25 to each dish. Control mosquitoes were exposed to 1ml/L acetone.

A total of 900 REC U larvae were exposed to the low dose of temephos, and 600 larvae were exposed to acetone only. For REC R, 550 larvae were exposed to the high dose of temephos and 400 were unexposed. This was the highest possible number of each strain that could be reared and exposed to temephos at the same time. To test the susceptibility of REC U to the high dose of temephos, 150 larvae were exposed, and mortality recorded, but were not included in the infection experiments. Larvae were exposed for 24 hours. After exposure, mortality was recorded, and surviving larvae were moved into

larval trays containing 1L distilled water at a maximum density of 100 larvae/tray. Larvae were provided with a yeast tablet every 48 hours and were reared to adults. Adults from each experimental group were housed in separate Bugdorm cages (MegaView Science Co Ltd, Taiwan) with access to 10% sugar and maintained at 27˚C (+/- 1), 80% relative humidity (+/- 5), and 12:12 hours light:dark.

5.3.3 Per os (PO) infections

Mosquitoes were sugar starved for 48h prior to blood feeding with access to water, and females were transferred to plastic pots in groups of 100. Stocks of ZIKV DAK AR 41524 (BEI Resources, NR-50338) were obtained from colleagues at the Liverpool School of Tropical Medicine, passaged once in Vero cells, and stored at -80˚C. The titre was confirmed by plaque assay ($2x10^7$ pfu/mL). ZIKV stocks were mixed 1:1 with whole human blood containing the phagostimulant adenosine triphosphate at a final concentration of 900µM. Samples of the blood meal were taken for back-titration. Under arthropod containment level 2 conditions, mosquitoes aged 5-7 days old were offered a blood meal via the Hemotek feeding system (Hemotek Ltd, UK). After 1 hour, the mosquitoes were cold anaesthetised, and unfed and partially fed females were discarded. Fully fed females were given access to 10% sucrose via cotton wool pads, and transferred to an incubator (27˚C +/- 1, relative humidity 80% +/- 5, and 12:12 hours light:dark) until further processing.

5.3.4 Dissections and forced salivations

Cohorts of mosquitoes from each experimental group were sampled at 5-, 7-, 14- or 21-days post infection (dpi). Mosquitoes were cold-anaesthetised for 5min at -20˚C and transferred to a cold plate. The tip of the proboscis of each mosquito was placed into a 20µL pipette tip containing mineral oil, and mosquitoes were salivated for 15 mins. Following salivation, each mosquito was dissected into head and body (thorax, abdomen and legs). Saliva was transferred into a tube containing 100 µL infection media (Dulbecco's Modified Eagle Media [DMEM]+ 2% foetal bovine serum [FBS], 1:1000 v/v 50mg/mL gentamicin, 1:200 v/v 10mg/mL Fungin [Invivogen]). Heads and bodies were stored in separate tubes containing 200µL infection media and a stainless-steel ball bearing (Dejay Distribution Limited). Samples were immediately frozen at -80˚C until homogenisation.

5.3.5 Plaque assays

Mosquito heads and bodies were homogenised at 26Hz for 5mins using a TissueLyser (Qiagen), and debris were pelleted by centrifugation prior to performing a serial 10-fold dilution series in infection media. Saliva samples were vortexed for 15 seconds prior to serial dilution. The dilution series for each sample was added in duplicate to confluent Vero cells in 24-well cell culture plates, prior to a 1hr incubation (37˚C, 5% CO_2). Following incubation, a 0.4% agarose overlay was applied, and the plates were incubated for 96 hours (37˚C, 5% CO_2). The plates were fixed using formaldehyde and stained using a 0.25% v/v crystal violet solution. Plaques were counted, and the mean of the replicates was used to calculate the viral titre of each sample. The analysis was performed sequentially whereby head samples were only

processed by plaque assay if the mosquito body was ZIKV-positive, and saliva was processed only if the mosquito body and head were positive for virus.

5.3.6 Statistical analysis

Infection prevalence was defined as the proportion of mosquitoes with established ZIKV infection of their bodies out of the total blood fed in that experimental group per timepoint. Dissemination prevalence was defined as the proportion of ZIKV-positive head samples out of the number of mosquitoes with established body infection in each experimental group per timepoint. Transmission was defined as the proportion of mosquitoes with ZIKV-positive saliva out of the total with disseminated infection in each experimental group per timepoint.

All statistical analysis was performed in the R environment (version 2.2.2). Chi-squared tests were conducted using the chisq.test function to determine independence of mortality data following exposure to different doses of temephos. At each timepoint, the infection, dissemination, or transmission prevalence was compared between exposed and unexposed mosquitoes of each Recife strain. As the overall sample size of count data was $n<1000$, these prevalence data was analysed using Fisher's exact test via the fisher.test function [363].

Binomial confidence intervals for these data were computed using the binconf function using the Wilson method. For each treatment group, the normality of viral titre data for each body part, at each timepoint, was assessed using a Shapiro-Wilks test via the shapiro.test function. Normally distributed data were analysed using an ANOVA with viral titre, temephos treatment and mosquito strain used as grouping variables. Non-Normally distributed data were analysed using a Kruskall-Wallis test (kruskal.test function) with the same grouping variables. Viral titre values of 0 were excluded from these calculations.

5.3.7 Sample sizes

A sample size of 30 mosquitoes per treatment group, per timepoint was the target for these experiments. This has previously been reported to be sufficiently powered to detect differences in infection, dissemination, and transmission prevalence between groups [339]. Sample size calculations conducted in the previous chapter (**Chapter 4**) show that this is sufficiently powered to detect a 30-40% difference in groups regardless of effect size, at $\beta \geq 0.8$ and $\alpha \leq 0.05$ thresholds.

5.4 Results

5.4.1 Mortality to temephos exposure

Larval exposure of REC U to a low dose (0.012mg/L) of temephos resulted in minimal mortality (1.7% [95% CI 1.0-2.7] n=900), but this was significantly higher than unexposed controls (0.3% [0.1-1.2] n=600, Chi-squared p=0.02, df=1) (**Figure 1**). Exposure of REC R to a high dose (0.12 mg/L) resulted in 23.3% ([19.9-27.0] n=550) mortality, which was significantly higher than unexposed controls (1.0% [0.3-2.5] n=400, Chi-squared p=2.2e-16, df=1). REC U exposed to the higher dose of temephos (0.12 mg/L) were not used for the infection experiments but demonstrated significantly higher mortality (49.3% [41.4-57.3] n=150, Chi-squared p=8.13e-10, df=1) than REC R (**Figure 1**).

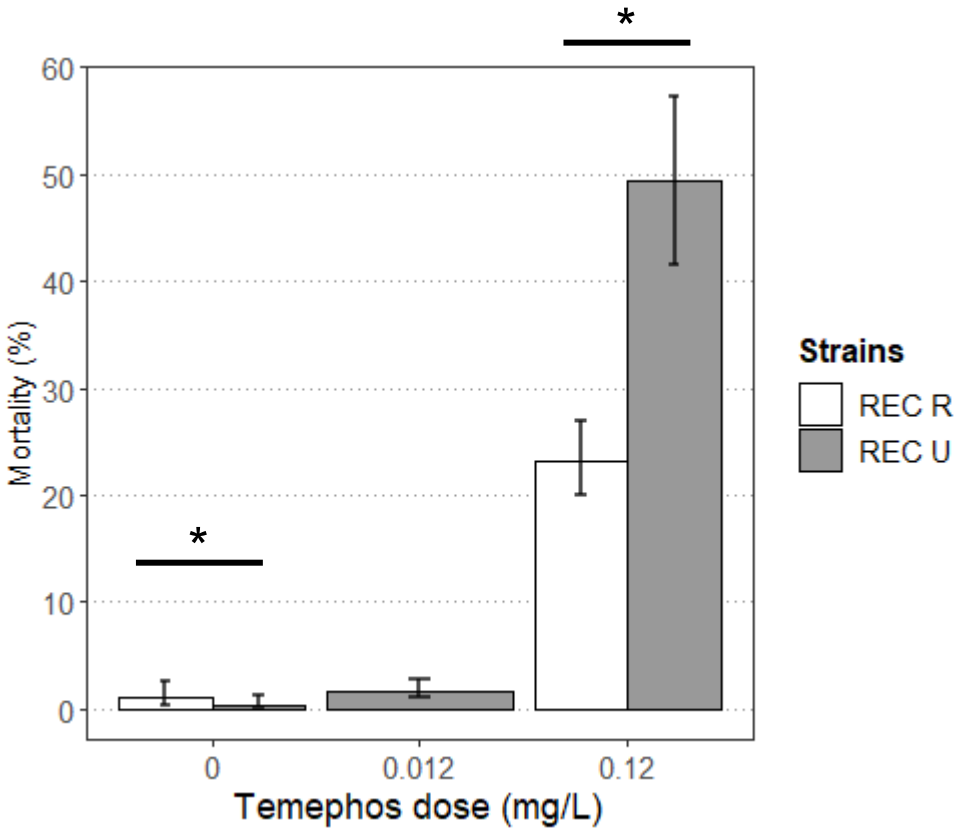


Figure 1 – Mortality after 24 hours of exposure to temephos.
Exposure of REC U to 0.012mg/L temephos resulted in 1.7% mortality [95% CI 1.0-2.7] n=900) which was significantly higher than REC-U exposed to acetone only (0.3% [0.1-1.2] n=600) (Chi-squared p=0.02, df=1). Exposure of REC-R to 0.12mg/L resulted in 23.3% mortality ([19.9-27.0] n=550) compared to 1.0% ([0.3-2.5] n=400) in unexposed controls. REC-U exposed to the higher dose had significantly higher mortality (49.3% [41.4-57.3] n=150) than REC-R but were not used for infection experiments.

Mosquitoes from all four experimental groups successfully blood fed resulting in a total of 122, 125, 50, and 40 mosquitoes in the REC U exposed, REC U unexposed, REC R exposed and REC U unexposed groups, respectively. The number of mosquitoes sampled at each timepoint per group is shown in **Supplementary Table 1**. The sample size target was not reached for all timepoints for each strain due to low blood feeding rates, which was lower in REC R despite attempts at optimisation. Plaque assays of blood meals confirmed the ZIKV titre as $1.0x10^7$ pfu/mL. There was no significant decrease in viral titre between the start and end of the 1h feeding period.

5.4.2 Infection, dissemination, and transmission prevalence

Infection prevalence was high (90-100%) in exposed and unexposed groups for both mosquito strains and remained relatively constant throughout the time-period tested (**Figure 2**, **Supplementary Table 1**). The prevalence of disseminated infection to the heads of mosquitoes was >90% for all experimental groups except REC R unexposed at 5dpi which was 75%. This difference was not statistically significant (Fisher's exact $p>0.05$) and was no longer present by the 7dpi timepoint. ZIKV was detected in the saliva of all experimental groups except REC R exposed, which remained negative at 21dpi. There was a difference in the prevalence of salivary infection at 14dpi between exposed REC U (30.0% [16.7-47.9]) and unexposed REC U (11.1% [3.9-28.1]), but this was not significant (Fisher's exact $p=0.11$). Virus was detected by 5dpi in the saliva of REC U unexposed, which was earlier than either REC U exposed (7dpi) or REC R exposed (14dpi). ZIKV was not found in the saliva of REC R unexposed at any timepoint (**Figure 2**, **Supplementary Table 1**).

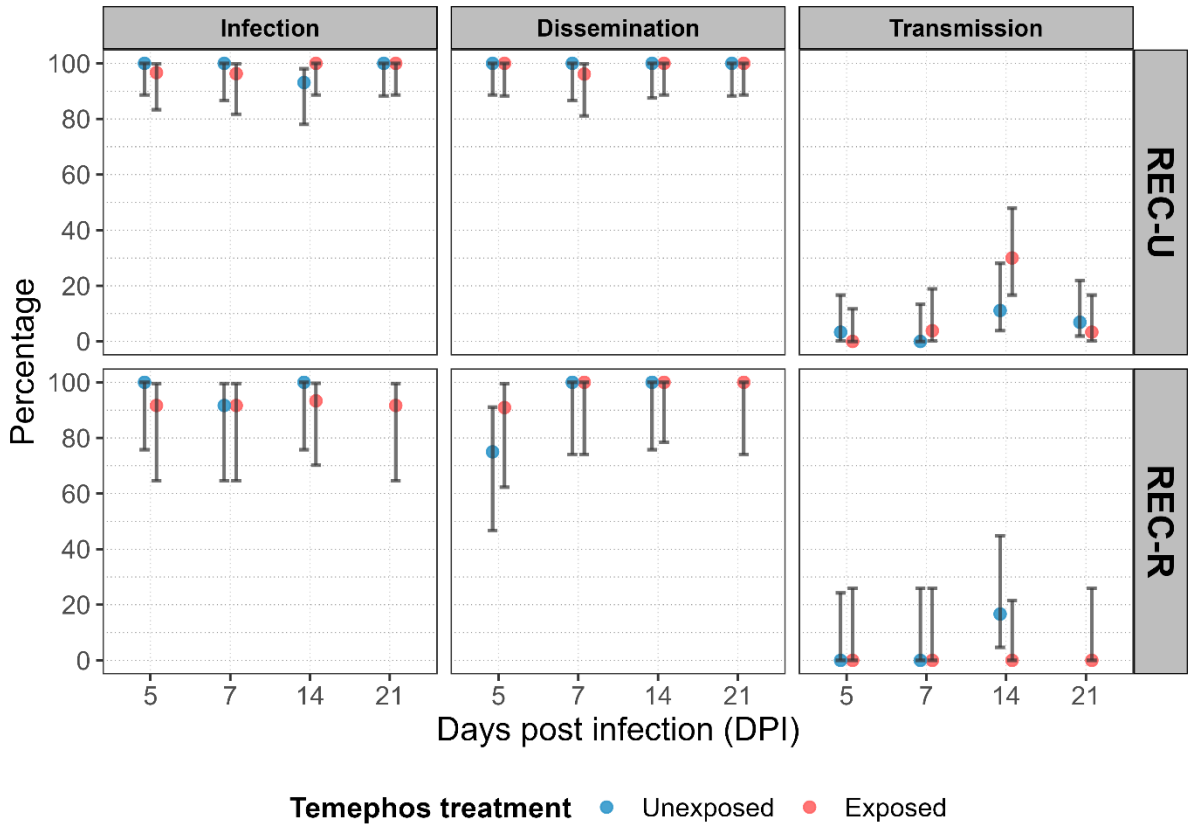


Figure 2– Infection, dissemination and transmission prevalence for REC U and REC R
Point estimates and 95% binomial confidence intervals (Wilson) are shown for the infection, dissemination, and transmission prevalence in two strains of mosquitoes that were either exposed or unexposed to temephos. Infection represents the proportion of blood fed mosquitoes that developed an infection in their bodies (thorax and abdomen). Dissemination represents the proportion of mosquitoes with body infections that developed infection of the head. Transmission represents the proportion of mosquitoes with body and head infections, that also had virus present in the saliva. No 21dpi data was collected for REC-R unexposed. The numbers of mosquitoes in each experimental group are shown in **Supplementary Table 1**.

5.4.2 ZIKV titres

Body titres remained relatively constant in all strains throughout the time period tested at approximately 5.5 $\log_{10}$ pfu/body (**Figure 3**). At 7dpi there was a significantly higher ZIKV titre in the bodies of temephos exposed REC R than the unexposed controls (REC R unexposed 5.46 [5.30-5.63] $\log_{10}$ pfu/body; REC R exposed 5.81 [5.62-6.00]; ANOVA p=0.01) (**Supplementary Table 3**). There were no significant differences in the ZIKV titres of heads between temephos exposed and unexposed groups at any timepoint. Prior to the peak prevalence in saliva at 14dpi, ZIKV was present in relatively high titres at 5dpi in REC U unexposed (2.28 $\log_{10}$pfu/sample), and 7dpi in REC U exposed (3.26 $\log_{10}$ pfu/sample) (**Supplementary Table 2**). There was a significantly higher ZIKV titre in the saliva of REC U exposed (2.14 [1.60-2.68] $\log_{10}$pfu/sample) at 14dpi than REC U unexposed (1.26 [0.66-1.86]; Kruskal-Wallis p=0.05).

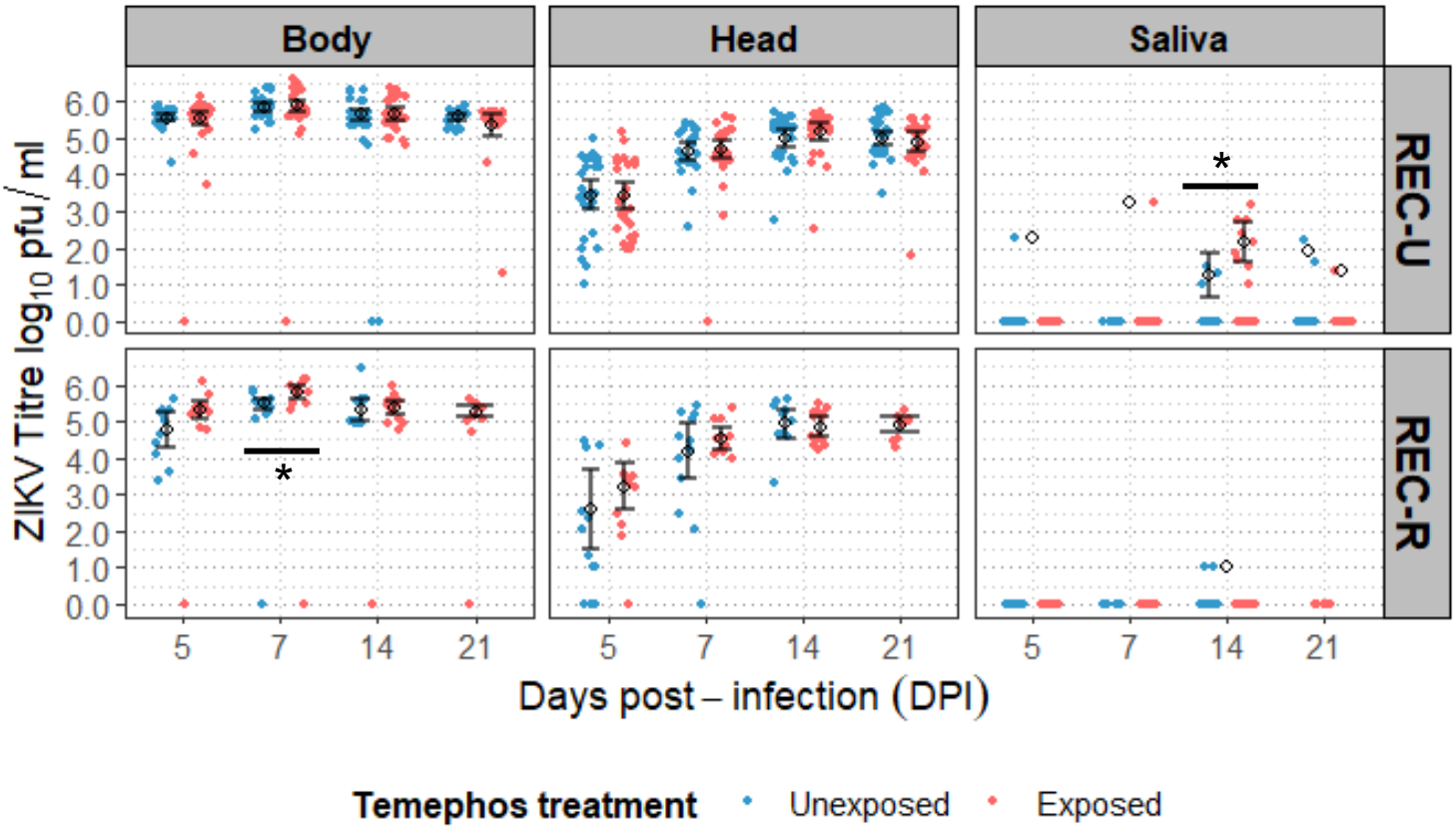


Figure 3– ZIKV titres for temephos exposed, and unexposed REC U and REC R
The mean viral titres of bodies, heads and saliva are shown for both mosquito strains, and temephos exposure groups. Viral titres were established by plaque assay. Mean titre values are indicated by the white circles. 95% confidence intervals are displayed in black. ZIKV titre values of 0 are not included in calculations of CI, and CI were not calculated when there are ≤2 data points per timepoint. No data was collected for REC-R unexposed at 21dpi.

5.5 Discussion

Mosquitoes receive sublethal doses of insecticides in multiple ways including environmental degradation of insecticides, mosquito avoidance behaviours, the presence of insecticide resistance mechanisms, and agricultural insecticide runoff into larval habitats. Whilst insufficient to cause mortality, sublethal insecticide exposure has been associated with profound physiological disturbances in both non-vector and vector arthropods [216]. Previous investigations with sublethal insecticide exposure and *Plasmodium* infection suggests it can lead to lower infection prevalence and burden [250, 355, 357-359]. Arboviral competence may also be altered following sublethal exposure of adult mosquitoes, with evidence suggesting that viral dissemination and replication may be enhanced [218, 222]. Few studies have investigated the effects of larval sublethal exposure to neurotoxic insecticides on vector competence for arboviruses [219]. Temephos is a widely used larvicide, yet the impacts of sublethal exposure on ZIKV competence have not been elucidated [361].

The data presented here show that there were no significant differences in the prevalence of ZIKV infection by temephos exposure status in either the temephos resistant REC R, or susceptible REC U strains, both of which were highly susceptible to oral infection with ZIKV. This finding is supported by previous investigations of adult [218, 223] and larval [219] exposure to neurotoxic insecticides. Taken together, these data suggest that sublethal insecticide exposure does not significantly influence infection.

We observed no significant effects of larval temephos exposure on the ability of ZIKV to disseminate to the heads of mosquitoes. There are no directly comparable studies, but larval exposure to malathion, which is in the same insecticide class as temephos, has previously been linked with increased dissemination of SINV to the heads of *Ae. aegypti* [219]. However, as SINV is an alphavirus, and is not a naturally vectored by *Ae. aegypti*, the results may not be generalisable to our findings. Exposure of adult mosquitoes to bifenthrin has also been associated with increased dissemination of ZIKV [218, 222]. Although not statistically significant, we found that at 5dpi the exposed REC R had a 15.9% higher prevalence of disseminated infection than unexposed. It is possible that with larger sample sizes, we may have detected a difference in dissemination between these groups.

There was a higher, but not statistically significant, proportion of REC U exposed (30.0%) with ZIKV in saliva than unexposed (11.1%) at 14dpi. It would be valuable to see if these effects were repeated with larger sample sizes as this could represent an important increase in transmission risk. Whilst few studies have directly measured salivary infection as an outcome, there is no existing evidence to suggest sublethal exposure can increase the prevalence of virus in saliva [218]. Interestingly, no virus was detected in the saliva of temephos exposed REC R strain at any timepoint, but, as the prevalence of ZIKV in saliva was also low in the unexposed REC R, it is uncertain whether this represents an effect of temephos exposure.

The ZIKV body titres were significantly higher in temephos exposed REC R than unexposed at 7dpi. Sublethally exposed *Ae. albopictus* have previously been demonstrated to have higher ZIKV body titres than unexposed [222]. The only previous study conducted with sublethal larval exposure to neurotoxic insecticides does not report differences in body titres between exposed and unexposed groups [219]. This was a transient phenomenon, with REC R unexposed having statistically equivalent body titres at other tested timepoints. This, coupled with a lack of difference in the prevalence of disseminated or salivary infection between exposed and unexposed groups, makes it unlikely that the higher body titres represent a marked difference in vector competence.

Interestingly, at 14dpi, mean saliva ZIKV titres were significantly higher in exposed REC U (2.14 log_{10}pfu/sample) than unexposed (1.26). To our knowledge, this has not been directly observed in previous studies. The extent to which a higher ZIKV inoculum delivered by a mosquito during blood feeding may increase the likelihood or virulence of a human infection is not clear. However, ZIKV challenge experiments in primates indicate that higher subcutaneous inocula result in more rapid

achievement of peak viraemia, which often correlates with the severity of disease [364]. As there were no notable differences in the ZIKV titres of other body parts between exposed and unexposed REC U at any timepoint, it may suggest that any potential impacts of temephos exposure on viral titre in saliva may be focused on the salivary glands. Further investigation with viral titration, and/or immunofluorescent assays of salivary glands would help establish if there were any differences in the characteristics of salivary gland infection or viral escape into saliva between temephos exposed and unexposed mosquitoes [266]. This would establish whether salivary glands can be successfully infected with ZIKV, and whether virus is able to enter to saliva.

Larger sample sizes, especially for REC R, would allow smaller differences between experimental groups to be detected. Furthermore, as infection and dissemination prevalence were high at all timepoints, use of a lower blood meal ZIKV titre would likely reduce infection and dissemination prevalence, and may reveal differences between strains that were not apparent here [261]. Use of higher doses of temephos may make any differences between experimental groups easier to detect, but as the observed mortality increases, there is an increased likelihood of observing a difference in competence that is due to a selection event, rather than insecticide exposure.

In summary, our data show that sublethal temephos exposure does not have significant impacts on the proportion of *Ae. aegypti*. demonstrating infection, dissemination or transmission of ZIKV virus. However, we did observe a significantly higher salivary ZIKV titre in temephos exposed REC U mosquitoes. This coupled with an increased, but not significantly higher, proportion of ZIKV-positive saliva samples, could represent an important difference in vector competence and warrants further investigation

5.6 Supplementary data

Supplementary Table 1: Infection, Dissemination, and Transmission Prevalence				
Days post-infection (dpi)	**Strain**	**Infection (%)** [95% CI]	**Dissemination (%)** [95% CI]	**Transmission (%)** [95% CI]
5	REC- U Exposed	96.7 [83.3-99.8] N=30	100.0 [88.3-100.0] N=29	0.0 [0.0-11.7] N=29
	REC-U Unexposed	100.0 [88.6-100.0] N=30	100.0 [88.6-100.0] N=30	3.3 [0.1-16.7] N=30
	REC-R Exposed	91.7 [64.6-99.6] N=12	90.9 [62.3-99.5] N=11	0.0 [0.0-25.8] N=11
	REC-R Unexposed	100.0 [75.8-100.0] N=12	75.0 [46.8-91.1] N=12	0.0 [0.0-24.2] N=12
7	REC- U Exposed	96.3 [81.7-99.8] N=27	96.2 [81.1-99.8] N=26	3.8 [0.1-18.9] N=26
	REC-U Unexposed	100.0 [86.7-100.0] N=25	100.0 [86.7-100.0] N=25	0.0 [0.0-13.3] N=25
	REC-R Exposed	91.7 [64.6-99.6] N=12	100.0 [74.1-100.0] N=11	0.0 [0.0-25.9] N=11
	REC-R Unexposed	91.7 [64.6-99.6] N=12	100.0 [74.1-100.0] N=11	0.0 [0.0-25.9] N=11
14	REC- U Exposed	100.0 [88.6-100.0] N=30	100.0 [88.6-100.0] N=30	30.0 [16.6-47.9] N=30
	REC-U Unexposed	93.1 [78.0-98.1] N=29	100.0 [87.5-100.0] N=27	11.1 [3.9-28.1] N=27
	REC-R Exposed	93.3 [70.2-99.7] N=15	100.0 [78.5-100.0] N=14	0.0 [0.0-21.5] N=14
	REC-R Unexposed	100.0 [75.8-100.0] N=12	100.0 [75.8-100.0] N=12	16.7 [4.7-44.8] N=12
21	REC- U Exposed	100.0 [88.6-100.0] N=30	100.0 [88.6-100.0] N=30	3.3 [0.2-16.7] N=30
	REC-U Unexposed	100.0 [88.3-100.0] N=29	100.0 [88.3-100.0] N=29	6.9 [1.9-22.0] N=29
	REC-R Exposed	91.7 [64.6-99.6] N=12	100.0 [74.1-100.0] N=11	0.0 [0.0-25.9] N=11
	REC-R Unexposed	NA	NA	NA

Supplementary Table 2: Mean ZIKV titres for temephos-exposed, and unexposed REC-U					
Part	**Treatment**	**Mean ZIKV titre (log_{10} pfu/sample)** [95% CI]			
		5dpi	**7dpi**	**14dpi**	**21dpi**
Body (Thorax, abdomen + legs)	REC- U Exposed	**5.53** [5.36-5.71]	**5.86** [5.71-6.02]	**5.64** [5.48-5.80]	**5.37** [5.07-5.66]
	REC-U Unexposed	**5.56** [5.45-5.66]	**5.85** [5.73-5.97]	**5.62** [5.48-5.77]	**5.57** [5.50-5.64]
Head	REC- U Exposed	**3.42** [3.07-3.80]	**4.68** [4.44-4.93]	**5.17** [4.93-5.41]	**4.87** [4.61-5.14]
	REC-U Unexposed	**3.44** [3.05-3.84]	**4.63** [4.37-4.89]	**5.00** [4.75-5.25]	**4.98** [4.78-5.19]
Saliva	REC- U Exposed	**0.00**	**3.26**	**2.14** [1.60-2.68]	**1.40**
	REC-U Unexposed	**2.28**	**0.00**	**1.26** [0.66-1.86]	**1.90** [0.00-5.73]

Supplementary Table 3: Mean ZIKV titres for temephos-exposed, and unexposed REC-R					
Part	**Treatment**	**Mean ZIKV titre (log_{10} pfu/sample) [95% CI]**			
		5dpi	**7dpi**	**14dpi**	**21dpi**
Body (Thorax, abdomen + legs)	REC-R Exposed	**5.32** [5.07-5.57]	**5.81** [5.62-6.00]	**5.35** [5.16-5.54]	**5.27** [5.10-5.44]
	REC-R Unexposed	**4.78** [4.31-5.26]	**5.46** [5.30-5.62]	**5.30** [5.01-5.59]	
Head	REC-R Exposed	**3.22** [2.60-3.84]	**4.54** [4.25-4.84]	**4.85** [4.60-5.10]	**4.91** [4.69-5.12]
	REC-R Unexposed	**2.59** [1.48-3.69]	**4.18** [3.43-4.94]	**4.93** [4.54-5.33]	
Saliva	REC-R Exposed	**0.00**	**0.00**	**0.00**	**0.00**
	REC-R Unexposed	**0.00**	**0.00**	**1.00**	

Chapter 6 – Discussion

Arboviruses spread by mosquitoes present an increasing threat to global health. Contrary to many other vector-borne diseases, the mortality and morbidity burden associated with these viruses has dramatically increased in the last few decades [365-367]. Despite their importance, control options remain limited. With few effective vaccines, and no specific medical treatments, control relies heavily on targeting the mosquito vector populations. Novel vector control strategies, such as *Wolbachia* and gene drive technologies, provide promising options for the future control of arbovirus vectors [39-41]. However, at present, insecticide-based vector control is the only option that is widely available.

Insecticide use has been associated with a marked increase in insecticide resistance amongst mosquito vectors of arboviruses [54-58]. Often this can result in alterations in life history traits and fitness costs, owing to changes in the normal physiology of vectors [174-179, 190-194]. Furthermore, contact with sublethal doses of insecticide can also affect traits including longevity, behaviour, and development [211-217]. Vector competence is a complex aggregation of multiple biotic and abiotic factors, many of which are not fully understood. As such, there are concerns that insecticide resistance and exposure may impact the ability of vectors to acquire and transmit arboviruses [58]. Though it has not received much research attention to date, there is evidence that these concerns may be warranted [181-183, 195, 218, 219, 222].

Understanding the effects of insecticide resistance and exposure on vector competence is important for the planning of vector control interventions. To manage insecticide resistant vectors, regular insecticide rotations are recommended, which are likely to lead to complex insecticide resistance and exposure combinations [238, 239]. The extent to which this may affect vector competence remains unclear. As arboviruses and vectors increasingly spread into new geographic areas, developing an understanding of how insecticide resistance profiles in local vector populations may interact with emerging arboviruses is important for outbreak planning.

Firstly, this study aimed to understand what effects differing insecticide selection pressures have on the important arbovirus vector *Ae. aegypti*, using transcriptomic analysis. Secondly it aimed to determine how insecticide resistance and exposure may alter the vector competence of mosquitoes for arboviruses. These aims were addressed in four experimental chapters, using *Aedes aegypti*-Zika virus and *Anopheles gambiae*-o'nyong nyong virus models. The progress of this thesis towards addressing these aims, the strengths and limitations of the approaches taken, and proposed next steps, will now be discussed.

6.1 Aim 1 – Investigate how insecticide selection pressures alter the transcriptome of mosquitoes, with a focus on metabolic insecticide resistance and innate immunity genes

Insecticide rotation is recommended to manage insecticide resistant *Aedes* populations [238, 239]. Changing insecticide selection pressures have been associated with alterations in life history traits, but the effects of changing insecticides on gene expression have not been well characterised [190, 282, 297]. In this study, the effects of different insecticide rotation scenarios were investigated in the Recife strain of *Ae. aegypti*, which had existing metabolic insecticide resistance to temephos [301]. Compared to maintenance of temephos selection, three scenarios were explored: 1) switching to malathion; 2) switching to permethrin; and 3) removal of insecticide. These data provide useful insights into the effects that insecticide rotations in the field can have on the physiology of mosquito vectors.

The data generated here show that within a small number of generations, changes in insecticide selection pressure can have widespread effects on the transcriptome of mosquitoes, across multiple biological domains. There was relatively limited overlap in the transcriptomic profiles between each scenario, showing that the choice of insecticide used in rotations can have a specific effect on a population of vectors. Changes in the expression of detoxification enzymes from cytochrome P450, carboxylesterase, and glutathione-s transferase families were associated with rapid changes in insecticide resistance phenotype. These data suggest that, at least under laboratory conditions, mosquito populations are capable of rapidly adapting to, and overcoming, insecticide rotation strategies.

The finding that immune gene expression was altered by changes in insecticide selection pressure provides important evidence that insecticide use has far reaching impacts on mosquito populations beyond causing mortality. Though under-researched, differences in immune gene expression, relating to insecticide resistance or selection, have previously been reported in a small number of studies [295, 298, 299]. In this thesis, removal of temephos selection pressure was associated with a decrease in the expression of a number of innate immunity genes. Infection experiments in this thesis demonstrated that these changes were associated with alterations in aspects of vector competence, including dissemination and transmission prevalence. If these findings also occur in response to insecticide rotation scenarios in the field, this could have important consequences for vector control. Temephos is a widely used larvicide to tackle *Aedes* arbovirus vectors [361]. As such, the data generated here suggest there may potentially be important implications if temephos-based vector control falters for several generations in the field, as this could be linked with a reduced immune response and increased vector competence. Further investigation by performing additional insecticide rotations replicates will be required to determine whether these effects are consistently observed.

6.2 Aim 2 – Investigate how insecticide resistance and exposure may alter the vector competence of mosquitoes for arboviruses

The second aim of this thesis investigated the effects of insecticide resistance and exposure on vector competence. The progress of this thesis in addressing these two aims, and the strengths and limitations of the approaches used, will now be discussed in turn.

6.2.1 Insecticide resistance

The effects of insecticide resistance on the vector competence of mosquitoes for arboviruses have not been well characterised to date. Two main experimental approaches were used to investigate this aim: 1) a genotype-focused approach, where the effects of a particular insecticide resistance allele were isolated and explored, was used in chapter 2; and 2) a phenotype-focused approach was used in chapter 4, where the relationship of insecticide resistance phenotypes and vector competence were explored.

6.2.1.1 Genotype-focused approach

Chapter 2 utilised a genotype-focused approach to determine whether the L1014F *kdr* allele was associated with alterations in vector competence for ONNV. Target-site insecticide resistance mutations lend themselves to a genotype-focused approach as it is relatively easy to identify important genes associated with the insecticide resistance phenotype [176]. Previous research has associated *kdr* alleles with alterations in measures of vector competence for *Plasmodium* [245, 251, 255] and arboviruses [175, 180, 182, 257]. However, the use of field caught, insecticide selected, or introgressed mosquito strains had not fully controlled for extraneous genetic differences between mosquito strains that could influence vector competence.

Here, a novel approach, using a CRISPR/Cas9 gene-edited mosquito strain, was used to minimise genetic differences between the *kdr* homozygotic and wildtype strains. The results of this experiment suggest that when the effects of the L1014F allele are isolated, there are no major impacts on vector competence. This approach provides a valuable method for systematically exploring the effects of particular insecticide resistance alleles of interest whilst excluding other potentially confounding alleles. Generation of a *kdr* mutant *Ae. aegypti* line using CRISPR/Cas9 would be an interesting next step, as this would allow competence testing for a wide range of arboviruses from different genera. Furthermore, evaluation of the effects of other *kdr* alleles, and target-site mutations affecting the acetylcholinesterase enzyme, would be interesting avenues for future research.

However, it is important to recognise that *kdr* alleles are likely to be in linkage disequilibrium with genes at other loci, and as such are not inherited in this isolated way in the field [249, 250]. This approach therefore cannot account for direct or epistatic effects of other loci on vector competence. If target site insecticide resistance alleles are commonly inherited with other genes that can influence competence *in vivo*, this genotype-focused approach may provide false reassurance that there is no effect on vector

competence. It does, however, provide a useful approach for excluding any direct effect of the *kdr* allele on vector competence.

6.2.1.2 Phenotype-focused approach

It is not always possible to determine a precise insecticide resistance genotype. For example, whilst the mechanisms underlying the metabolic insecticide resistance phenotypes have previously been explored in the Recife strains using gene expression and biochemical analyses [283, 301], it is not possible to determine a precise genotype for the observed resistance phenotype. The genetic determinants of metabolic insecticide resistance are not fully understood, and the relative importance of different detoxification enzymes, and their interactions with each other, are complex and not well characterised [56, 57]. Furthermore, putative mechanisms for interactions between metabolic insecticide resistance and vector competence, such as reactive oxygen species-mediated immune pathway activation, and resource trade-offs, are at the level of metabolic pathways, rather than due to the direct effects of individual genes [58, 191, 197]. As such, a higher level approach that explores the impact of insecticide resistance phenotypes on vector competence, is more appropriate than focusing on isolating particular genotypes. A phenotype-focused approach was therefore used in chapter 4 to explore how alterations in insecticide selection pressure, resulting in changes in insecticide resistance phenotype, can be associated with alterations in the vector competence of *Ae. aegypti* for ZIKV.

The advantage of using insecticides to select for particular insecticide resistance phenotypes is that it is more similar to how insecticide resistance develops in the field. By selecting for an insecticide resistance phenotype over generations, other competence-modifying genes that are in linkage disequilibrium with resistance alleles, are also likely to be represented. As such, the data produced provides useful insights into how changes in insecticide resistance phenotypes in the field may result in alterations in vector competence. The data from Chapter 4 show that temephos selection is associated with decreased viral dissemination and transmission prevalence compared to the unselected strain. A particular strength of the experimental design used here was the common genetic background of the Recife mosquito strains which were derived from the same parent strain. This minimises genetic variability between strains that is unrelated to changes in the insecticide resistance phenotype.

It is important to note, however, that similar resistance phenotypes can result from markedly different underlying mechanisms [56]. There can be significant overlap in detoxification pathways for different insecticides, and high variability in the suite of detoxification enzymes observed in different vector populations with similar insecticide resistance phenotypes [286, 287, 294]. This means findings from studies that have examined the association of an insecticide resistance phenotype with vector competence, are hard to generalise to other vector populations, even when insecticide resistance phenotypes may appear similar. Therefore, it is essential that an understanding of the underlying detoxification mechanisms is gained in addition to assessment of phenotype. In this thesis, the

transcriptomic data for the Recife strains provides important information about the nature of the potential metabolic insecticide resistance mechanisms present and permits greater generalisability of the vector competence data.

6.2.2 Sublethal insecticide exposure

In addition to investigating the impact of insecticide resistance on vector competence, this thesis also aimed to investigate the effects of insecticide exposure on competence. Though the effects of sublethal insecticide exposure on life history traits are well established, the impacts it may have on vector competence have been poorly characterised [211-217, 351]. Despite the fact that temephos is a widely used larvicide for *Aedes* control, the impacts of sublethal larval exposure to temephos have not previously been investigated.

Chapter 5 explored the potential for sublethal insecticide exposure to modulate vector competence. There was a slightly higher prevalence of salivary infection in the temephos exposed REC U than unexposed, but this was not statistically significant. However, the salivary titres of the REC U strain were significantly higher in those that had been sublethally exposed to temephos. Together this could represent a difference in the transmission potential for ZIKV relating to temephos exposure, however larger sample sizes will be needed to explore this further. It would also be interesting to use of a range of temephos doses to determine whether there is a dose-dependent effect of temephos exposure on vector competence. Furthermore, gene expression analysis could be performed to determine whether sublethal insecticide exposure is associated with differences in innate immune gene expression. Future investigation in this area should focus on exploring the effects of commonly used insecticides on vectors of medically important arboviruses.

6.2.3 Limitations of vector competence experiments

6.2.3.1 Sample sizes

Obtaining adequate sample sizes for statistically meaningful comparisons can be difficult in vector competence experiments [167, 368, 369]. This proved a considerable challenge in the experiments conducted here. The primary outcomes of vector competence experiments are generally to determine differences in the prevalence of infection, dissemination, and transmission, between treatment groups. These data are binomial in nature, and the statistical confidence in comparisons between treatment groups are dependent on both the effect size and the total population size (see **Chapter 4**). Even in highly competent vectors, not every infected mosquito will necessarily develop a disseminated and salivary infection [349]. Therefore, studies that are adequately powered for differences in infection prevalence are likely to be underpowered for comparisons in transmission prevalence between groups. Furthermore, the expected prevalence of infection, dissemination, and transmission in different treatment groups may not be known *a priori*, which makes proactive sample size calculations difficult.

A number of other factors contributed to difficulties in obtaining larger sample sizes in this thesis. Firstly, blood feeding rates were highly variable and difficult to predict, even between closely related mosquito strains. Multiple factors are known to influence blood feeding rates using artificial feeders [370]. During my PhD, there have been very few other people who have been conducting vector competence work, and there was no prior established practice for successful blood feeding. Therefore, there has been a great deal of problem solving, and learning by trial and error. I carried out extensive optimisation over several months, but despite this, it was not always possible to collect sufficient data for some of the mosquito strains. It may be the case that poor blood feeding rates could represent a fitness cost of insecticide resistance in the strains used. Differences in longevity and mortality rates between mosquito strains posed another challenge to achieving large sample sizes. It was difficult to predict the number of individuals that were likely to still be alive for sampling at later timepoints post-infection. This could reflect known fitness costs of insecticide resistance on longevity that have been previously reported in the Kis-kdr [176] and Recife [190] strains used in these experiments. Using mosquito colonies that underwent insecticide selections every three generations posed a further challenge to obtaining large sample sizes. Following selection, the colony sizes were often reduced for 1-2 generations, limiting the availability of females for infection experiments.

Sample size calculations that take all these factors into account are likely to produce targets that are logistically unattainable [349]. There are few individuals who can work with virally infected mosquitoes at LSTM, therefore I carried out all infections, dissections, sample processing, and virological assays for the presented data in this thesis. As such, focusing on achieving large sample size targets would have been at the expense of exploring multiple treatment options or different strains in one experiment. Intrathoracic injections were used here to provide larger sample sizes and circumvent poor feeding rates. Whilst useful for ensuring infection, excluding midgut factors, and exploring salivary gland barriers, the unnatural infection route limits the conclusions that can be made from these data.

6.2.3.2 Vector-virus pairings

A second challenge faced when performing vector competence experiments is the availability of different vectors and viruses. This was particularly the case for the ONNV-*Anopheles* model used to investigate the impacts of the L1014F allele. The main limitation of this experiment related to the lack of viral dissemination from the midgut following an infected blood meal. The Kis-kdr strain of *Anopheles gambiae* was not initially developed for vector competence experiments, but rather to validate the role of the L1014F allele in pyrethroid resistance, and to understand the related fitness costs [176]. Unfortunately, there are limited options of arboviruses for infection experiments in *Anopheles* species. Given the poor dissemination seen with the ONNV UgMp30 strain, several attempts were made to obtain different viral strains for which the Kisumu line may have been more competent. Attempts were made to rescue a different ONNV strain (SG650) from an infectious clone, as this was previously reported to disseminate more readily in *An. gambiae* [264]. However, viral stocks of sufficient titres were not successfully

produced. There are very few isolates of ONNV available in the UK, which makes working with this virus challenging. Furthermore, importation of viral stocks from abroad can be challenging and prohibitively expensive due to biosafety requirements, and the regulations of the Convention on the International Trade in Endangered Species of Wild Fauna and Flora (CITES), which applies to the Vero cell (African green monkey kidney) lines used to culture the virus.

6.3 What are the implications for vectorial capacity?

The evidence to date suggests there is not a universal effect of insecticide resistance or exposure on the vector competence of mosquitoes for arboviruses [175, 181, 183, 195, 218, 219, 222, 223, 336, 337]. This is perhaps unsurprising given the multifactorial nature of vector competence, the variety of insecticide resistance mechanisms, the differences in experimental design, and the multiple vector-virus combinations used. However, whilst there is no consistency in the direction of effect, it is clear that these factors can have impacts on vector competence. Therefore, it is important to consider what changes in vector competence, due to insecticide resistance or exposure, could cause tangible differences in disease transmission dynamics in the field. The vectorial capacity equation, in which vector competence is one parameter, provides a useful theoretical tool to consider how different scenarios could affect arbovirus transmission (**Figure 1**).

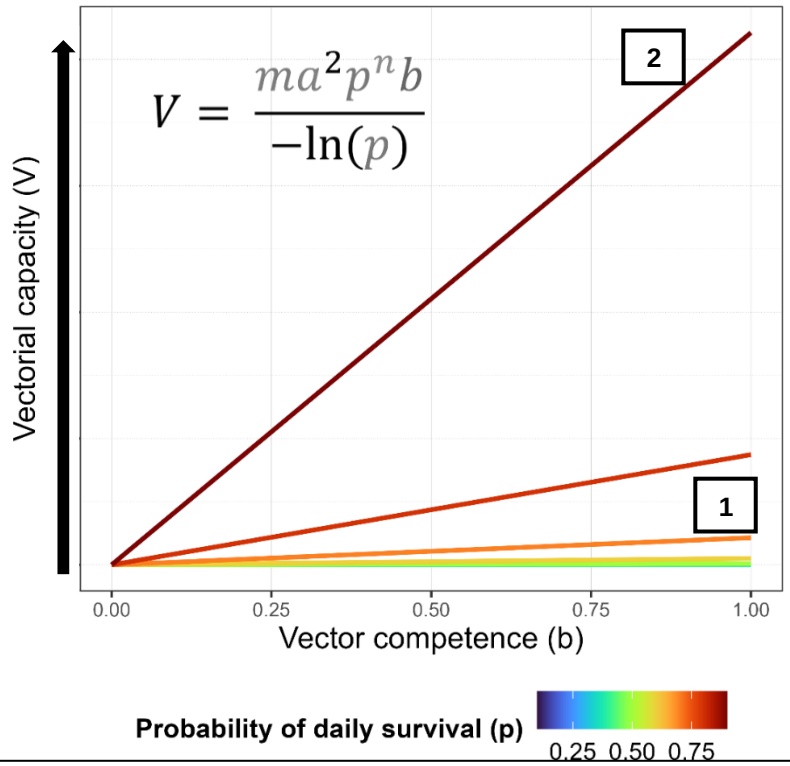


Figure 1– The effects of vector competence on vectorial capacity for situations with different probabilities of daily survival

Changes in vector competence due to insecticide resistance or exposure have different effects on vectorial capacity depending on the probability of daily survival of mosquitoes. In areas where the probability of daily survival is low [1], large changes in vector competence make negligible changes to vectorial capacity. This is due to the necessity for a mosquito to survive through the EIP of a virus. In a situation where the probability of daily survival is high [2] (which could result from insecticide resistance in areas of insecticide use) small changes in vector competence due to insecticide resistance or exposure have marked effects on vectorial capacity.

Clearly, if insecticide resistance or exposure caused a population of non-permissive vectors to become competent for an arbovirus, this would increase transmission potential, and vectorial capacity would rise above zero. This could pose a significant concern for disease transmission in the field. Conversely, if a previously competent vector population was no longer able to acquire and transmit a virus due to insecticide resistance or exposure, then vectorial capacity would fall to zero. Whilst there is not currently evidence to suggest either of these two more extreme scenarios arise, the evidence does suggest that there may be more subtle effects on vector competence which may nonetheless affect vectorial capacity.

There are data to suggest that insecticide usage and resistance can produce changes in the dissemination efficiency of viruses, which could result in alterations in the extrinsic incubation period (EIP) (parameter *n*) [175, 182, 183] (**Chapter 4**). Furthermore, Chapter 4 of this thesis showed that different insecticide selection pressures, resulting in different resistance phenotypes, may be associated with decreased transmission prevalence. Transmission prevalence is mathematically equivalent to the vector competence (*b*) parameter in the vectorial capacity equation. The impacts of both of these changes are

heavily dependent on the probability of daily survival of vectors, denoted in the vectorial capacity equation as *p* (**Figure 1**).

Changes in vector competence (*b*) due to insecticide resistance or exposure can only affect vectorial capacity linearly, and the gradient is determined primarily by the probability of daily survival (*p*) (**Figure 1**). The length of the EIP (*n*) also has a minor influence on the gradient, mostly when values for *p* are low, where a shorter incubation time can partially offset the poor probability of daily survival (*p*). Therefore, in a population where the probability of daily survival is low, changes in vector competence (*b*) for an arbovirus due to insecticide resistance or exposure would have minimal effect on the vectorial capacity, assuming all other parameters remained constant. It is clearly of little importance what proportion of mosquitoes would develop salivary infection if the probability of surviving to that point is negligible (**Figure 1**). However, if the probability of daily survival is high, alterations in the proportion of mosquitoes with salivary infection resulting from insecticide resistance or exposure, could have much greater impacts on the vectorial capacity (**Figure 1**). If, as the evidence from Chapter 4 suggests, insecticide resistance is associated with decreased transmission prevalence, then this could have a significant impact on reducing transmission in an environment where daily survival rates are high.

Daily survival remains the most important factor for vector capacity and transmission rates, and therefore efforts to restore operational vector control and maintain high vector mortality such as insecticide rotations, the use of synergists, and non-insecticide based interventions, should be prioritised over concerns relating to the pleiotropic effects of insecticide use on vector competence. However, in situations where insecticide resistance results in increases in daily survival, even subtle changes in competence resulting from the interaction with insecticides could have an impact upon overall transmission (**Figure 1**). Therefore, even small changes in vector competence brought about by insecticide resistance or exposure, could become increasingly important in areas where vector control is failing, and vector mortality associated with insecticides is decreasing.

Given the challenge insecticide resistance presents to maintaining effective vector control, and the potential for alterations in vector competence due to insecticide resistance and exposure to alter vectorial capacity, further research into the effects of insecticide use and resistance on arbovirus competence is urgently required. To have maximum translational impact, this should focus on medically important vector-virus pairings and insecticides that are commonly used in the field.

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Appendix 1 – Other work conducted during thesis

During the Covid-19 pandemic I undertook a 6-month secondment with the Centre for Drugs & Diagnostics at LSTM to evaluate novel diagnostic tools for SARS-CoV-2. My main research focus was the evaluation of novel enzyme-linked immunosorbent assays (ELISAs) through comparison with

plaque reduction neutralisation tests (PRNTs). Other roles included the training of staff to work in containment level 3 laboratories and developing protocols for new experiments using live virus.

This work resulted in a number of publications.

- **Kay, Grant A**., Sophie I. Owen, Emanuele Giorgi, David J. Clark, Christopher T. Williams, Stefanie Menzies, Luis E. Cuevas et al. "SARS-CoV-2 enzyme-linked immunosorbent assays as proxies for plaque reduction neutralisation tests." Scientific reports 12, no. 1 (2022): 1-9.
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Appendix 2 – R scripts

Chapter 2: Knockdown resistance allele L1014F introduced by CRISPR/Cas9 is not associated with altered infection susceptibility for o'nyong nyong virus in *Anopheles gambiae*

```
################ Fishers exact for proportions. Injection data ########

DRl5dpi <- data.frame("Disseminated" = c(20,21), "Not disseminated"=c(2,1), row.names=c("KIS", "KDR"))
DRl5dpi
fisher.test(DRl5dpi)
```

```
DRl7dpi <- data.frame("Disseminated" = c(23,20), "Not disseminated"=c(0,0), row.names=c("KIS", "KDR"))
DRl7dpi
fisher.test(DRl7dpi)
DRl10dpi <- data.frame("Disseminated" = c(22,13), "Not disseminated"=c(1,1), row.names=c("KIS", "KDR"))
DRl10dpi
fisher.test(DRl10dpi)
######ANOVA comparisons at each timepoint x body part
 #testing for normality with shapiro test
#running Kruskall-wallis non para if not normally dist - only legs
#bodies 5dpi
Bodies5dpi <- filter(KISKDR_titre_NaR2,Part =="Heads", DPI == "5")
Bodies5dpi_KIS <- filter(Bodies5dpi, Strain =="KIS")
Bodies5dpi_KDR <- filter(Bodies5dpi, Strain =="KDR")
shapiro.test(Bodies5dpi_KIS$Titre) #if p-value >0.05
shapiro.test(Bodies5dpi_KDR$Titre) # p>.05
aov_B_5dpi <- aov(Titre ~ Strain, data = Bodies5dpi)
summary(aov_B_5dpi) #p=0.68

#legs 5dpi
Legs5dpi <- filter(KISKDR_titre_NaR2, Part == "Legs" & DPI == "5" & Titre > 0)
Legs5dpi_KIS <- filter(Legs5dpi, Strain =="KIS")
Legs5dpi_KDR <- filter(Legs5dpi, Strain =="KDR")
shapiro.test(Legs5dpi_KIS$Titre) ##p=0.72
shapiro.test(Legs5dpi_KDR$Titre) ##p=0.005
aov_L_5dpi <- aov(Titre ~ Strain, data = Legs5dpi)
summary(aov_L_5dpi) #p=0.73
  #non parametric
kruskal.test(Titre~Strain, data = Legs5dpi)#p=0.86

#heads 5dpi
Heads5dpi <- filter(KISKDR_titre_NaR2, Part == "Heads" & DPI == "5")
Heads5dpi_KIS <- filter(Heads5dpi, Strain =="KIS")
Heads5dpi_KDR <- filter(Heads5dpi, Strain =="KDR")
shapiro.test(Heads5dpi_KIS$Titre) #p>.05
shapiro.test(Heads5dpi_KDR$Titre) #p>.05
aov_H_5dpi <- aov(Titre ~ Strain, data = Heads5dpi)
summary(aov_H_5dpi) #p=0.68

#bodies 7dpi
Bodies7dpi <- filter(KISKDR_titre_NaR2, Part == "Bodies" & DPI == "7")
Bodies7dpi_KIS <- filter(Bodies7dpi, Strain =="KIS")
Bodies7dpi_KDR <- filter(Bodies7dpi, Strain =="KDR")
shapiro.test(Bodies7dpi_KIS$Titre) # p>.05
shapiro.test(Bodies7dpi_KDR$Titre) # p>.05
aov_B_7dpi <- aov(Titre ~ Strain, data = Bodies7dpi)
summary(aov_B_7dpi) #p=0.31

#legs 7dpi
Legs7dpi <- filter(KISKDR_titre_NaR2, Part == "Legs" & DPI == "7")
Legs7dpi_KIS <- filter(Legs7dpi, Strain =="KIS")
Legs7dpi_KDR <- filter(Legs7dpi, Strain =="KDR")
shapiro.test(Legs7dpi_KIS$Titre) #p>.05
shapiro.test(Legs7dpi_KDR$Titre) #p>.05
aov_L_7dpi <- aov(Titre ~ Strain, data = Legs7dpi)
summary(aov_L_7dpi) #p=0.049

#heads 7dpi
Heads7dpi <- filter(KISKDR_titre_NaR2, Part == "Heads" & DPI == "7")
Heads7dpi_KIS <- filter(Heads7dpi, Strain =="KIS")
Heads7dpi_KDR <- filter(Heads7dpi, Strain =="KDR")
```

```
shapiro.test(Heads7dpi_KIS$Titre) #p>.05
shapiro.test(Heads7dpi_KDR$Titre) #p0.049*
aov_H_7dpi <- aov(Titre ~ Strain, data = Heads7dpi)
summary(aov_H_7dpi) #p=0.49
#non parametric
kruskal.test(Titre~Strain, data = Heads7dpi)#p=0.48

#bodies 10dpi
Bodies10dpi <- filter(KISKDR_titre_NaR2, Part == "Bodies" & DPI == "10")
Bodies10dpi_KIS <- filter(Bodies10dpi, Strain =="KIS")
Bodies10dpi_KDR <- filter(Bodies10dpi, Strain =="KDR")
shapiro.test(Bodies10dpi_KIS$Titre) # p>.05
shapiro.test(Bodies10dpi_KDR$Titre) # p>.05
aov_B_10dpi <- aov(Titre ~ Strain, data = Bodies10dpi)
summary(aov_B_10dpi) #p=0.56
#legs 10dpi
#again removing 0s from legs 10dpi
Legs10dpi <- filter(KISKDR_titre_NaR2, Part == "Legs" & DPI == "10" & Titre >0)
Legs10dpi_KIS <- filter(Legs10dpi, Strain =="KIS")
Legs10dpi_KDR <- filter(Legs10dpi, Strain =="KDR")
shapiro.test(Legs10dpi_KIS$Titre) #sig
shapiro.test(Legs10dpi_KDR$Titre) #sig

kruskal.test(Titre~Strain, data = Legs10dpi) #p=0.13

#heads 10dpi
Heads10dpi <- filter(KISKDR_titre_NaR2, Part == "Heads" & DPI == "10")

Heads10dpi_KIS <- filter(Heads10dpi, Strain =="KIS")
Heads10dpi_KDR <- filter(Heads10dpi, Strain =="KDR")
shapiro.test(Heads10dpi_KIS$Titre) #p>.05
shapiro.test(Heads10dpi_KDR$Titre) #p>.05
aov_H_10dpi <- aov(Titre ~ Strain, data = Heads10dpi)
summary(aov_H_10dpi) #p=0.84

################fisher's exact PO feed data ##############################
IR7 <- data.frame("Infected"=c(9,8), "Uninfected"=c(11,12), row.names=c("KIS", "KDR"))
IR7
fisher.test(IR7) #p = 1

DRh7 <- data.frame("Disseminated"=c(1,0), "Non disseminated"=c(8,8), row.names=c("KIS","KDR"))
DRh7
fisher.test(DRh7) #p = 1

######################significantly different body titres at 7dpi?##############
POBodies7dpi <- filter(KISKDR_PO_Titre, Part == "Bodies" & DPI == "7" & Titre >0)
  #remove 0s for this bit
POBodies7dpi
POBodies7dpi_KIS <- filter(POBodies7dpi, Strain =="KIS")
POBodies7dpi_KDR <- filter(POBodies7dpi, Strain =="KDR")
shapiro.test(POBodies7dpi_KIS$Titre) # p<0.05
shapiro.test(POBodies7dpi_KDR$Titre) # p<0.05

aov_POB_7dpi <- aov(Titre ~ Strain, data = POBodies7dpi)
summary(aov_POB_7dpi)

################Blood meal volume analysis###########

BMV_KIS <- filter(BMV_ONNV, Strain =="KIS")
BMV_KDR <- filter(BMV_ONNV, Strain == "KDR")
```

```
shapiro.test(BMV_KIS$Titre) # p>0.05
shapiro.test(BMV_KDR$Titre) # p>0.05

aov_BMV <- aov(Titre ~ Strain, data = BMV_ONNV)
summary(aov_BMV)

Mean_BMV <- BMV_ONNV %>%
  group_by(Strain) %>%
  summarise(mean.titre = mean(Titre, na.rm = TRUE),
            sd.titre = sd(Titre, na.rm = TRUE),
            n.titre = n()) %>%
  mutate(se.titre = sd.titre / sqrt(n.titre),
         lower.ci.titre = mean.titre - qt(1 - (0.05 / 2), n.titre - 1) * se.titre,
         upper.ci.titre = mean.titre + qt(1 - (0.05 / 2), n.titre - 1) * se.titre)
Mean_BMV
```

Chapter 3: Altering insecticide selection pressure is associated with changes in the expression of metabolic insecticide resistance and immune genes in *Aedes aegypti*

```
###############plotting just GO terms that are Bonferroni p <.5################

install.packages("readxl")
library(readxl)
install.packages("dplyr")
library(dplyr)
install.packages("tidyverse")
library(tidyverse)
install.packages("ggplot2")
library(ggplot2)
#works off the files outputted by Vectorbase


#RvB GO terms
```

```
RvB_sig <- filter(GO_master, Comparison=="RvB" & Bonferroni <0.05)
RvB_sig$Expression <- factor(RvB_sig$Expression, levels = c("DOWN", "UP"))
RvB_sig$Ontology <- factor(RvB_sig$Ontology, levels = c("BP", "MF"))
RvB <- ggplot(RvB_sig, aes(fill=Ontology, x=Result.count, y=Name)) +
 geom_bar(position="dodge", stat="identity") +
 scale_fill_manual(values=c("#000000","#999999")) +
 facet_wrap(~Expression) +
 theme_bw() + xlab("Number of Genes") + ylab("GO term") +
 theme(panel.grid.major.y = element_line(colour = "#999999", size = 0.1, linetype=3),
                                panel.grid.minor.y = element_line(colour = "#CCCCCC", size = 0.05, linetype=3),
                                panel.background = element_rect(colour = "black", fill="white"),
                                panel.grid.major.x = element_line(colour="#CCCCCC", size =0.1, linetype=3),
                                panel.grid.minor.x = element_line(colour ="#CCCCCC", size=0.1, linetype=3),
                                strip.background = element_rect(colour = "black", fill = "grey"),
                                strip.text.x = element_text(colour = "black", face = "bold", size=12),
                                strip.text.y=element_text(colour="black", face="bold", size=15),
                                axis.title.y = element_text(size = rel(1.25)),
                                axis.title.x = element_text(size=rel(1.25)),
                                axis.text.x = element_text(size=rel(1)),
                                axis.text.y = element_text(size=rel(1)),
                                legend.title = element_text(face="bold",size=rel(1.1)),
                                legend.text = element_text(size=rel(1)),
                                legend.position="right")

RvB


#RvU GO terms

RvU_sig <- filter(GO_master, Comparison=="RvU" & Bonferroni <0.05)
RvU_sig$Expression <- factor(RvU_sig$Expression, levels = c("DOWN", "UP"))
RvU_sig$Ontology <- factor(RvU_sig$Ontology, levels = c("BP", "MF", "CC"))
RvU<- ggplot(RvU_sig, aes(fill=Ontology, x=Result.count, y=Name)) +
 geom_bar(position="dodge", stat="identity", colour="black") +
 facet_wrap(~Expression) +  scale_fill_manual(values=c("#000000","#999999", "#FFFFFF")) +
 theme_bw() + xlab("Number of Genes") + ylab("GO term") +
 theme(panel.grid.major.y = element_line(colour = "#999999", size = 0.1, linetype=3),
      panel.grid.minor.y = element_line(colour = "#CCCCCC", size = 0.05, linetype=3),
      panel.background = element_rect(colour = "black", fill="white"),
      panel.grid.major.x = element_line(colour="#CCCCCC", size =0.1, linetype=3),
      panel.grid.minor.x = element_line(colour ="#CCCCCC", size=0.1, linetype=3),
      strip.background = element_rect(colour = "black", fill = "grey"),
      strip.text.x = element_text(colour = "black", face = "bold", size=12),
      strip.text.y=element_text(colour="black", face="bold", size=15),
      axis.title.y = element_text(size = rel(1.25)),
      axis.title.x = element_text(size=rel(1.25)),
      axis.text.x = element_text(size=rel(1), color="black"),
      axis.text.y = element_text(size=rel(1), color="black"),
      legend.title = element_text(face="bold",size=rel(1.1)),
      legend.text = element_text(size=rel(1)),
      legend.position="right")
RvU
ggsave("RvUGO.tiff", RvU, height = 5, width = 7, dpi = 600)
#RvP GO terms

RvP_sig <- filter(GO_master, Comparison=="RvP" & Bonferroni <0.05)
RvP_sig$Expression <- factor(RvP_sig$Expression, levels = c("DOWN", "UP"))

RvP<- ggplot(RvP_sig, aes(fill=Ontology, x=Result.count, y=Name)) +
 geom_bar(position="dodge", stat="identity", color="black") +
```

```
  facet_wrap(~Expression) + scale_fill_manual(values=c("#000000","#ffffff")) +
  theme_bw() + xlab("Number of Genes") + ylab("GO Term") +
  theme(panel.grid.major.y = element_line(colour = "#999999", size = 0.1, linetype=3),
        panel.grid.minor.y = element_line(colour = "#CCCCCC", size = 0.05, linetype=3),
        panel.background = element_rect(colour = "black", fill="white"),
        panel.grid.major.x = element_line(colour="#CCCCCC", size =0.1, linetype=3),
        panel.grid.minor.x = element_line(colour ="#CCCCCC", size=0.1, linetype=3),
        strip.background = element_rect(colour = "black", fill = "grey"),
        strip.text.x = element_text(colour = "black", face = "bold", size=12),
        strip.text.y=element_text(colour="black", face="bold", size=15),
        axis.title.y = element_text(size = rel(1.25)),
        axis.title.x = element_text(size=rel(1.25)),
        axis.text.x = element_text(size=rel(1)),
        axis.text.y = element_text(size=rel(1), color="black"),
        legend.title = element_text(face="bold",size=rel(1.1)),
        legend.text = element_text(size=rel(1)),
        legend.position="right")
RvP
ggsave("RvPGO04.03.tiff", RvP, height = 5, width = 7, dpi = 600)


#RvM GO terms

RvM_sig <- filter(GO_master, Comparison=="RvM" & Bonferroni <0.05)
RvM_sig$Expression <- factor(RvM_sig$Expression, levels = c("DOWN", "UP"))
RvM_sig
RvM<- ggplot(RvM_sig, aes(fill=Ontology, x=Result.count, y=Name)) +
  geom_bar(position="dodge", stat="identity") +
  facet_wrap(~Expression) +
  theme_bw() + xlab("Number of Genes") +
  theme(panel.grid.major.y = element_line(colour = "#999999", size = 0.1, linetype=3),
        panel.grid.minor.y = element_line(colour = "#CCCCCC", size = 0.05, linetype=3),
        panel.background = element_rect(colour = "black", fill="white"),
        panel.grid.major.x = element_line(colour="#CCCCCC", size =0.1, linetype=3),
        panel.grid.minor.x = element_line(colour ="#CCCCCC", size=0.1, linetype=3),
        strip.background = element_rect(colour = "black", fill = "grey"),
        strip.text.x = element_text(colour = "black", face = "bold", size=12),
        strip.text.y=element_text(colour="black", face="bold", size=15),
        axis.title.y = element_text(size = rel(1.25)),
        axis.title.x = element_text(size=rel(1.25)),
        axis.text.x = element_text(size=rel(1)),
        axis.text.y = element_text(size=rel(1)),
        legend.title = element_text(face="bold",size=rel(1.1)),
        legend.text = element_text(size=rel(1)),
        legend.position="right")
RvM



####Heatmap for detox genes####
#detox genes identified by product annotation or associated GO term
if (!requireNamespace("BiocManager", quietly = TRUE))
  #      install.packages("BiocManager")
  BiocManager::install("DESeq2")
if (!requireNamespace("BiocManager", quietly=TRUE))
  install.packages("BiocManager")


install.packages("readxl")
library(readxl)
```

```
install.packages("dplyr")
library(dplyr)
install.packages("tidyverse")
library(tidyverse)
install.packages("ggplot2")
library(ggplot2)
install.packages("circlize")
library(circlize)
install.packages("pheatmap")
library(pheatmap)
BiocManager::install("ComplexHeatmap")
library(ComplexHeatmap)

#manually compiled matrix of FC in each comparison from premade list of detox-related GENEIDs
Called heatmap.detox
heatmap.detox <- as.data.frame(heatmap.detox)
rownames(heatmap.detox) <- heatmap.detox$GeneID

heatmap.detox <- heatmap.detox[ ,2:5]
heatmap.detox <- heatmap.detox*-1 #to show relative to REC R, rather than what REC R is relative to each of the other strains
heatmap.detox <- transform(heatmap.detox,
                           REC_B = as.numeric(REC_B),
                           REC_U = as.numeric(REC_U),
                           REC_M = as.numeric(REC_M),
                           REC_P = as.numeric(REC_P))
heatmap.detox <- as.matrix(heatmap.detox)

col_fun = colorRamp2(c(-3.65, -1.5, -1, 1, 1.5, 3.65), c( "#283593", "#C5CAE9","snow2", "snow2", "#FFE0B2", "#E60510"))
col_fun(seq(-10, 10))

heatmap_detoxgenes <- Heatmap(heatmap.detox, name = "Log2 Fold Change",
                              cluster_columns=T,
                              cluster_rows=T,
                              cluster_row_slices = TRUE,
                              cluster_column_slices=TRUE,
                              col = col_fun,
                              row_names_gp = gpar(fontsize = 10))



#manually compiled matrix of FC in each comparison from premade list of immune-related GENEIDs
Called heatmap.immune
heatmap.immune <- heatmap.immune[ ,2:5]
heatmap.immune <- heatmap.immune*-1 #to show relative to REC R, rather than what REC R is relative to each of the other strains

heatmap.immune <- transform(heatmap.immune,
                            REC_B = as.numeric(REC_B),
                            REC_U = as.numeric(REC_U),
                            REC_M = as.numeric(REC_M),
                            REC_P = as.numeric(REC_P))
heatmap.immune <- as.matrix(heatmap.immune)

col_fun = colorRamp2(c(-6, -2, -1, 1, 2, 6), c( "#283593", "#C5CAE9","snow2", "snow2", "#FFE0B2", "#E60510"))
col_fun(seq(-10, 10))
```

```
heatmap_immunegenes <- Heatmap(heatmap.immune, name = "Log2 Fold Change",
                cluster_columns=T,
                cluster_rows=T,
                cluster_row_slices = TRUE,
                cluster_column_slices=TRUE,
                col = col_fun,
                row_names_gp = gpar(fontsize = 10))

heatmap_immunegenes
```

###Volcano plots###

```
if (!requireNamespace('BiocManager', quietly = TRUE))
  install.packages('BiocManager')

BiocManager::install('EnhancedVolcano')
library(EnhancedVolcano)


RvU_volc <- RvU %>% mutate(gene_type = case_when(log2FoldChange >= log2(2) & padj <= 0.05 ~
"Upregulated",
                                   log2FoldChange <= log2(0.5) & padj <= 0.05 ~ "Downregulated",
                                   TRUE ~ "Not significant"))
RvU_volc_labs_UP <- filter(RvU_volc, gene_type=="Upregulated") %>%
  arrange(desc(log2FoldChange)) %>%  slice(1:10)
RvU_volc_labs_DOWN <- filter(RvU_volc, gene_type=="Downregulated") %>%
  arrange(log2FoldChange) %>% slice(1:10)
RvU_volc_labs <- rbind(RvU_volc_labs_UP, RvU_volc_labs_DOWN)
RvU_volc_genes <- as.vector(RvU_volc_labs$GeneID) #creates vector of GeneIDs to pass to Enhanced volcano

EnhancedVolcano(RvU,
        lab = RvU$GeneID,
        x = 'log2FoldChange',
        y = 'padj',
        title="REC-R vs REC-U",
        pCutoff = 0.05,
        FCcutoff = 2,
        pointSize = 2,
        labSize=3.5,
        legendPosition = "right",
        legendLabels = c("Not significant", "Log2FC", "p-value", "p-value & Log2FC"),
        legendLabSize = 12.5,
        colAlpha = 3/5,
        legendIconSize = 3,
        #selectLab = RvU_volc_genes, #can also supply vector of GeneIDs here for specifics
        selectLab = c("AAEL025856", "AAEL028175", 'AAEL027839', "AAEL028095",
                "AAEL022121", "AAEL021292", "AAEL027453",
                "AAEL026974", "AAEL020244", "AAEL022058", "AAEL002389",
                "AAEL025617", "AAEL028663", "AAEL002622", "AAEL025333",
                "AAEL029041"),
        xlab = bquote(~Log[2]~ 'fold change'),
        boxedLabels = TRUE,
        drawConnectors = TRUE,
        widthConnectors=0.6,
        lengthConnectors = unit(0.01, 'npc'),
        colConnectors="#333333")

###RvM###
RvM_volc <- RvM %>% mutate(gene_type = case_when(log2FoldChange >= log2(2) & padj <= 0.05 ~
"Upregulated",
```

```
                                log2FoldChange <= log2(0.5) & padj <= 0.05 ~ "Downregulated",
                                TRUE ~ "Not significant"))
RvM_volc_labs_UP <- filter(RvM_volc, gene_type=="Upregulated") %>%
  arrange(desc(log2FoldChange)) %>%  slice(1:10)
RvM_volc_labs_DOWN <- filter(RvM_volc, gene_type=="Downregulated") %>%
  arrange(log2FoldChange) %>% slice(1:10)
RvM_volc_labs <- rbind(RvM_volc_labs_UP, RvM_volc_labs_DOWN)
RvM_volc_genes <- as.vector(RvM_volc_labs$GeneID)

EnhancedVolcano(RvM,
        lab = RvM$GeneID,
        x = 'log2FoldChange',
        y = 'padj',
        title="REC-R vs REC-M",
        pCutoff = 0.05,
        FCcutoff = 2,
        pointSize = 2,
        labSize=3.5,
        legendPosition = "right",
        legendLabels = c("Not significant", "Log2FC", "p-value", "p-value & Log2FC"),
        legendLabSize = 12.5,
        colAlpha = 3/5,
        legendIconSize = 3,
        #selectLab = RvU_volc_genes, #can also supply vector of GeneIDs here for specifics
        selectLab = c("AAEL028175", "AAEL027537", "AAEL022665",
                "AAEL027045", "AAEL022708", "AAEL026471",
                "AAEL025617", "AAEL008861", "AAEL000477", "AAEL020569",
                "AAEL026222", "AAEL028095", "AAEL020244",
                "AAEL008798", "AAEL024176"),
        xlab = bquote(~Log[2]~ 'fold change'),
        boxedLabels = TRUE,
        drawConnectors = TRUE,
        widthConnectors=0.6,
        lengthConnectors = unit(0.01, 'npc'),
        colConnectors="#333333")


###RvP###
RvP_volc <- RvP %>% mutate(gene_type = case_when(log2FoldChange >= log2(2) & padj <= 0.05 ~
"Upregulated",
                                log2FoldChange <= log2(0.5) & padj <= 0.05 ~ "Downregulated",
                                TRUE ~ "Not significant"))
RvP_volc_labs_UP <- filter(RvP_volc, gene_type=="Upregulated") %>%
  arrange(desc(log2FoldChange)) %>%  slice(1:10)
RvP_volc_labs_DOWN <- filter(RvP_volc, gene_type=="Downregulated") %>%
  arrange(log2FoldChange) %>% slice(1:10)
RvP_volc_labs <- rbind(RvP_volc_labs_UP, RvP_volc_labs_DOWN)
RvP_volc_genes <- as.vector(RvP_volc_labs$GeneID)

EnhancedVolcano(RvP,
        lab = RvP$GeneID,
        x = 'log2FoldChange',
        y = 'padj',
        title="REC-R vs REC-P",
        pCutoff = 0.05,
        FCcutoff = 2,
        pointSize = 2,
        labSize=3.5,
        legendPosition = "right",
        legendLabels = c("Not significant", "Log2FC", "p-value", "p-value & Log2FC"),
```

```
        legendLabSize = 12.5,
        colAlpha = 3/5,
        legendIconSize = 3,
        #selectLab = RvU_volc_genes, #can also supply vector of GeneIDs here for specifics
        selectLab = c("AAEL028175", "AAEL020244", "AAEL022058", "AAEL022499",
                "AAEL025856", "AAEL007879", "AAEL027839", "AAEL019643",
                "AAEL025060", "AAEL018095", "AAEL010500", "AAEL027045",
                "AAEL002389", "AAEL024591", "AAEL028095"),
        xlab = bquote(~Log[2]~ 'fold change'),
        boxedLabels = TRUE,
        drawConnectors = TRUE,
        widthConnectors=0.6,
        lengthConnectors = unit(0.01, 'npc'),
        colConnectors="#333333")
###RvB###
RvB_volc <- RvB %>% mutate(gene_type = case_when(log2FoldChange >= log2(2) & padj <= 0.05 ~
"Upregulated",
                                log2FoldChange <= log2(0.5) & padj <= 0.05 ~ "Downregulated",
                                TRUE ~ "Not significant"))
RvB_volc_labs_UP <- filter(RvB_volc, gene_type=="Upregulated") %>%
 arrange(desc(log2FoldChange)) %>%  slice(1:10)
RvB_volc_labs_DOWN <- filter(RvB_volc, gene_type=="Downregulated") %>%
 arrange(log2FoldChange) %>% slice(1:10)
RvB_volc_labs <- rbind(RvB_volc_labs_UP, RvB_volc_labs_DOWN)
RvB_volc_genes <- as.vector(RvB_volc_labs$GeneID)

EnhancedVolcano(RvB,
        lab = RvB$GeneID,
        x = 'log2FoldChange',
        y = 'padj',
        title="REC-R vs REC-B",
        pCutoff = 0.05,
        FCcutoff = 2,
        pointSize = 2,
        labSize=3.5,
        legendPosition = "right",
        legendLabels = c("Not significant", "Log2FC", "p-value", "p-value & Log2FC"),
        legendLabSize = 12.5,
        colAlpha = 3/5,
        legendIconSize = 3,
        #selectLab = RvU_volc_genes, #can also supply vector of GeneIDs here for specifics
        selectLab = c("AAEL028175", "AAEL020244", "AAEL027453",
        "AAEL022499", "AAL35858", "AAEL026974",
        "AAEL027839", "AAEL025856", "AAEL027045",
        "AAEL020373", "AAEL003004", "AAEL028095", "AAEL025743"),
        xlab = bquote(~Log[2]~ 'fold change'),
        boxedLabels = TRUE,
        drawConnectors = TRUE,
        widthConnectors=0.6,
        lengthConnectors = unit(0.01, 'npc'),
        colConnectors="#333333")
```

Chapter 4: Insecticide selection of *Aedes aegypti* is associated with alterations in vector competence for Zika virus

```
####################
##Propotions Data###
```

```
###################

REC_INJ_Master <- select(REC_INJ_Master,
                  "MosquitoID",
                  "Strain",
                  "Part",
                  "DPI",
                  "log10pfu_sample")

bodies <- filter(REC_INJ_Master, Part =="Bodies")
bodies <- rename(bodies, Body_Titre = log10pfu_sample)
bodies <- select(bodies, MosquitoID, Strain, DPI, Body_Titre)
bodies

heads <- filter(REC_INJ_Master, Part =="Heads")
heads <- rename(heads, Head_Titre = log10pfu_sample)
heads <- select(heads, MosquitoID, Strain, DPI, Head_Titre)
heads

legs <- filter(REC_INJ_Master, Part =="Legs")
legs <- rename(legs, Leg_Titre = log10pfu_sample)
legs <- select(legs, MosquitoID, Strain, DPI, Leg_Titre)
legs

saliva<- filter(REC_INJ_Master, Part =="Saliva")
saliva <- rename(saliva, Saliva_Titre = log10pfu_sample)
saliva <- select(saliva, MosquitoID, Strain, DPI, Saliva_Titre)
saliva


a <-left_join(bodies, heads)
b <- left_join(a, legs)
Titrebysample <- left_join(b, saliva)

#makes a dataframe showing all 3 titres by mosquito ID

b$Body_Titre <- as.numeric(b$Body_Titre)
b$Head_Titre <- as.numeric(b$Head_Titre)
b$Leg_Titre <- as.numeric(b$Leg_Titre)
b$Saliva_Titre <- as.numeric(b$Saliva_Titre)


########################################
############infection prevalence data#####
########################################

###REC U 5dpi###
UB5_total <- nrow(b[b$Body_Titre >=0 & b$Strain == 'REC U' & b$DPI == '5', ])
UB5_pos <- nrow(b[b$Strain == 'REC U' & b$DPI == '5' & b$Body_Titre >0, ])
UB5_CI <- data.frame(binconf(UB5_pos,UB5_total))
UB5_CI['Positive'] <- UB5_pos #add in Ns to make it easier
UB5_CI['Total'] <- UB5_total
Identity <- "UB_5dpi"
UB5_CI['Identity'] <- Identity
UB5_CI
```

```
###REC R 5dpi###
RB5_total <- nrow(b[b$Body_Titre >=0 & b$Strain == 'REC R' & b$DPI == '5', ])
RB5_pos <- nrow(b[b$Strain == 'REC R' & b$DPI == '5' & b$Body_Titre >0, ])
RB5_CI <- data.frame(binconf(RB5_pos,RB5_total))
RB5_CI['Positive'] <- RB5_pos
RB5_CI['Total'] <- RB5_total
Identity <- "RB_5dpi"
RB5_CI['Identity'] <- Identity
RB5_CI

###REC M 5dpi###
MB5_total <- nrow(b[b$Body_Titre >=0 & b$Strain == 'REC M' & b$DPI == '5', ])
MB5_pos <- nrow(b[b$Strain == 'REC M' & b$DPI == '5' & b$Body_Titre >0, ])
MB5_CI <- data.frame(binconf(MB5_pos,MB5_total))
MB5_CI['Positive'] <- MB5_pos
MB5_CI['Total'] <- MB5_total
Identity <- "MB_5dpi"
MB5_CI['Identity'] <- Identity
MB5_CI

###REC P 5dpi###
PB5_total <- nrow(b[b$Body_Titre >=0 & b$Strain == 'REC P' & b$DPI == '5', ])
PB5_pos <- nrow(b[b$Strain == 'REC P' & b$DPI == '5' & b$Body_Titre >0, ])
PB5_CI <- data.frame(binconf(PB5_pos,PB5_total))
PB5_CI['Positive'] <- PB5_pos
PB5_CI['Total'] <- PB5_total
Identity <- "PB_5dpi"
PB5_CI['Identity'] <- Identity
PB5_CI

###REC U 7dpi###
UB7_total <- nrow(b[b$Body_Titre >=0 & b$Strain == 'REC U' & b$DPI == '7', ])
UB7_pos <- nrow(b[b$Strain == 'REC U' & b$DPI == '7' & b$Body_Titre >0, ])
UB7_CI <- data.frame(binconf(UB7_pos,UB7_total))
UB7_CI['Positive'] <- UB7_pos
UB7_CI['Total'] <- UB7_total
Identity <- "UB_7dpi"
UB7_CI['Identity'] <- Identity
UB7_CI

###REC R 7dpi###
RB7_total <- nrow(b[b$Body_Titre >=0 & b$Strain == 'REC R' & b$DPI == '7', ])
RB7_pos <- nrow(b[b$Strain == 'REC R' & b$DPI == '7' & b$Body_Titre >0, ])
RB7_CI <- data.frame(binconf(RB7_pos,RB7_total))
RB7_CI['Positive'] <- RB7_pos
RB7_CI['Total'] <- RB7_total
Identity <- "RB_7dpi"
RB7_CI['Identity'] <- Identity
RB7_CI

###REC M 7dpi###
MB7_total <- nrow(b[b$Body_Titre >=0 & b$Strain == 'REC M' & b$DPI == '7', ])
MB7_pos <- nrow(b[b$Strain == 'REC M' & b$DPI == '7' & b$Body_Titre >0, ])
MB7_CI <- data.frame(binconf(MB7_pos,MB7_total))
```

```
MB7_CI['Positive'] <- MB7_pos
MB7_CI['Total'] <- MB7_total
Identity <- "MB_7dpi"
MB7_CI['Identity'] <- Identity
MB7_CI

###REC P 7dpi###
PB7_total <- nrow(b[b$Body_Titre >=0 & b$Strain == 'REC P' & b$DPI == '7', ])
PB7_pos <- nrow(b[b$Strain == 'REC P' & b$DPI == '7' & b$Body_Titre >0, ])
PB7_CI <- data.frame(binconf(PB7_pos,PB7_total))
PB7_CI['Positive'] <- PB7_pos
PB7_CI['Total'] <- PB7_total
Identity <- "PB_7dpi"
PB7_CI['Identity'] <- Identity
PB7_CI

###REC U 10dpi###
UB10_total <- nrow(b[b$Body_Titre >=0 & b$Strain == 'REC U' & b$DPI == '10', ])
UB10_pos <- nrow(b[b$Strain == 'REC U' & b$DPI == '10' & b$Body_Titre >0, ])
UB10_CI <- data.frame(binconf(UB10_pos,UB10_total))
UB10_CI['Positive'] <- UB10_pos
UB10_CI['Total'] <- UB10_total
Identity <- "UB_10dpi"
UB10_CI['Identity'] <- Identity
UB10_CI

###REC R 10dpi###
RB10_total <- nrow(b[b$Body_Titre >=0 & b$Strain == 'REC R' & b$DPI == '10', ])
RB10_pos <- nrow(b[b$Strain == 'REC R' & b$DPI == '10' & b$Body_Titre >0, ])
RB10_CI <- data.frame(binconf(RB10_pos,RB10_total))
RB10_CI['Positive'] <- RB10_pos
RB10_CI['Total'] <- RB10_total
Identity <- "RB_10dpi"
RB10_CI['Identity'] <- Identity
RB10_CI

###REC M 10dpi###
MB10_total <- nrow(b[b$Body_Titre >=0 & b$Strain == 'REC M' & b$DPI == '10', ])
MB10_pos <- nrow(b[b$Strain == 'REC M' & b$DPI == '10' & b$Body_Titre >0, ])
MB10_CI <- data.frame(binconf(MB10_pos,MB10_total))
MB10_CI['Positive'] <- MB10_pos
MB10_CI['Total'] <- MB10_total
Identity <- "MB_10dpi"
MB10_CI['Identity'] <- Identity
MB10_CI

###REC P 10dpi###
PB10_total <- nrow(b[b$Body_Titre >=0 & b$Strain == 'REC P' & b$DPI == '10', ])
PB10_pos <- nrow(b[b$Strain == 'REC P' & b$DPI == '10' & b$Body_Titre >0, ])
PB10_CI <- data.frame(binconf(PB10_pos,PB10_total))
PB10_CI['Positive'] <- PB10_pos
PB10_CI['Total'] <- PB10_total
Identity <- "PB_10dpi"
PB10_CI['Identity'] <- Identity
PB10_CI #checked
```

```
#combining all into one csv
IR_ZIKVINJ <-
rbind(UB5_CI,UB7_CI,UB10_CI,RB5_CI,RB7_CI,RB10_CI,MB5_CI,MB7_CI,MB10_CI,PB5_CI,PB7_
CI,PB10_CI)
IR_ZIKVINJ <- IR_ZIKVINJ%>% select("Identity", "Positive", "Total", "PointEst", "Lower", "Upper")
IR_ZIKVINJ



####DISSEMINATION TO HEADS###

###REC U 5dpi###
UH5_total <- nrow(b[b$Strain== 'REC U' & b$DPI == '5' & b$Body_Titre >0 & b$Head_Titre>=0, ])
UH5_pos <- (b[b$Strain == 'REC U' & b$DPI == '5' & b$Body_Titre >0 & b$Head_Titre>0, ])
UH5_pos<-na.omit(UH5_pos)
UH5_pos
UH5_pos<-nrow(UH5_pos)
UH5_CI <- data.frame(binconf(UH5_pos,UH5_total))
UH5_CI['Positive'] <- UH5_pos
UH5_CI['Total'] <- UH5_total
Identity <- "UH_5dpi"
UH5_CI['Identity'] <- Identity
UH5_CI

###REC R 5dpi###
RH5_total <- nrow(b[b$Strain== 'REC R' & b$DPI == '5' & b$Body_Titre >0 & b$Head_Titre>=0, ])
RH5_pos <- (b[b$Strain == 'REC R' & b$DPI == '5' & b$Body_Titre >0 & b$Head_Titre>0, ])
RH5_pos<-na.omit(RH5_pos)
RH5_pos
RH5_pos<-nrow(RH5_pos)
RH5_CI <- data.frame(binconf(RH5_pos,RH5_total))
RH5_CI['Positive'] <- RH5_pos
RH5_CI['Total'] <- RH5_total
Identity <- "RH_5dpi"
RH5_CI['Identity'] <- Identity
RH5_CI

###REC M 5dpi###
MH5_total <- nrow(b[b$Strain== 'REC M' & b$DPI == '5' & b$Body_Titre >0 & b$Head_Titre>=0, ])
MH5_pos <- (b[b$Strain == 'REC M' & b$DPI == '5' & b$Body_Titre >0 & b$Head_Titre>0, ])
MH5_pos<-na.omit(MH5_pos)
MH5_pos
MH5_pos<-nrow(MH5_pos)
MH5_CI <- data.frame(binconf(MH5_pos,MH5_total))
MH5_CI['Positive'] <- MH5_pos
MH5_CI['Total'] <- MH5_total
Identity <- "MH_5dpi"
MH5_CI['Identity'] <- Identity
MH5_CI

###REC P 5dpi###
PH5_total <- nrow(b[b$Strain== 'REC P' & b$DPI == '5' & b$Body_Titre >0 & b$Head_Titre>=0, ])
PH5_pos <- (b[b$Strain == 'REC P' & b$DPI == '5' & b$Body_Titre >0 & b$Head_Titre>0, ])
PH5_pos<-na.omit(PH5_pos)
```

```
PH5_pos
PH5_pos<-nrow(PH5_pos)
PH5_CI <- data.frame(binconf(PH5_pos,PH5_total))
PH5_CI['Positive'] <- PH5_pos
PH5_CI['Total'] <- PH5_total
Identity <- "PH_5dpi"
PH5_CI['Identity'] <- Identity
PH5_CI
###REC U 7dpi###
UH7_total <- nrow(b[b$Strain== 'REC U' & b$DPI == '7' & b$Body_Titre >0 & b$Head_Titre>=0, ])
UH7_pos <- (b[b$Strain == 'REC U' & b$DPI == '7' & b$Body_Titre >0 & b$Head_Titre>0, ])
UH7_pos<-na.omit(UH7_pos)
UH7_pos
UH7_pos<-nrow(UH7_pos)
UH7_CI <- data.frame(binconf(UH7_pos,UH7_total))
UH7_CI['Positive'] <- UH7_pos
UH7_CI['Total'] <- UH7_total
Identity <- "UH_7dpi"
UH7_CI['Identity'] <- Identity
UH7_CI

###REC R 7dpi###
RH7_total <- nrow(b[b$Strain== 'REC R' & b$DPI == '7' & b$Body_Titre >0 & b$Head_Titre>=0, ])
RH7_pos <- (b[b$Strain == 'REC R' & b$DPI == '7' & b$Body_Titre >0 & b$Head_Titre>0, ])
RH7_pos<-na.omit(RH7_pos)
RH7_pos
RH7_pos<-nrow(RH7_pos)
RH7_CI <- data.frame(binconf(RH7_pos,RH7_total))
RH7_CI['Positive'] <- RH7_pos
RH7_CI['Total'] <- RH7_total
Identity <- "RH_7dpi"
RH7_CI['Identity'] <- Identity
RH7_CI

###REC M 7dpi###
MH7_total <- nrow(b[b$Strain== 'REC M' & b$DPI == '7' & b$Body_Titre >0 & b$Head_Titre>=0, ])
MH7_pos <- (b[b$Strain == 'REC M' & b$DPI == '7' & b$Body_Titre >0 & b$Head_Titre>0, ])
MH7_pos<-na.omit(MH7_pos)
MH7_pos
MH7_pos<-nrow(MH7_pos)
MH7_CI <- data.frame(binconf(MH7_pos,MH7_total))
MH7_CI['Positive'] <- MH7_pos
MH7_CI['Total'] <- MH7_total
Identity <- "MH_7dpi"
MH7_CI['Identity'] <- Identity
MH7_CI


###REC P 7dpi###
PH7_total <- nrow(b[b$Strain== 'REC P' & b$DPI == '7' & b$Body_Titre >0 & b$Head_Titre>=0, ])
PH7_pos <- (b[b$Strain == 'REC P' & b$DPI == '7' & b$Body_Titre >0 & b$Head_Titre>0, ])
PH7_pos<-na.omit(PH7_pos)
PH7_pos
PH7_pos<-nrow(PH7_pos)
PH7_CI <- data.frame(binconf(PH7_pos,PH7_total))
```

```
PH7_CI['Positive'] <- PH7_pos
PH7_CI['Total'] <- PH7_total
Identity <- "PH_7dpi"
PH7_CI['Identity'] <- Identity
PH7_CI

###REC U 10dpi###
UH10_total <- nrow(b[b$Strain== 'REC U' & b$DPI == '10' & b$Body_Titre >0 & b$Head_Titre>=0, ])
UH10_pos <- (b[b$Strain == 'REC U' & b$DPI == '10' & b$Body_Titre >0 & b$Head_Titre>0, ])
UH10_pos<-na.omit(UH10_pos)
UH10_pos
UH10_pos<-nrow(UH10_pos)
UH10_CI <- data.frame(binconf(UH10_pos,UH10_total))
UH10_CI['Positive'] <- UH10_pos
UH10_CI['Total'] <- UH10_total
Identity <- "UH_10dpi"
UH10_CI['Identity'] <- Identity
UH10_CI
###REC R 10dpi###
RH10_total <- nrow(b[b$Strain== 'REC R' & b$DPI == '10' & b$Body_Titre >0 & b$Head_Titre>=0, ])
RH10_pos <- (b[b$Strain == 'REC R' & b$DPI == '10' & b$Body_Titre >0 & b$Head_Titre>0, ])
RH10_pos<-na.omit(RH10_pos)
RH10_pos
RH10_pos<-nrow(RH10_pos)
RH10_CI <- data.frame(binconf(RH10_pos,RH10_total))
RH10_CI['Positive'] <- RH10_pos
RH10_CI['Total'] <- RH10_total
Identity <- "RH_10dpi"
RH10_CI['Identity'] <- Identity
RH10_CI

###REC M 10dpi###
MH10_total <- nrow(b[b$Strain== 'REC M' & b$DPI == '10' & b$Body_Titre >0 & b$Head_Titre>=0, ])
MH10_pos <- (b[b$Strain == 'REC M' & b$DPI == '10' & b$Body_Titre >0 & b$Head_Titre>0, ])
MH10_pos<-na.omit(MH10_pos)
MH10_pos
MH10_pos<-nrow(MH10_pos)
MH10_CI <- data.frame(binconf(MH10_pos,MH10_total))
MH10_CI['Positive'] <- MH10_pos
MH10_CI['Total'] <- MH10_total
Identity <- "MH_10dpi"
MH10_CI['Identity'] <- Identity
MH10_CI

###REC P 10dpi###
PH10_total <- nrow(b[b$Strain== 'REC P' & b$DPI == '10' & b$Body_Titre >0 & b$Head_Titre>=0, ])
PH10_pos <- (b[b$Strain == 'REC P' & b$DPI == '10' & b$Body_Titre >0 & b$Head_Titre>0, ])
PH10_pos<-na.omit(PH10_pos)
PH10_pos
PH10_pos<-nrow(PH10_pos)
PH10_CI <- data.frame(binconf(PH10_pos,PH10_total))
PH10_CI['Positive'] <- PH10_pos
PH10_CI['Total'] <- PH10_total
Identity <- "PH_10dpi"
PH10_CI['Identity'] <- Identity
```

```
PH10_CI

#COMBINING ALL DRH INTO ONE CSV
#combining all into one csv
DRH_ZIKVINJ <-
rbind(UH5_CI,UH7_CI,UH10_CI,RH5_CI,RH7_CI,RH10_CI,MH5_CI,MH7_CI,MH10_CI,PH5_CI,PH
7_CI,PH10_CI)
DRH_ZIKVINJ <- DRH_ZIKVINJ%>% select("Identity", "Positive", "Total", "PointEst", "Lower",
"Upper")
DRH_ZIKVINJ


####DISSEMINATION TO LEGS###

###REC U 5dpi###
UL5_total <- nrow(b[b$Strain== 'REC U' & b$DPI == '5' & b$Body_Titre >0 & b$Leg_Titre>=0, ])
UL5_pos <- (b[b$Strain == 'REC U' & b$DPI == '5' & b$Body_Titre >0 & b$Leg_Titre>0, ])
UL5_pos<-na.omit(UL5_pos)
UL5_pos
UL5_pos<-nrow(UL5_pos)
UL5_CI <- data.frame(binconf(UL5_pos,UL5_total))
UL5_CI['Positive'] <- UL5_pos
UL5_CI['Total'] <- UL5_total
Identity <- "UL_5dpi"
UL5_CI['Identity'] <- Identity
UL5_CI

###REC R 5dpi###
RL5_total <- nrow(b[b$Strain== 'REC R' & b$DPI == '5' & b$Body_Titre >0 & b$Leg_Titre>=0, ])
RL5_pos <- (b[b$Strain == 'REC R' & b$DPI == '5' & b$Body_Titre >0 & b$Leg_Titre>0, ])
RL5_pos<-na.omit(RL5_pos)
RL5_pos
RL5_pos<-nrow(RL5_pos)
RL5_CI <- data.frame(binconf(RL5_pos,RL5_total))
RL5_CI['Positive'] <- RL5_pos
RL5_CI['Total'] <- RL5_total
Identity <- "RL_5dpi"
RL5_CI['Identity'] <- Identity
RL5_CI

###REC M 5dpi###
ML5_total <- nrow(b[b$Strain== 'REC M' & b$DPI == '5' & b$Body_Titre >0 & b$Leg_Titre>=0, ])
ML5_pos <- (b[b$Strain == 'REC M' & b$DPI == '5' & b$Body_Titre >0 & b$Leg_Titre>0, ])
ML5_pos<-na.omit(ML5_pos)
ML5_pos
ML5_pos<-nrow(ML5_pos)
ML5_CI <- data.frame(binconf(ML5_pos,ML5_total))
ML5_CI['Positive'] <- ML5_pos
ML5_CI['Total'] <- ML5_total
Identity <- "ML_5dpi"
ML5_CI['Identity'] <- Identity
ML5_CI


###REC P 5dpi###
```

```
PL5_total <- nrow(b[b$Strain== 'REC P' & b$DPI == '5' & b$Body_Titre >0 & b$Leg_Titre>=0, ])
PL5_pos <- (b[b$Strain == 'REC P' & b$DPI == '5' & b$Body_Titre >0 & b$Leg_Titre>0, ])
PL5_pos<-na.omit(PL5_pos)
PL5_pos
PL5_pos<-nrow(PL5_pos)
PL5_CI <- data.frame(binconf(PL5_pos,PL5_total))
PL5_CI['Positive'] <- PL5_pos
PL5_CI['Total'] <- PL5_total
Identity <- "PL_5dpi"
PL5_CI['Identity'] <- Identity
PL5_CI

###REC U 7dpi###
UL7_total <- nrow(b[b$Strain== 'REC U' & b$DPI == '7' & b$Body_Titre >0 & b$Leg_Titre>=0, ])
UL7_pos <- (b[b$Strain == 'REC U' & b$DPI == '7' & b$Body_Titre >0 & b$Leg_Titre>0, ])
UL7_pos<-na.omit(UL7_pos)
UL7_pos
UL7_pos<-nrow(UL7_pos)
UL7_CI <- data.frame(binconf(UL7_pos,UL7_total))
UL7_CI['Positive'] <- UL7_pos
UL7_CI['Total'] <- UL7_total
Identity <- "UL_7dpi"
UL7_CI['Identity'] <- Identity
UL7_CI

###REC R 7dpi###
RL7_total <- nrow(b[b$Strain== 'REC R' & b$DPI == '7' & b$Body_Titre >0 & b$Leg_Titre>=0, ])
RL7_pos <- (b[b$Strain == 'REC R' & b$DPI == '7' & b$Body_Titre >0 & b$Leg_Titre>0, ])
RL7_pos<-na.omit(RL7_pos)
RL7_pos
RL7_pos<-nrow(RL7_pos)
RL7_CI <- data.frame(binconf(RL7_pos,RL7_total))
RL7_CI['Positive'] <- RL7_pos
RL7_CI['Total'] <- RL7_total
Identity <- "RL_7dpi"
RL7_CI['Identity'] <- Identity
RL7_CI

###REC M 7dpi###
ML7_total <- nrow(b[b$Strain== 'REC M' & b$DPI == '7' & b$Body_Titre >0 & b$Leg_Titre>=0, ])
ML7_pos <- (b[b$Strain == 'REC M' & b$DPI == '7' & b$Body_Titre >0 & b$Leg_Titre>0, ])
ML7_pos<-na.omit(ML7_pos)
ML7_pos
ML7_pos<-nrow(ML7_pos)
ML7_CI <- data.frame(binconf(ML7_pos,ML7_total))
ML7_CI['Positive'] <- ML7_pos
ML7_CI['Total'] <- ML7_total
Identity <- "ML_7dpi"
ML7_CI['Identity'] <- Identity
ML7_CI

###REC P 7dpi###
PL7_total <- nrow(b[b$Strain== 'REC P' & b$DPI == '7' & b$Body_Titre >0 & b$Leg_Titre>=0, ])
PL7_pos <- (b[b$Strain == 'REC P' & b$DPI == '7' & b$Body_Titre >0 & b$Leg_Titre>0, ])
PL7_pos<-na.omit(PL7_pos)
```

```
PL7_pos
PL7_pos<-nrow(PL7_pos)
PL7_CI <- data.frame(binconf(PL7_pos,PL7_total))
PL7_CI['Positive'] <- PL7_pos
PL7_CI['Total'] <- PL7_total
Identity <- "PL_7dpi"
PL7_CI['Identity'] <- Identity
PL7_CI

###REC U 10dpi###
UL10_total <- nrow(b[b$Strain== 'REC U' & b$DPI == '10' & b$Body_Titre >0 & b$Leg_Titre>=0, ])
UL10_pos <- (b[b$Strain == 'REC U' & b$DPI == '10' & b$Body_Titre >0 & b$Leg_Titre>0, ])
UL10_pos<-na.omit(UL10_pos)
UL10_pos
UL10_pos<-nrow(UL10_pos)
UL10_CI <- data.frame(binconf(UL10_pos,UL10_total))
UL10_CI['Positive'] <- UL10_pos
UL10_CI['Total'] <- UL10_total
Identity <- "UL_10dpi"
UL10_CI['Identity'] <- Identity
UL10_CI

###REC R 10dpi###
RL10_total <- nrow(b[b$Strain== 'REC R' & b$DPI == '10' & b$Body_Titre >0 & b$Leg_Titre>=0, ])
RL10_pos <- (b[b$Strain == 'REC R' & b$DPI == '10' & b$Body_Titre >0 & b$Leg_Titre>0, ])
RL10_pos<-na.omit(RL10_pos)
RL10_pos
RL10_pos<-nrow(RL10_pos)
RL10_CI <- data.frame(binconf(RL10_pos,RL10_total))
RL10_CI['Positive'] <- RL10_pos
RL10_CI['Total'] <- RL10_total
Identity <- "RL_10dpi"
RL10_CI['Identity'] <- Identity
RL10_CI

###REC M 10dpi###
ML10_total <- nrow(b[b$Strain== 'REC M' & b$DPI == '10' & b$Body_Titre >0 & b$Leg_Titre>=0, ])
ML10_pos <- (b[b$Strain == 'REC M' & b$DPI == '10' & b$Body_Titre >0 & b$Leg_Titre>0, ])
ML10_pos<-na.omit(ML10_pos)
ML10_pos
ML10_pos<-nrow(ML10_pos)
ML10_CI <- data.frame(binconf(ML10_pos,ML10_total))
ML10_CI['Positive'] <- ML10_pos
ML10_CI['Total'] <- ML10_total
Identity <- "ML_10dpi"
ML10_CI['Identity'] <- Identity
ML10_CI

###REC M 10dpi###
PL10_total <- nrow(b[b$Strain== 'REC P' & b$DPI == '10' & b$Body_Titre >0 & b$Leg_Titre>=0, ])
PL10_pos <- (b[b$Strain == 'REC P' & b$DPI == '10' & b$Body_Titre >0 & b$Leg_Titre>0, ])
PL10_pos<-na.omit(PL10_pos)
PL10_pos
PL10_pos<-nrow(PL10_pos)
PL10_CI <- data.frame(binconf(PL10_pos,PL10_total))
```

```
PL10_CI['Positive'] <- PL10_pos
PL10_CI['Total'] <- PL10_total
Identity <- "PL_10dpi"
PL10_CI['Identity'] <- Identity
PL10_CI

#combining all into one csv
DRL_ZIKVINJ <-
rbind(UL5_CI,UL7_CI,UL10_CI,RL5_CI,RL7_CI,RL10_CI,ML5_CI,ML7_CI,ML10_CI,PL5_CI,PL7_CI,
PL10_CI)
DRL_ZIKVINJ <- DRL_ZIKVINJ%>% select("Identity", "Positive", "Total", "PointEst", "Lower", "Upper")
#just reorder for ease of reading
DRL_ZIKVINJ

###**********###
####TRANSMISSION###

###REC U 5dpi###
US5_total <- nrow(b[b$Strain== 'REC U' & b$DPI == '5' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
US5_pos <- (b[b$Strain == 'REC U' & b$DPI == '5' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
US5_pos<-na.omit(US5_pos)
US5_pos
US5_pos<-nrow(US5_pos)
US5_CI <- data.frame(binconf(US5_pos,US5_total))
US5_CI['Positive'] <- US5_pos
US5_CI['Total'] <- US5_total
Identity <- "US_5dpi"
US5_CI['Identity'] <- Identity
US5_CI

###REC R 5dpi###
RS5_total <- nrow(b[b$Strain== 'REC R' & b$DPI == '5' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
RS5_pos <- (b[b$Strain == 'REC R' & b$DPI == '5' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
RS5_pos<-na.omit(RS5_pos)
RS5_pos
RS5_pos<-nrow(RS5_pos)
RS5_CI <- data.frame(binconf(RS5_pos,RS5_total))
RS5_CI['Positive'] <- RS5_pos
RS5_CI['Total'] <- RS5_total
Identity <- "RS_5dpi"
RS5_CI['Identity'] <- Identity
RS5_CI

###REC M 5dpi###
MS5_total <- nrow(b[b$Strain== 'REC M' & b$DPI == '5' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
MS5_pos <- (b[b$Strain == 'REC M' & b$DPI == '5' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
MS5_pos<-na.omit(MS5_pos)
MS5_pos
MS5_pos<-nrow(MS5_pos)
MS5_CI <- data.frame(binconf(MS5_pos,MS5_total))
MS5_CI['Positive'] <- MS5_pos
MS5_CI['Total'] <- MS5_total
Identity <- "MS_5dpi"
MS5_CI['Identity'] <- Identity
MS5_CI
```

```
###REC P 5dpi###
PS5_total <- nrow(b[b$Strain== 'REC P' & b$DPI == '5' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
PS5_pos <- (b[b$Strain == 'REC P' & b$DPI == '5' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
PS5_pos<-na.omit(PS5_pos)
PS5_pos
PS5_pos<-nrow(PS5_pos)
PS5_CI <- data.frame(binconf(PS5_pos,PS5_total))
PS5_CI['Positive'] <- PS5_pos
PS5_CI['Total'] <- PS5_total
Identity <- "PS_5dpi"
PS5_CI['Identity'] <- Identity
PS5_CI


###REC U 7dpi###
US7_total <- nrow(b[b$Strain== 'REC U' & b$DPI == '7' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
US7_pos <- (b[b$Strain == 'REC U' & b$DPI == '7' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
US7_pos<-na.omit(US7_pos)
US7_pos
US7_pos<-nrow(US7_pos)
US7_CI <- data.frame(binconf(US7_pos,US7_total))
US7_CI['Positive'] <- US7_pos
US7_CI['Total'] <- US7_total
Identity <- "US_7dpi"
US7_CI['Identity'] <- Identity
US7_CI


###REC R 7dpi###
RS7_total <- nrow(b[b$Strain== 'REC R' & b$DPI == '7' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
RS7_pos <- (b[b$Strain == 'REC R' & b$DPI == '7' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
RS7_pos<-na.omit(RS7_pos)
RS7_pos
RS7_pos<-nrow(RS7_pos)
RS7_CI <- data.frame(binconf(RS7_pos,RS7_total))
RS7_CI['Positive'] <- RS7_pos
RS7_CI['Total'] <- RS7_total
Identity <- "RS_7dpi"
RS7_CI['Identity'] <- Identity
RS7_CI


###REC M 7dpi###
MS7_total <- nrow(b[b$Strain== 'REC M' & b$DPI == '7' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
MS7_pos <- (b[b$Strain == 'REC M' & b$DPI == '7' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
MS7_pos<-na.omit(MS7_pos)
MS7_pos
MS7_pos<-nrow(MS7_pos)
MS7_CI <- data.frame(binconf(MS7_pos,MS7_total))
MS7_CI['Positive'] <- MS7_pos
MS7_CI['Total'] <- MS7_total
Identity <- "MS_7dpi"
MS7_CI['Identity'] <- Identity
MS7_CI
```

```
###REC P 7dpi###
PS7_total <- nrow(b[b$Strain== 'REC P' & b$DPI == '7' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
PS7_pos <- (b[b$Strain == 'REC P' & b$DPI == '7' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
PS7_pos<-na.omit(PS7_pos)
PS7_pos
PS7_pos<-nrow(PS7_pos)
PS7_CI <- data.frame(binconf(PS7_pos,PS7_total))
PS7_CI['Positive'] <- PS7_pos
PS7_CI['Total'] <- PS7_total
Identity <- "PS_7dpi"
PS7_CI['Identity'] <- Identity
PS7_CI

###REC U 10dpi###
US10_total <- nrow(b[b$Strain== 'REC U' & b$DPI == '10' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
US10_pos <- (b[b$Strain == 'REC U' & b$DPI == '10' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
US10_pos<-na.omit(US10_pos)
US10_pos
US10_pos<-nrow(US10_pos)
US10_CI <- data.frame(binconf(US10_pos,US10_total))
US10_CI['Positive'] <- US10_pos
US10_CI['Total'] <- US10_total
Identity <- "US_10dpi"
US10_CI['Identity'] <- Identity
US10_CI

###REC R 10dpi###
RS10_total <- nrow(b[b$Strain== 'REC R' & b$DPI == '10' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
RS10_pos <- (b[b$Strain == 'REC R' & b$DPI == '10' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
RS10_pos<-na.omit(RS10_pos)
RS10_pos
RS10_pos<-nrow(RS10_pos)
RS10_CI <- data.frame(binconf(RS10_pos,RS10_total))
RS10_CI['Positive'] <- RS10_pos
RS10_CI['Total'] <- RS10_total
Identity <- "RS_10dpi"
RS10_CI['Identity'] <- Identity
RS10_CI

###REC M 10dpi###
MS10_total <- nrow(b[b$Strain== 'REC M' & b$DPI == '10' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
MS10_pos <- (b[b$Strain == 'REC M' & b$DPI == '10' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
MS10_pos<-na.omit(MS10_pos)
MS10_pos
MS10_pos<-nrow(MS10_pos)
MS10_CI <- data.frame(binconf(MS10_pos,MS10_total))
MS10_CI['Positive'] <- MS10_pos
MS10_CI['Total'] <- MS10_total
Identity <- "MS_10dpi"
MS10_CI['Identity'] <- Identity
MS10_CI

###REC P 10dpi###
PS10_total <- nrow(b[b$Strain== 'REC P' & b$DPI == '10' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
```

```
PS10_pos <- (b[b$Strain == 'REC P' & b$DPI == '10' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
PS10_pos<-na.omit(PS10_pos)
PS10_pos
PS10_pos<-nrow(PS10_pos)
PS10_CI <- data.frame(binconf(PS10_pos,PS10_total))
PS10_CI['Positive'] <- PS10_pos
PS10_CI['Total'] <- PS10_total
Identity <- "PS_10dpi"
PS10_CI['Identity'] <- Identity
PS10_CI


#combining all into one csv
TR_ZIKVINJ <-
rbind(US5_CI,US7_CI,US10_CI,RS5_CI,RS7_CI,RS10_CI,MS5_CI,MS7_CI,MS10_CI,PS5_CI,PS7_
CI,PS10_CI)
TR_ZIKVINJ <- TR_ZIKVINJ%>% select("Identity", "Positive", "Total", "PointEst", "Lower", "Upper")
#just reorder for ease of reading
TR_ZIKVINJ

###################plotting proportion data##################

Proportion_plot <- read_excel("C:/Users/Owner/Documents/Grant/PhD/Chapters/ZIKV REC
Injections/Data analysis/Proportion_plot_IT.xlsx")
Proportion_plot$DPI<- factor(Proportion_plot$DPI, levels = c("5", "7", "10"))
Proportion_plot$Strain<- factor(Proportion_plot$Strain, levels = c("REC U", "REC R","REC M", "REC
P"))
Proportion_plot$Index<- factor(Proportion_plot$Index, levels = c("Infection", "Dissemination to
heads","Dissemination to legs", "Transmission"))
Proportion_plot$Proportion <- as.numeric(Proportion_plot$Proportion)
Proportion_plot$Lower <- as.numeric(Proportion_plot$Lower)
Proportion_plot$Upper <- as.numeric(Proportion_plot$Upper)

prop <- ggplot(Proportion_plot, aes(x = DPI, y=Proportion, colour=Strain, group=Strain)) +
  geom_point(aes(), shape=19, size=2.0, position=position_dodge(width = 0.75), alpha = 0.9) +
  scale_color_manual(name = "Strain", values=c("#FF9900","#CC0000","#0066CC", "#00CC00")) +
  theme_bw() + facet_grid(~Index)
prop
prop2 <- prop + geom_errorbar(aes(ymin = Lower, ymax = Upper),
                        colour = "black", width = 0.4, linewidth=0.8,
                        position=position_dodge(width = 0.75), alpha = 0.8)
prop3 <-prop2 + geom_point(aes(), shape=19, size=2.0, position=position_dodge(width = 0.75), alpha
= 0.9)
prop4 <- prop3 + theme(panel.grid.major.y = element_line(colour = "#999999", size = 0.2, linetype=3),
                    panel.grid.minor.y = element_line(colour="#999999", size =0.1, linetype=3),
                    panel.background = element_rect(colour = "black", fill="white"),
                    panel.grid.major.x = element_line(colour="#CCCCCC", size =0.2, linetype=3),
                    panel.grid.minor.x = element_line(colour =NA),
                    strip.background = element_rect(colour = "black", fill = "grey"),
                    strip.text.x = element_text(colour = "black", face = "bold", size=9),
                    strip.text.y=element_text(colour="black", face="bold", size=15),
                    axis.title.y = element_text(size = rel(1.25)),
                    axis.title.x = element_text(size=rel(1.25)),
                    axis.text.x = element_text(size=rel(1.25)),
                    axis.text.y = element_text(size=rel(1.25)),
```

```
                legend.title = element_text(face="bold",size=rel(1.1)),
                legend.text = element_text(size=rel(1)),
                legend.position="bottom") +
  scale_y_continuous(breaks=seq(0, 1, 0.20)) + labs(x = "Days post infection (DPI)", y = "Proportion")
 prop4

 ggsave("prop_IT.tiff", prop4, height = 5, width = 7, dpi = 600)

 ###################################fisher's exact tests############################
#TRANSMISSION#
#RvU 5dpi
 RvU5dpiTR <- matrix(c(5,2,30,29), nrow=2)
 fisher.test(RvU5dpiTR) #p=0.4334

#RvM 5dpi
 RvM5dpiTR <- matrix(c(3,2,20,29), nrow=2)
 RvM5dpiTR
 fisher.test(RvM5dpiTR) #p=0.6404

#RvP5dpi
 RvP5dpiTR <- matrix(c(1,2,26,29), nrow=2)
 RvP5dpiTR
 fisher.test(RvP5dpiTR) #p=1

#UvM 5dpi
 UvM5dpiTR <- matrix(c(5,3,30,20), nrow=2)
 UvM5dpiTR
 fisher.test(UvM5dpiTR) #p=1

#PvM 5dpi
 PvM5dpiTR <- matrix(c(3,1,20,26), nrow=2)
 PvM5dpiTR
 fisher.test(PvM5dpiTR) #p=0.3223

#PvU 5dpi
 PvU5dpiTR <- matrix(c(5,1,30,26), nrow=2)
 PvU5dpiTR
 fisher.test(PvU5dpiTR) #p=0.2198

 #****************#

 #RvU 7dpi
 RvU7dpiTR <- matrix(c(11,1,17,25), nrow=2)
 fisher.test(RvU7dpiTR) #p=0.002375 #significant**********

 #RvM 7dpi
 RvM7dpiTR <- matrix(c(12,1,13,25), nrow=2)
 fisher.test(RvM7dpiTR) #p=0.0003167 #significant**********

 #RvP 7dpi
 RvP7dpiTR <- matrix(c(5,1,22,25), nrow=2)
 fisher.test(RvP7dpiTR) #p=0.1917

 #UvM 7dpi
 UvM7dpiTR <- matrix(c(11,12,17,13), nrow=2)
```

```
fisher.test(UvM7dpiTR) #p=0.5862

#PvU 7dpi
PvU7dpiTR <- matrix(c(11,5,17,22), nrow=2)
PvU7dpiTR
fisher.test(PvU7dpiTR) #p=0.1379

#****************#

#RvU 10dpi
RvU10dpiTR <- matrix(c(13,2,18,36), nrow=2)
fisher.test(RvU10dpiTR) #p=0.0003014 #significant**********

#RvM 10dpi
RvM10dpiTR <- matrix(c(2,13,36,12), nrow=2)
fisher.test(RvM10dpiTR) #p=0.3.125e-05 #significant**********

#RvP 10dpi
RvP10dpiTR <- matrix(c(2,5,36,25), nrow=2)
fisher.test(RvP10dpiTR) #p=0.2272

#UvM 10dpi
UvM10dpiTR <- matrix(c(13,13,18,12), nrow=2)
fisher.test(UvM10dpiTR) #p=0.591

#PvU 10dpi
PvU10dpiTR <- matrix(c(13,5,18,25), nrow=2)
PvU10dpiTR
fisher.test(PvU10dpiTR) #p=0.04 #significant*******

#PvM 10dpi
PvM10dpiTR <- matrix(c(5,13,25,12), nrow=2)
PvM10dpiTR
fisher.test(PvM10dpiTR) #P=0.00889 #SIGNIFICANT***



#Plotting Viral titre data#
REC_INJ_Master <- select(REC_INJ_Master,
                         "MosquitoID",
                         "Strain",
                         "Part",
                         "DPI",
                         "log10pfu_sample")

REC_INJ_Master$log10pfu_sample <- as.numeric(REC_INJ_Master$log10pfu_sample)
REC_INJ_Master$DPI<- factor(REC_INJ_Master$DPI, levels = c("5", "7", "10"))
REC_INJ_Master$Strain<- factor(REC_INJ_Master$Strain, levels = c("REC U", "REC R", "REC M",
"REC P"))
REC_INJ_Master$Part <- factor(REC_INJ_Master$Part, levels = c("Bodies", "Heads", "Legs",
"Saliva"))


#creating data sets for no 0 and only 0
```

```
Titre_no0 <- filter(REC_INJ_Master, log10pfu_sample >0) #no 0s for plots calculating means
Titre_no0
Titre_only0 <- filter(REC_INJ_Master, log10pfu_sample ==0) #only 0s to plot on after calculating
means

#creating a dataset that doesn't have the small number of salivas present - allows plotting of errorbars
that aren't massive
Titre_noS_no0 <- Titre_no0 %>% filter(!(Part=='Saliva' & DPI=='5' & Strain =="REC R" |
                                (Part=='Saliva' & DPI=='5' & Strain =="REC P" |
                                 Part=='Saliva' & DPI=='5' & Strain =='REC M' |
                                 Part=='Saliva' & DPI=='7' & Strain =="REC R" |
                                 Part=='Saliva' & DPI=='10'& Strain =='REC R')))

Titre_no0$log10pfu_sample <- as.numeric(Titre_no0$log10pfu_sample)
Titre_no0$DPI<- factor(Titre_no0$DPI, levels = c("5", "7", "10"))
Titre_no0$Part <- factor(Titre_no0$Part, levels = c("Bodies", "Heads","Legs", "Saliva"))
Titre_no0$Strain<-factor(Titre_no0$Strain, levels=c("REC U", "REC R", "REC M", "REC P"))


#factors and levels for only 0
Titre_only0$log10pfu_sample <- as.numeric(Titre_only0$log10pfu_sample)
Titre_only0$DPI<- factor(Titre_only0$DPI, levels = c("5", "7", "10"))
Titre_only0$Part <- factor(Titre_only0$Part, levels = c("Bodies", "Heads","Legs", "Saliva"))
Titre_only0$Strain<-factor(Titre_only0$Strain, levels=c("REC U", "REC R", "REC M", "REC P"))


#factors and levels for no 0 (if you forget this then means can be plotted wrong way around)
Titre_noS_no0$log10pfu_sample <- as.numeric(Titre_noS_no0$log10pfu_sample)
Titre_noS_no0$DPI<- factor(Titre_noS_no0$DPI, levels = c("5", "7", "10"))
Titre_noS_no0$Part <- factor(Titre_noS_no0$Part, levels = c("Bodies", "Heads","Legs", "Saliva"))
Titre_noS_no0$Strain<-factor(Titre_noS_no0$Strain, levels=c("REC U", "REC R", "REC M", "REC
P"))


a <- ggplot(Titre_no0, aes(x = Strain, y=log10pfu_sample, colour=Strain, group=Strain)) +
  geom_point(aes(), size=0.4, shape=19, position=position_jitterdodge(jitter.width= 0.5,
                                                jitter.height=0, dodge.width = 0.5)) +
  scale_color_manual(name = "Strain", values=c("#FF9900","#CC0000","#0066CC", "#00CC00")) +
  facet_grid(DPI~Part)
a1 <- a + theme_bw()
a1
a2 <- a1 + xlab(expression(Days~post-infection~(DPI))) +
  ylab(expression(ZIKV~Titre~(log[10]~pfu/sample)))
a2
a3 <- a2 + theme(panel.grid.major.y = element_line(colour = "#999999", size = 0.2, linetype=3),
                 panel.grid.minor.y = element_line(colour="#999999", size =0.1, linetype=3),
                 panel.background = element_rect(colour = "black", fill="white"),
                 panel.grid.major.x = element_line(colour="#CCCCCC", size =0.2, linetype=3),
                 panel.grid.minor.x = element_line(colour =NA),
                 strip.background = element_rect(colour = "black", fill = "grey"),
                 strip.text.x = element_text(colour = "black", face = "bold", size=10),
                 strip.text.y=element_text(colour="black", face="bold", size=15),
                 axis.title.y = element_text(size = rel(1.25)),
                 axis.title.x = element_text(size=rel(1.25)),
                 axis.text.x = element_text(size=rel(1.25), colour="white"),
```

```
            axis.text.y = element_text(size=rel(1.25)),
            legend.title = element_text(face="bold",size=rel(1.1)),
            legend.text = element_text(size=rel(1)),
            legend.position="bottom") +
            scale_y_continuous(breaks=seq(0, 5 ,1),
            labels = scales::number_format(accuracy = 0.1, decimal.mark = '.'))
 a3

 #I need a dataset that excludes all 0s and saliva samples with <=2 titres
 a4 <- a3 + stat_summary(data=Titre_noS_no0,
                  aes(x=Strain, y=log10pfu_sample),
                  fun.data = "mean_cl_normal",
                  geom="errorbar",
                  color="#000000",
                  width=0.25, size=0.5, alpha=.65,
                  position=position_dodge(width = 0.7)) +
  stat_summary(data=Titre_no0,
           aes(x=Strain, y=log10pfu_sample),
           fun ="mean", geom="point",
           shape=1, color="#000000", size=1,
           position=position_dodge(width=0.7)) #calculating and plotting means from dataset with no 0
titres
 a4

 #need to plot 0s back in

 a5 <- a4 + geom_point(data=Titre_only0, aes(x=Strain, y=log10pfu_sample),
                  size = 0.4, position=position_jitterdodge(jitter.width= 0.5,
                  jitter.height=0, dodge.width = 0.5))
 a5




#Creating table of means and CIs
  Titre <- filter(REC_INJ_Master, log10pfu_sample>0)

  Mean_titre <- Titre %>%
    group_by(Strain, DPI, Part) %>%
    summarise(mean.titre = mean(log10pfu_sample, na.rm = TRUE),
           sd.titre = sd(log10pfu_sample, na.rm = TRUE),
           n.titre = n()) %>%
    mutate(se.titre = sd.titre / sqrt(n.titre),
         lower.ci.titre = mean.titre - qt(1 - (0.05 / 2), n.titre - 1) * se.titre,
         upper.ci.titre = mean.titre + qt(1 - (0.05 / 2), n.titre - 1) * se.titre)
  write.csv(Mean_titre, "C:/Users/Owner/Documents/Grant/PhD/Chapters/ZIKV REC Injections/Data
analysis/Titres/Mean_titre_IT.csv", row.names=F)


  ######CALCULATING MEANS AND DOING ANOVAs######

  REC_INJ_Master <- select(REC_INJ_Master,
                    "MosquitoID",
```

```
                  "Strain",
                  "Part",
                  "DPI",
                  "log10pfu_sample")
###***************###
#BODIES at 5dpi
Bods5 <- filter(REC_INJ_Master, Part =="Bodies" & DPI =="5")
Bods5 <- filter(Bods5, log10pfu_sample >0) #removing 0s from this calculation
Bods5$log10pfu_sample <- as.numeric(Bods5$log10pfu_sample)
shapiro.test(Bods5$log10pfu_sample) # p>0.05 so ANOVA
#significantly different from normal distribution, so need to Kruskal
kruskal.test(Strain~log10pfu_sample, data = Bods5) #no significant difference by strain - don't need
pairwise

#BODIES at 7dpi
Bods7 <- filter(REC_INJ_Master, Part =="Bodies" & DPI =="7")
Bods7 <- filter(Bods7, log10pfu_sample >0) #removing 0s from this calculation
Bods7$log10pfu_sample <- as.numeric(Bods7$log10pfu_sample)
shapiro.test(Bods7$log10pfu_sample) # p<0.05 so ANOVA
# not significantly different from normal distribution, so can aov
aov_Bods7 <- aov(log10pfu_sample ~ Strain, data = Bods7)
summary(aov_Bods7) #strain is significant - need to run Dunn's to work out which comparisons

dunn_Bods7 <- Bods7 %>%
  dunn_test(log10pfu_sample ~ Strain, p.adjust.method = "bonferroni")
dunn_Bods7 #RvU is just not significant after correction for multiple testing 0.0564

#BODIES at 10dpi
Bods10 <- filter(REC_INJ_Master, Part =="Bodies" & DPI =="10")
Bods10 <- filter(Bods10, log10pfu_sample >0) #removing 0s from this calculation
Bods10$log10pfu_sample <- as.numeric(Bods10$log10pfu_sample)
shapiro.test(Bods10$log10pfu_sample) # p<0.05 so ANOVA
# not significantly different from normal distribution, so can aov
aov_Bods10 <- aov(log10pfu_sample ~ Strain, data = Bods10)
summary(aov_Bods10) #strain is not significant



###***************###
#HEADS at 5dpi
Heads5 <- filter(REC_INJ_Master, Part =="Heads" & DPI=="5")
Heads5 <- filter(Heads5, log10pfu_sample >0) #removing 0s from this calculation
Heads5$log10pfu_sample <- as.numeric(Heads5$log10pfu_sample)
shapiro.test(Heads5$log10pfu_sample) # p<0.05 so ANOVA
aov_Heads5 <- aov(log10pfu_sample ~ Strain, data = Heads5)
summary(aov_Heads5)
#Strain not sig

#HEADS at 7dpi
Heads7 <- filter(REC_INJ_Master, Part =="Heads" & DPI=="7")
Heads7 <- filter(Heads7, log10pfu_sample >0) #removing 0s from this calculation
Heads7$log10pfu_sample <- as.numeric(Heads7$log10pfu_sample)
shapiro.test(Heads7$log10pfu_sample) # p<0.05 so ANOVA
aov_Heads7 <- aov(log10pfu_sample ~ Strain, data = Heads7)
summary(aov_Heads7)
```

```
#Strain not sig

#HEADS at 10dpi
Heads10 <- filter(REC_INJ_Master, Part =="Heads" & DPI=="10")
Heads10 <- filter(Heads10, log10pfu_sample >0) #removing 0s from this calculation
Heads10$log10pfu_sample <- as.numeric(Heads10$log10pfu_sample)
shapiro.test(Heads10$log10pfu_sample) # p<0.05 so ANOVA
aov_Heads10 <- aov(log10pfu_sample ~ Strain, data = Heads10)
summary(aov_Heads10)
#Strain not sig


  ###**********###
#LEGS at all time points
Legs <- filter(REC_INJ_Master, Part =="Legs")
Legs <- filter(Legs, log10pfu_sample >0) #removing 0s from this calculation
Legs$log10pfu_sample <- as.numeric(Legs$log10pfu_sample)
shapiro.test(Legs$log10pfu_sample) # p<0.05 so ANOVA
#not significantly different from normal distribution, so can ANOVA them
aov_Legs <- aov(log10pfu_sample ~ Strain + DPI, data = Legs)
summary(aov_Legs) # DPI and strain are significant - need to do pairwise for these :(

  #if i split legs by DPI then I can do Dunn test does multiple pairwise comparisons and corrects for
multiple testing
Legs5 <- filter(REC_INJ_Master, Part =="Legs" & DPI =="5")
dunn_Legs5 <- Legs5 %>%
  dunn_test(log10pfu_sample ~ Strain, p.adjust.method = "bonferroni")
dunn_Legs5
  #P is significantly higher than R (Bonferroni p=0.00665) and U (0.0206) at 5dpi
Legs7 <- filter(REC_INJ_Master, Part =="Legs" & DPI =="7")
dunn_Legs7 <- Legs7 %>%
  dunn_test(log10pfu_sample ~ Strain, p.adjust.method = "bonferroni")
dunn_Legs7
  #no significant differences
Legs10 <- filter(REC_INJ_Master, Part =="Legs" & DPI =="10")
dunn_Legs10 <- Legs10 %>%
  dunn_test(log10pfu_sample ~ Strain, p.adjust.method = "bonferroni")
dunn_Legs10
#M is significantly higher than P (p=0.00608)
#M is significantly higher than U (0.0103)


###**********###

#SALIVA at 7dpi
Saliva7 <- filter(REC_INJ_Master, Part =="Saliva" & DPI =="7")
Saliva7 <- filter(Saliva7, log10pfu_sample >0) #removing 0s from this calculation
Saliva7$log10pfu_sample <- as.numeric(Saliva7$log10pfu_sample)
shapiro.test(Saliva7$log10pfu_sample) # p<0.05 so ANOVA
aov_Saliva7 <- aov(log10pfu_sample ~ Strain, data = Saliva7)
summary(aov_Saliva7)
#strain is significant
dunn_Saliva7 <- Saliva10 %>%
  dunn_test(log10pfu_sample ~ Strain, p.adjust.method = "bonferroni")
dunn_Saliva7
```

```
#not significant after correction for multiple comparisons


#SALIVA at 10dpi
Saliva10 <- filter(REC_INJ_Master, Part =="Saliva" & DPI =="10")
Saliva10 <- filter(Saliva10, log10pfu_sample >0) #removing 0s from this calculation
Saliva10$log10pfu_sample <- as.numeric(Saliva10$log10pfu_sample)
shapiro.test(Saliva10$log10pfu_sample) # p<0.05 so ANOVA
aov_Saliva10 <- aov(log10pfu_sample ~ Strain, data = Saliva10)
summary(aov_Saliva10)
  #strain is significant
dunn_Saliva10 <- Saliva10 %>%
  dunn_test(log10pfu_sample ~ Strain, p.adjust.method = "bonferroni")
dunn_Saliva10
#not significant after correction for multiple comparisons
```

Chapter 5: Can sublethal larval exposure to temephos alter the vector competence of *Aedes aegypti* for Zika virus?

```
SLE_master <- select(SLE_master,
                     "MosquitoID",
                     "Parent_strain",
                     "Part",
                     "Strain_code",
                     "Treatment",
                     "DPI",
                     "log10pfu",
                     "Infection_status")
SLE_master<- na.omit(SLE_master)

bodies <- filter(SLE_master, Part =="Body" )
bodies <- rename(bodies, Body_Titre = log10pfu)
bodies <- select(bodies, MosquitoID, Strain_code, DPI, Body_Titre)
bodies
heads <- filter(SLE_master, Part =="Head")
heads <- rename(heads, Head_Titre = log10pfu)
heads <- select(heads, MosquitoID, Strain_code, DPI, Head_Titre)
heads
saliva <- filter(SLE_master, Part =="Saliva")
```

```
saliva <- rename(saliva, Saliva_Titre = log10pfu)
saliva <- select(saliva, MosquitoID, Strain_code, DPI, Saliva_Titre)
a <-left_join(bodies, heads)
b <- left_join(a, saliva) #makes a dataframe showing all 3 titres by mosquito ID

####################################
#INFECTION PREVALENCE
###UE###
#UEI5dpi
UEIB5_total <- nrow(b[b$Strain_code == 'UEI' & b$DPI == '5' & b$Body_Titre >=0, ])
UEIB5_pos <- nrow(b[b$Strain_code == 'UEI' & b$DPI == '5' & b$Body_Titre >0, ])
UEIB5_CI <- data.frame(binconf(UEIB5_pos,UEIB5_total))
UEIB5_CI['Positive'] <- UEIB5_pos #add in Ns to make it easier
UEIB5_CI['Total'] <- UEIB5_total
Identity <- "UEI_5dpi"
UEIB5_CI['Identity'] <- Identity
UEIB5_CI
#UEI7dpi
UEIB7_total <- nrow(b[b$Strain_code == 'UEI' & b$DPI == '7' & b$Body_Titre >=0, ])
UEIB7_total
UEIB7_pos <- nrow(b[b$Strain_code == 'UEI' & b$DPI == '7' & b$Body_Titre >0, ])
UEIB7_pos
UEIB7_CI <- data.frame(binconf(UEIB7_pos,UEIB7_total))
UEIB7_CI['Positive'] <- UEIB7_pos
UEIB7_CI['Total'] <- UEIB7_total
Identity <- "UEI_7dpi"
UEIB7_CI['Identity'] <- Identity
UEIB7_CI
#UEI14dpi
UEIB14_total <- nrow(b[b$Strain_code == 'UEI' & b$DPI == '14' & b$Body_Titre >=0, ])
UEIB14_total
UEIB14_pos <- nrow(b[b$Strain_code == 'UEI' & b$DPI == '14' & b$Body_Titre >0, ])
UEIB14_pos
UEIB14_CI <- data.frame(binconf(UEIB14_pos,UEIB14_total))
UEIB14_CI['Positive'] <- UEIB14_pos
UEIB14_CI['Total'] <- UEIB14_total
Identity <- "UEI_14dpi"
UEIB14_CI['Identity'] <- Identity
UEIB14_CI
#UEI21dpi
UEIB21_total <- nrow(b[b$Strain_code == 'UEI' & b$DPI == '21' & b$Body_Titre >=0, ])
UEIB21_total
UEIB21_pos <- nrow(b[b$Strain_code == 'UEI' & b$DPI == '21' & b$Body_Titre >0, ])
UEIB21_pos
UEIB21_CI <- data.frame(binconf(UEIB21_pos,UEIB21_total))
UEIB21_CI['Positive'] <- UEIB21_pos
UEIB21_CI['Total'] <- UEIB21_total
Identity <- "UEI_21dpi"
UEIB21_CI['Identity'] <- Identity
UEIB21_CI
IR_UEI <- rbind(UEIB5_CI, UEIB7_CI, UEIB14_CI, UEIB21_CI)
IR_UEI
###UUI###
#UUI5dpi
UUIB5_total <- nrow(b[b$Strain_code == 'UUI' & b$DPI == '5' & b$Body_Titre >=0, ])
UUIB5_total
UUIB5_pos <- nrow(b[b$Strain_code == 'UUI' & b$DPI == '5' & b$Body_Titre >0, ])
UUIB5_pos
UUIB5_CI <- data.frame(binconf(UUIB5_pos,UUIB5_total))
UUIB5_CI['Positive'] <- UUIB5_pos #add in Ns to make it easier
```

```
UUIB5_CI['Total'] <- UUIB5_total
Identity <- "UUI_5dpi"
UUIB5_CI['Identity'] <- Identity
UUIB5_CI
#UUI7dpi
UUIB7_total <- nrow(b[b$Strain_code == 'UUI' & b$DPI == '7' & b$Body_Titre >=0, ])
UUIB7_total
UUIB7_pos <- nrow(b[b$Strain_code == 'UUI' & b$DPI == '7' & b$Body_Titre >0, ])
UUIB7_pos
UUIB7_CI <- data.frame(binconf(UUIB7_pos,UUIB7_total))
UUIB7_CI['Positive'] <- UUIB7_pos
UUIB7_CI['Total'] <- UUIB7_total
Identity <- "UUI_7dpi"
UUIB7_CI['Identity'] <- Identity
UUIB7_CI
#UUI14dpi
UUIB14_total <- nrow(b[b$Strain_code == 'UUI' & b$DPI == '14' & b$Body_Titre >=0, ])
UUIB14_total
UUIB14_pos <- nrow(b[b$Strain_code == 'UUI' & b$DPI == '14' & b$Body_Titre >0, ])
UUIB14_pos
UUIB14_CI <- data.frame(binconf(UUIB14_pos,UUIB14_total))
UUIB14_CI['Positive'] <- UUIB14_pos
UUIB14_CI['Total'] <- UUIB14_total
Identity <- "UUI_14dpi"
UUIB14_CI['Identity'] <- Identity
UUIB14_CI
#UUI21dpi
UUIB21_total <- nrow(b[b$Strain_code == 'UUI' & b$DPI == '21' & b$Body_Titre >=0, ])
UUIB21_total
UUIB21_pos <- nrow(b[b$Strain_code == 'UUI' & b$DPI == '21' & b$Body_Titre >0, ])
UUIB21_pos
UUIB21_CI <- data.frame(binconf(UUIB21_pos,UUIB21_total))
UUIB21_CI['Positive'] <- UUIB21_pos
UUIB21_CI['Total'] <- UUIB21_total
Identity <- "UUI_21dpi"
UUIB21_CI['Identity'] <- Identity
UUIB21_CI
#rbind these together
IR_UUI <- rbind(UUIB5_CI, UUIB7_CI, UUIB14_CI, UUIB21_CI)
IR_UUI
###REI###
#REI5dpi
REIB5_total <- nrow(b[b$Strain_code == 'REI' & b$DPI == '5' & b$Body_Titre >=0, ])
REIB5_pos <- nrow(b[b$Strain_code == 'REI' & b$DPI == '5' & b$Body_Titre >0, ])
REIB5_CI <- data.frame(binconf(REIB5_pos,REIB5_total))
REIB5_CI['Positive'] <- REIB5_pos #add in Ns to make it easier
REIB5_CI['Total'] <- REIB5_total
Identity <- "REI_5dpi"
REIB5_CI['Identity'] <- Identity
REIB5_CI
#REI7dpi
REIB7_total <- nrow(b[b$Strain_code == 'REI' & b$DPI == '7' & b$Body_Titre >=0, ])
REIB7_pos <- nrow(b[b$Strain_code == 'REI' & b$DPI == '7' & b$Body_Titre >0, ])
REIB7_CI <- data.frame(binconf(REIB7_pos,REIB7_total))
REIB7_CI['Positive'] <- REIB7_pos
REIB7_CI['Total'] <- REIB7_total
Identity <- "REI_7dpi"
REIB7_CI['Identity'] <- Identity
REIB7_CI
#REI14dpi
```

```
REIB14_total <- nrow(b[b$Strain_code == 'REI' & b$DPI == '14' & b$Body_Titre >=0, ])
REIB14_pos <- nrow(b[b$Strain_code == 'REI' & b$DPI == '14' & b$Body_Titre >0, ])
REIB14_CI <- data.frame(binconf(REIB14_pos,REIB14_total))
REIB14_CI['Positive'] <- REIB14_pos
REIB14_CI['Total'] <- REIB14_total
Identity <- "REI_14dpi"
REIB14_CI['Identity'] <- Identity
REIB14_CI
#REI21dpi
REIB21_total <- nrow(b[b$Strain_code == 'REI' & b$DPI == '21' & b$Body_Titre >=0, ])
REIB21_pos <- nrow(b[b$Strain_code == 'REI' & b$DPI == '21' & b$Body_Titre >0, ])
REIB21_CI <- data.frame(binconf(REIB21_pos,REIB21_total))
REIB21_CI['Positive'] <- REIB21_pos
REIB21_CI['Total'] <- REIB21_total
Identity <- "REI_21dpi"
REIB21_CI['Identity'] <- Identity
REIB21_CI
#rbind these together
IR_REI <- rbind(REIB5_CI, REIB7_CI, REIB14_CI, REIB21_CI)
IR_REI

###RUI###
#RUI5dpi
RUIB5_total <- nrow(b[b$Strain_code == 'RUI' & b$DPI == '5' & b$Body_Titre >=0, ])
RUIB5_pos <- nrow(b[b$Strain_code == 'RUI' & b$DPI == '5' & b$Body_Titre >0, ])
RUIB5_CI <- data.frame(binconf(RUIB5_pos,RUIB5_total))
RUIB5_CI['Positive'] <- RUIB5_pos #add in Ns to make it easier
RUIB5_CI['Total'] <- RUIB5_total
Identity <- "RUI_5dpi"
RUIB5_CI['Identity'] <- Identity
RUIB5_CI
#RUI7dpi
RUIB7_total <- nrow(b[b$Strain_code == 'RUI' & b$DPI == '7' & b$Body_Titre >=0, ])
RUIB7_pos <- nrow(b[b$Strain_code == 'RUI' & b$DPI == '7' & b$Body_Titre >0, ])
RUIB7_CI <- data.frame(binconf(RUIB7_pos,RUIB7_total))
RUIB7_CI['Positive'] <- RUIB7_pos
RUIB7_CI['Total'] <- RUIB7_total
Identity <- "RUI_7dpi"
RUIB7_CI['Identity'] <- Identity
RUIB7_CI
#RUI14dpi
RUIB14_total <- nrow(b[b$Strain_code == 'RUI' & b$DPI == '14' & b$Body_Titre >=0, ])
RUIB14_pos <- nrow(b[b$Strain_code == 'RUI' & b$DPI == '14' & b$Body_Titre >0, ])
RUIB14_CI <- data.frame(binconf(RUIB14_pos,RUIB14_total))
RUIB14_CI['Positive'] <- RUIB14_pos
RUIB14_CI['Total'] <- RUIB14_total
Identity <- "RUI_14dpi"
RUIB14_CI['Identity'] <- Identity
RUIB14_CI
#RUI21dpi
RUIB21_total <- nrow(b[b$Strain_code == 'RUI' & b$DPI == '21' & b$Body_Titre >=0, ])
RUIB21_pos <- nrow(b[b$Strain_code == 'RUI' & b$DPI == '21' & b$Body_Titre >0, ])
RUIB21_CI <- data.frame(binconf(RUIB21_pos,RUIB21_total))
RUIB21_CI['Positive'] <- RUIB21_pos
RUIB21_CI['Total'] <- RUIB21_total
Identity <- "RUI_21dpi"
RUIB21_CI['Identity'] <- Identity
RUIB21_CI
#rbind these together
IR_RUI <- rbind(RUIB5_CI, RUIB7_CI, RUIB14_CI, RUIB21_CI)
```

```
IR_RUI


##############OVERALL TABLE OF IRS######
IR_master <- rbind(IR_UEI, IR_UUI, IR_REI, IR_RUI)
IR_master <- IR_master%>% select("Identity", "Positive", "Total", "PointEst", "Lower", "Upper") #just reorder for
ease of reading
IR_master
#########################################################################
############Dissemination prevalence to heads###################
#UEI DR
UEIH5_total <- nrow(b[b$Strain_code == 'UEI' & b$DPI == '5' &
                      b$Body_Titre >0 & b$Head_Titre>=0, ])
UEIH5_pos <- (b[b$Strain_code == 'UEI' & b$DPI == '5' &
                    b$Body_Titre >0 & b$Head_Titre>0, ])
UEIH5_pos<-na.omit(UEIH5_pos)
UEIH5_pos
UEIH5_pos<-nrow(UEIH5_pos)
UEIH5_CI <- data.frame(binconf(UEIH5_pos,UEIH5_total))
UEIH5_CI['Positive'] <- UEIH5_pos
UEIH5_CI['Total'] <- UEIH5_total
Identity <- "UEI_5dpi"
UEIH5_CI['Identity'] <- Identity
UEIH5_CI

UEIH7_total <- nrow(b[b$Strain_code == 'UEI' & b$DPI == '7' & b$Body_Titre >0 & b$Head_Titre>=0, ])
UEIH7_pos <- (b[b$Strain_code == 'UEI' & b$DPI == '7' & b$Body_Titre >0 & b$Head_Titre>0, ])
UEIH7_pos<-na.omit(UEIH7_pos)
UEIH7_pos
UEIH7_pos<-nrow(UEIH7_pos)
UEIH7_CI <- data.frame(binconf(UEIH7_pos,UEIH7_total))
UEIH7_CI['Positive'] <- UEIH7_pos #
UEIH7_CI['Total'] <- UEIH7_total
Identity <- "UEI_7dpi"
UEIH7_CI['Identity'] <- Identity
UEIH7_CI

UEIH14_total <- nrow(b[b$Strain_code == 'UEI' & b$DPI == '14' & b$Body_Titre >0 & b$Head_Titre>=0, ])
UEIH14_pos <- (b[b$Strain_code == 'UEI' & b$DPI == '14' & b$Body_Titre >0 & b$Head_Titre>0, ])
UEIH14_pos<-na.omit(UEIH14_pos)
UEIH14_pos<-nrow(UEIH14_pos)
UEIH14_CI <- data.frame(binconf(UEIH14_pos,UEIH14_total))
UEIH14_CI['Positive'] <- UEIH14_pos
UEIH14_CI['Total'] <- UEIH14_total
Identity <- "UEI_14dpi"
UEIH14_CI['Identity'] <- Identity
UEIH14_CI

UEIH21_total <- nrow(b[b$Strain_code == 'UEI' & b$DPI == '21' & b$Body_Titre >0 & b$Head_Titre>=0, ])
UEIH21_pos <- (b[b$Strain_code == 'UEI' & b$DPI == '21' & b$Body_Titre >0 & b$Head_Titre>0, ])
UEIH21_pos<-na.omit(UEIH21_pos)
UEIH21_pos
UEIH21_pos<-nrow(UEIH21_pos)
UEIH21_CI <- data.frame(binconf(UEIH21_pos,UEIH21_total))
UEIH21_CI['Positive'] <- UEIH21_pos
UEIH21_CI['Total'] <- UEIH21_total
Identity <- "UEI_21dpi"
UEIH21_CI['Identity'] <- Identity
UEIH21_CI
```

```
DR_UEI <- rbind(UEIH5_CI, UEIH7_CI, UEIH14_CI, UEIH21_CI)
DR_UEI


#UUI DR
UUIH5_total <- nrow(b[b$Strain_code == 'UUI' & b$DPI == '5' & b$Body_Titre >0 & b$Head_Titre>=0, ])
UUIH5_pos <- (b[b$Strain_code == 'UUI' & b$DPI == '5' & b$Body_Titre >0 & b$Head_Titre>0, ])
UUIH5_pos<-na.omit(UUIH5_pos)
UUIH5_pos
UUIH5_pos<-nrow(UUIH5_pos)
UUIH5_CI <- data.frame(binconf(UUIH5_pos,UUIH5_total))
UUIH5_CI['Positive'] <- UUIH5_pos
UUIH5_CI['Total'] <- UUIH5_total
Identity <- "UUI_5dpi"
UUIH5_CI['Identity'] <- Identity
UUIH5_CI

UUIH7_total <- nrow(b[b$Strain_code == 'UUI' & b$DPI == '7' & b$Body_Titre >0 & b$Head_Titre>=0, ])
UUIH7_pos <- (b[b$Strain_code == 'UUI' & b$DPI == '7' & b$Body_Titre >0 & b$Head_Titre>0, ])
UUIH7_pos
UUIH7_pos<-nrow(UUIH7_pos) #WATCH OUT FOR NAS in saliva but not in heads - na.omit gets rid of all of
them
UUIH7_CI <- data.frame(binconf(UUIH7_pos,UUIH7_total))
UUIH7_CI['Positive'] <- UUIH7_pos #
UUIH7_CI['Total'] <- UUIH7_total
Identity <- "UUI_7dpi"
UUIH7_CI['Identity'] <- Identity
UUIH7_CI

UUIH14_total <- nrow(b[b$Strain_code == 'UUI' & b$DPI == '14' & b$Body_Titre >0 & b$Head_Titre>=0, ])
UUIH14_pos <- (b[b$Strain_code == 'UUI' & b$DPI == '14' & b$Body_Titre >0 & b$Head_Titre>0, ])
UUIH14_pos<-na.omit(UUIH14_pos)
UUIH14_pos
UUIH14_pos<-nrow(UUIH14_pos)
UUIH14_CI <- data.frame(binconf(UUIH14_pos,UUIH14_total))
UUIH14_CI['Positive'] <- UUIH14_pos
UUIH14_CI['Total'] <- UUIH14_total
Identity <- "UUI_14dpi"
UUIH14_CI['Identity'] <- Identity
UUIH14_CI

UUIH21_total <- nrow(b[b$Strain_code == 'UUI' & b$DPI == '21' & b$Body_Titre >0 & b$Head_Titre>=0, ])
UUIH21_pos <- (b[b$Strain_code == 'UUI' & b$DPI == '21' & b$Body_Titre >0 & b$Head_Titre>0, ])
UUIH21_pos<-na.omit(UUIH21_pos)
UUIH21_pos
UUIH21_pos<-nrow(UUIH21_pos)
UUIH21_CI <- data.frame(binconf(UUIH21_pos,UUIH21_total))
UUIH21_CI['Positive'] <- UUIH21_pos
UUIH21_CI['Total'] <- UUIH21_total
Identity <- "UUI_21dpi"
UUIH21_CI['Identity'] <- Identity
UUIH21_CI

DR_UUI <- rbind(UUIH5_CI, UUIH7_CI, UUIH14_CI, UUIH21_CI)
DR_UUI

#REI DR
REIH5_total <- nrow(b[b$Strain_code == 'REI' & b$DPI == '5' & b$Body_Titre >0 & b$Head_Titre>=0, ])
REIH5_pos <- (b[b$Strain_code == 'REI' & b$DPI == '5' & b$Body_Titre >0 & b$Head_Titre>0, ])
REIH5_pos<-na.omit(REIH5_pos)
```

```
REIH5_pos
REIH5_pos<-nrow(REIH5_pos)
REIH5_CI <- data.frame(binconf(REIH5_pos,REIH5_total))
REIH5_CI['Positive'] <- REIH5_pos
REIH5_CI['Total'] <- REIH5_total
Identity <- "REI_5dpi"
REIH5_CI['Identity'] <- Identity
REIH5_CI

REIH7_total <- nrow(b[b$Strain_code == 'REI' & b$DPI == '7' & b$Body_Titre >0 & b$Head_Titre>=0,
])
REIH7_pos <- (b[b$Strain_code == 'REI' & b$DPI == '7' & b$Body_Titre >0 & b$Head_Titre>0, ])
REIH7_pos<-na.omit(REIH7_pos)
REIH7_pos
REIH7_pos<-nrow(REIH7_pos)
REIH7_CI <- data.frame(binconf(REIH7_pos,REIH7_total))
REIH7_CI['Positive'] <- REIH7_pos #
REIH7_CI['Total'] <- REIH7_total
Identity <- "REI_7dpi"
REIH7_CI['Identity'] <- Identity
REIH7_CI

REIH14_total <- nrow(b[b$Strain_code == 'REI' & b$DPI == '14' & b$Body_Titre >0 & b$Head_Titre>=0, ])
REIH14_pos <- (b[b$Strain_code == 'REI' & b$DPI == '14' & b$Body_Titre >0 & b$Head_Titre>0, ])
REIH14_pos<-na.omit(REIH14_pos)
REIH14_pos
REIH14_pos<-nrow(REIH14_pos)
REIH14_CI <- data.frame(binconf(REIH14_pos,REIH14_total))
REIH14_CI['Positive'] <- REIH14_pos
REIH14_CI['Total'] <- REIH14_total
Identity <- "REI_14dpi"
REIH14_CI['Identity'] <- Identity
REIH14_CI

REIH21_total <- nrow(b[b$Strain_code == 'REI' & b$DPI == '21' & b$Body_Titre >0 & b$Head_Titre>=0, ])
REIH21_pos <- (b[b$Strain_code == 'REI' & b$DPI == '21' & b$Body_Titre >0 & b$Head_Titre>0, ])
REIH21_pos<-na.omit(REIH21_pos)
REIH21_pos
REIH21_pos<-nrow(REIH21_pos)
REIH21_CI <- data.frame(binconf(REIH21_pos,REIH21_total))
REIH21_CI['Positive'] <- REIH21_pos
REIH21_CI['Total'] <- REIH21_total
Identity <- "REI_21dpi"
REIH21_CI['Identity'] <- Identity
REIH21_CI

DR_REI <- rbind(REIH5_CI, REIH7_CI, REIH14_CI, REIH21_CI)
DR_REI
#RUI DR
RUIH5_total <- nrow(b[b$Strain_code == 'RUI' & b$DPI == '5' & b$Body_Titre >0 & b$Head_Titre>=0, ])
RUIH5_pos <-(b[b$Strain_code == 'RUI' & b$DPI == '5' & b$Body_Titre >0 & b$Head_Titre>0, ])
RUIH5_pos<-na.omit(RUIH5_pos)
RUIH5_pos
RUIH5_pos<-nrow(RUIH5_pos)
RUIH5_CI <- data.frame(binconf(RUIH5_pos,RUIH5_total))
RUIH5_CI['Positive'] <- RUIH5_pos
RUIH5_CI['Total'] <- RUIH5_total
Identity <- "RUI_5dpi"
RUIH5_CI['Identity'] <- Identity
RUIH5_CI
```

```
RUIH7_total <- nrow(b[b$Strain_code == 'RUI' & b$DPI == '7' & b$Body_Titre >0 & b$Head_Titre>=0, ])
RUIH7_pos <- (b[b$Strain_code == 'RUI' & b$DPI == '7' & b$Body_Titre >0 & b$Head_Titre>0, ])
RUIH7_pos<-na.omit(RUIH7_pos)
RUIH7_pos
RUIH7_pos<-nrow(RUIH7_pos)
RUIH7_CI <- data.frame(binconf(RUIH7_pos,RUIH7_total))
RUIH7_CI['Positive'] <- RUIH7_pos #
RUIH7_CI['Total'] <- RUIH7_total
Identity <- "RUI_7dpi"
RUIH7_CI['Identity'] <- Identity
RUIH7_CI

RUIH14_total <- nrow(b[b$Strain_code == 'RUI' & b$DPI == '14' & b$Body_Titre >0 & b$Head_Titre>=0, ])
RUIH14_pos <- (b[b$Strain_code == 'RUI' & b$DPI == '14' & b$Body_Titre >0 & b$Head_Titre>0, ])
RUIH14_pos<-na.omit(RUIH14_pos)
RUIH14_pos
RUIH14_pos<-nrow(RUIH14_pos)
RUIH14_CI <- data.frame(binconf(RUIH14_pos,RUIH14_total))
RUIH14_CI['Positive'] <- RUIH14_pos
RUIH14_CI['Total'] <- RUIH14_total
Identity <- "RUI_14dpi"
RUIH14_CI['Identity'] <- Identity
RUIH14_CI

RUIH21_total <- nrow(b[b$Strain_code == 'RUI' & b$DPI == '21' & b$Body_Titre >0 & b$Head_Titre>=0, ])
RUIH21_pos <- nrow(b[b$Strain_code == 'RUI' & b$DPI == '21' & b$Body_Titre >0 & b$Head_Titre>0, ])
RUIH21_CI <- data.frame(binconf(RUIH21_pos,RUIH21_total))
RUIH21_CI['Positive'] <- RUIH21_pos
RUIH21_CI['Total'] <- RUIH21_total
Identity <- "RUI_21dpi"
RUIH21_CI['Identity'] <- Identity
RUIH21_CI
DR_RUI <- rbind(RUIH5_CI, RUIH7_CI, RUIH14_CI, RUIH21_CI)
DR_RUI
##############OVERALL TABLE OF DRS#####
DR_master <- rbind(DR_UEI, DR_UUI, DR_REI, DR_RUI)
DR_master <- DR_master%>% select("Identity", "Positive", "Total", "PointEst", "Lower", "Upper")
########################################
#####Prevalence in saliva#############
#UEI
UEIS5_total <- nrow(b[b$Strain_code == 'UEI' & b$DPI == '5' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
UEIS5_pos <- nrow(b[b$Strain_code == 'UEI' & b$DPI == '5' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
UEIS5_CI <- data.frame(binconf(UEIS5_pos,UEIS5_total))
UEIS5_CI['Positive'] <- UEIS5_pos
UEIS5_CI['Total'] <- UEIS5_total
Identity <- "UEI_5dpi"
UEIS5_CI['Identity'] <- Identity
UEIS5_CI
UEIS7_total <- nrow(b[b$Strain_code == 'UEI' & b$DPI == '7' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
UEIS7_total
UEIS7_pos <- (b[b$Strain_code == 'UEI' & b$DPI == '7' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
UEIS7_pos<-na.omit(UEIS7_pos) #can't get filter to omit NAs in saliva titre so doing it another way
UEIS7_pos
UEIS7_pos<-nrow(UEIS7_pos)
UEIS7_CI <- data.frame(binconf(UEIS7_pos,UEIS7_total))
UEIS7_CI['Positive'] <- UEIS7_pos
UEIS7_CI['Total'] <- UEIS7_total
Identity <- "UEI_7dpi"
UEIS7_CI['Identity'] <- Identity
```

```
UEIS7_CI

UEIS14_total <- nrow(b[b$Strain_code == 'UEI' & b$DPI == '14' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
UEIS14_pos <- (b[b$Strain_code == 'UEI' & b$DPI == '14' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
UEIS14_pos<-na.omit(UEIS14_pos)
UEIS14_pos
UEIS14_pos<-nrow(UEIS14_pos)
UEIS14_CI <- data.frame(binconf(UEIS14_pos,UEIS14_total))
UEIS14_CI['Positive'] <- UEIS14_pos
UEIS14_CI['Total'] <- UEIS14_total
Identity <- "UEI_14dpi"
UEIS14_CI['Identity'] <- Identity
UEIS14_CI

UEIS21_total <- nrow(b[b$Strain_code == 'UEI' & b$DPI == '21' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
UEIS21_pos <- (b[b$Strain_code == 'UEI' & b$DPI == '21' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
UEIS21_pos<-na.omit(UEIS21_pos)
UEIS21_pos
UEIS21_pos<-nrow(UEIS21_pos)
UEIS21_CI <- data.frame(binconf(UEIS21_pos,UEIS21_total))
UEIS21_CI['Positive'] <- UEIS21_pos
UEIS21_CI['Total'] <- UEIS21_total
Identity <- "UEI_21dpi"
UEIS21_CI['Identity'] <- Identity
UEIS21_CI

TR_UEI <- rbind(UEIS5_CI, UEIS7_CI, UEIS14_CI, UEIS21_CI)
TR_UEI




#UUI
UUIS5_total <- nrow(b[b$Strain_code == 'UUI' & b$DPI == '5' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
UUIS5_pos <- (b[b$Strain_code == 'UUI' & b$DPI == '5' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
UUIS5_pos<-na.omit(UUIS5_pos)
UUIS5_pos
UUIS5_pos<-nrow(UUIS5_pos)
UUIS5_CI <- data.frame(binconf(UUIS5_pos,UUIS5_total))
UUIS5_CI['Positive'] <- UUIS5_pos
UUIS5_CI['Total'] <- UUIS5_total
Identity <- "UUI_5dpi"
UUIS5_CI['Identity'] <- Identity
UUIS5_CI

UUIS7_total <- nrow(b[b$Strain_code == 'UUI' & b$DPI == '7' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
UUIS7_pos <- (b[b$Strain_code == 'UUI' & b$DPI == '7' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
UUIS7_pos<-na.omit(UUIS7_pos)
UUIS7_pos
UUIS7_pos<-nrow(UUIS7_pos)
UUIS7_CI <- data.frame(binconf(UUIS7_pos,UUIS7_total))
UUIS7_CI['Positive'] <- UUIS7_pos
UUIS7_CI['Total'] <- UUIS7_total
Identity <- "UUI_7dpi"
UUIS7_CI['Identity'] <- Identity
UUIS7_CI

UUIS14_total <- nrow(b[b$Strain_code == 'UUI' & b$DPI == '14' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
UUIS14_pos <- (b[b$Strain_code == 'UUI' & b$DPI == '14' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
```

```
UUIS14_pos<-na.omit(UUIS14_pos)
UUIS14_pos
UUIS14_pos<-nrow(UUIS14_pos)
UUIS14_pos
UUIS14_CI <- data.frame(binconf(UUIS14_pos,UUIS14_total))
UUIS14_CI['Positive'] <- UUIS14_pos
UUIS14_CI['Total'] <- UUIS14_total
Identity <- "UUI_14dpi"
UUIS14_CI['Identity'] <- Identity
UUIS14_CI

UUIS21_total <- nrow(b[b$Strain_code == 'UUI' & b$DPI == '21' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
UUIS21_pos <- (b[b$Strain_code == 'UUI' & b$DPI == '21' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
UUIS21_pos<-na.omit(UUIS21_pos)
UUIS21_pos
UUIS21_pos<-nrow(UUIS21_pos)
UUIS21_CI <- data.frame(binconf(UUIS21_pos,UUIS21_total))
UUIS21_CI['Positive'] <- UUIS21_pos
UUIS21_CI['Total'] <- UUIS21_total
Identity <- "UUI_21dpi"
UUIS21_CI['Identity'] <- Identity
UUIS21_CI

TR_UUI <- rbind(UUIS5_CI, UUIS7_CI, UUIS14_CI, UUIS21_CI)
TR_UUI

#REI
REIS5_total <- nrow(b[b$Strain_code == 'REI' & b$DPI == '5' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
REIS5_pos <- (b[b$Strain_code == 'REI' & b$DPI == '5' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
REIS5_pos<-na.omit(REIS5_pos)
REIS5_pos
REIS5_pos<-nrow(REIS5_pos)
REIS5_CI <- data.frame(binconf(REIS5_pos,REIS5_total))
REIS5_CI['Positive'] <- REIS5_pos
REIS5_CI['Total'] <- REIS5_total
Identity <- "REI_5dpi"
REIS5_CI['Identity'] <- Identity
REIS5_CI

REIS7_total <- nrow(b[b$Strain_code == 'REI' & b$DPI == '7' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
REIS7_pos <- (b[b$Strain_code == 'REI' & b$DPI == '7' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
REIS7_pos<-na.omit(REIS7_pos)
REIS7_pos
REIS7_pos<-nrow(REIS7_pos)
REIS7_CI <- data.frame(binconf(REIS7_pos,REIS7_total))
REIS7_CI['Positive'] <- REIS7_pos
REIS7_CI['Total'] <- REIS7_total
Identity <- "REI_7dpi"
REIS7_CI['Identity'] <- Identity
REIS7_CI

REIS14_total <- nrow(b[b$Strain_code == 'REI' & b$DPI == '14' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
REIS14_pos <- (b[b$Strain_code == 'REI' & b$DPI == '14' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
REIS14_pos<-na.omit(REIS14_pos)
REIS14_pos
REIS14_pos<-nrow(REIS14_pos)
REIS14_CI <- data.frame(binconf(REIS14_pos,REIS14_total))
REIS14_CI['Positive'] <- REIS14_pos
REIS14_CI['Total'] <- REIS14_total
Identity <- "REI_14dpi"
```

```
REIS14_CI['Identity'] <- Identity
REIS14_CI

REIS21_total <- nrow(b[b$Strain_code == 'REI' & b$DPI == '21' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
REIS21_pos <- (b[b$Strain_code == 'REI' & b$DPI == '21' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
REIS21_pos<-na.omit(REIS21_pos)
REIS21_pos
REIS21_pos<-nrow(REIS21_pos)
REIS21_CI <- data.frame(binconf(REIS21_pos,REIS21_total))
REIS21_CI['Positive'] <- REIS21_pos
REIS21_CI['Total'] <- REIS21_total
Identity <- "REI_21dpi"
REIS21_CI['Identity'] <- Identity
REIS21_CI

TR_REI <- rbind(REIS5_CI, REIS7_CI, REIS14_CI, REIS21_CI)
TR_REI

#RUI
RUIS5_total <- nrow(b[b$Strain_code == 'RUI' & b$DPI == '5' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
RUIS5_pos <- (b[b$Strain_code == 'RUI' & b$DPI == '5' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
RUIS5_pos<-na.omit(RUIS5_pos)
RUIS5_pos
RUIS5_pos<-nrow(RUIS5_pos)
RUIS5_pos
RUIS5_CI <- data.frame(binconf(RUIS5_pos,RUIS5_total))
RUIS5_CI['Positive'] <- RUIS5_pos
RUIS5_CI['Total'] <- RUIS5_total
Identity <- "RUI_5dpi"
RUIS5_CI['Identity'] <- Identity
RUIS5_CI

RUIS7_total <- nrow(b[b$Strain_code == 'RUI' & b$DPI == '7' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
RUIS7_pos <- (b[b$Strain_code == 'RUI' & b$DPI == '7' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
RUIS7_pos<-na.omit(RUIS7_pos)
RUIS7_pos
RUIS7_pos<-nrow(RUIS7_pos)
RUIS7_CI <- data.frame(binconf(RUIS7_pos,RUIS7_total))
RUIS7_CI['Positive'] <- RUIS7_pos
RUIS7_CI['Total'] <- RUIS7_total
Identity <- "RUI_7dpi"
RUIS7_CI['Identity'] <- Identity
RUIS7_CI

RUIS14_total <- nrow(b[b$Strain_code == 'RUI' & b$DPI == '14' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
RUIS14_pos <- (b[b$Strain_code == 'RUI' & b$DPI == '14' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
RUIS14_pos<-na.omit(RUIS14_pos)
RUIS14_pos
RUIS14_pos<-nrow(RUIS14_pos)
RUIS14_CI <- data.frame(binconf(RUIS14_pos,RUIS14_total))
RUIS14_CI['Positive'] <- RUIS14_pos
RUIS14_CI['Total'] <- RUIS14_total
Identity <- "RUI_14dpi"
RUIS14_CI['Identity'] <- Identity
RUIS14_CI

#isn't a 21dpi
RUIS21_total <- nrow(b[b$Strain_code == 'RUI' & b$DPI == '21' & b$Body_Titre >0 & b$Saliva_Titre>=0, ])
RUIS21_pos <- nrow(b[b$Strain_code == 'RUI' & b$DPI == '21' & b$Body_Titre >0 & b$Saliva_Titre>0, ])
RUIS21_CI <- data.frame(binconf(RUIS21_pos,RUIS21_total))
```

```
RUIS21_CI['Positive'] <- RUIS21_pos
RUIS21_CI['Total'] <- RUIS21_total
Identity <- "RUI_21dpi"
RUIS21_CI['Identity'] <- Identity
RUIS21_CI

TR_RUI <- rbind(RUIS5_CI, RUIS7_CI, RUIS14_CI, RUIS21_CI)

###############OVERALL TABLE OF IRS#####
TR_master <- rbind(TR_UEI, TR_UUI, TR_REI, TR_RUI)
TR_master <- TR_master%>% select("Identity", "Positive", "Total", "PointEst", "Lower", "Upper")

#########################################################################################################
##########significance testing of proportions###############
####fishers exact tests###########
  # infected and uninfected in contingency
####Infection prevalence#####
#UEIvUUI IR 5dpi
Fisher_U5_IR <- matrix(c(UEIB5_pos, UUIB5_pos, UEIB5_total-UEIB5_pos, UUIB5_total-UUIB5_pos), nrow = 2)
Fisher_U5_IR
fisher.test(Fisher_U5_IR) #not sig
#UEIvUUI IR 7dpi
Fisher_U7_IR <- matrix(c(UEIB7_pos, UUIB7_pos, UEIB7_total-UEIB7_pos, UUIB7_total-UUIB7_pos), nrow = 2)
Fisher_U7_IR
fisher.test(Fisher_U7_IR) #not sig
#UEIvUUI IR 14dpi
Fisher_U14_IR <- matrix(c(UEIB14_pos, UUIB14_pos, UEIB14_total-UEIB14_pos, UUIB14_total-UUIB14_pos),
nrow = 2)
Fisher_U14_IR
fisher.test(Fisher_U14_IR) #p=0.2
#UEIvUUI IR 21dpi
Fisher_U21_IR <- matrix(c(UEIB21_pos, UUIB21_pos, UEIB21_total-UEIB21_pos, UUIB21_total-UUIB21_pos),
nrow = 2)
Fisher_U21_IR
fisher.test(Fisher_U21_IR) #not sig
#REIvRUI IR 5dpi
Fisher_R5_IR <- matrix(c(REIB5_pos, RUIB5_pos, REIB5_total-REIB5_pos, RUIB5_total-RUIB5_pos), nrow = 2)
Fisher_R5_IR
fisher.test(Fisher_R5_IR) #not sig
#REIvRUI IR 7dpi
Fisher_R7_IR <- matrix(c(REIB7_pos, RUIB7_pos, REIB7_total-REIB7_pos, RUIB7_total-RUIB7_pos), nrow = 2)
Fisher_R7_IR
fisher.test(Fisher_R7_IR) #not sig
#REIvRUI IR 14dpi
Fisher_R14_IR <- matrix(c(REIB14_pos, RUIB14_pos, REIB14_total-REIB14_pos, RUIB14_total-RUIB14_pos),
nrow = 2)
Fisher_R14_IR
fisher.test(Fisher_R14_IR) #not sig
#no 21dpi
#########DR#######
#UEIvUUI DR 5dpi
Fisher_U5_DR <- matrix(c(UEIH5_pos, UUIH5_pos, UEIH5_total-UEIH5_pos, UUIH5_total-UUIH5_pos), nrow =
2)
Fisher_U5_DR
fisher.test(Fisher_U5_DR) #not sig
#UEIvUUI DR 7dpi
Fisher_U7_DR <- matrix(c(UEIH7_pos, UUIH7_pos, UEIH7_total-UEIH7_pos, UUIH7_total-UUIH7_pos), nrow =
2)
Fisher_U7_DR
fisher.test(Fisher_U7_DR) #not sig
```

```
#UEIvUUI DR 14dpi
Fisher_U14_DR <- matrix(c(UEIH14_pos, UUIH14_pos, UEIH14_total-UEIH14_pos, UUIH14_total-
UUIH14_pos), nrow = 2)
Fisher_U14_DR
fisher.test(Fisher_U14_DR) #not sig
#UEIvUUI DR 21dpi
Fisher_U21_DR <- matrix(c(UEIH21_pos, UUIH21_pos, UEIH21_total-UEIH21_pos, UUIH21_total-
UUIH21_pos), nrow = 2)
Fisher_U21_DR
fisher.test(Fisher_U21_DR) #not sig
#REIvRUI DR 5dpi
Fisher_R5_DR <- matrix(c(REIH5_pos, RUIH5_pos, REIH5_total-REIH5_pos, RUIH5_total-RUIH5_pos), nrow =
2)
Fisher_R5_DR
fisher.test(Fisher_R5_DR) #P=0.59
#REIvRUI DR 7dpi
Fisher_R7_DR <- matrix(c(REIH7_pos, RUIH7_pos, REIH7_total-REIH7_pos, RUIH7_total-RUIH7_pos), nrow =
2)
Fisher_R7_DR
fisher.test(Fisher_R7_DR) #not sig
#REIvRUI DR 14dpi
Fisher_R14_DR <- matrix(c(REIH14_pos, RUIH14_pos, REIH14_total-REIH14_pos, RUIH14_total-
RUIH14_pos), nrow = 2)
Fisher_R14_DR
fisher.test(Fisher_R14_DR) #not sig
#no 21dpi

########TR#######
#UEIvUUI TR 5dpi
Fisher_U5_TR <- matrix(c(UEIS5_pos, UUIS5_pos, UEIS5_total-UEIS5_pos, UUIS5_total-UUIS5_pos), nrow =
2)
Fisher_U5_TR
fisher.test(Fisher_U5_TR) #not sig
#UEIvUUI TR 7dpi
Fisher_U7_TR <- matrix(c(UEIS7_pos, UUIS7_pos, UEIS7_total-UEIS7_pos, UUIS7_total-UUIS7_pos), nrow =
2)
Fisher_U7_TR
fisher.test(Fisher_U7_TR) #not sig
#UEIvUUI TR 14dpi
Fisher_U14_TR <- matrix(c(UEIS14_pos, UUIS14_pos, UEIS14_total-UEIS14_pos, UUIS14_total-UUIS14_pos),
nrow = 2)
Fisher_U14_TR
fisher.test(Fisher_U14_TR) #P=0.11
#UEIvUUI TR 21dpi
Fisher_U21_TR <- matrix(c(UEIS21_pos, UUIS21_pos, UEIS21_total-UEIS21_pos, UUIS21_total-UUIS21_pos),
nrow = 2)
Fisher_U21_TR
UEIS21_pos
UUIS21_pos
UEIS21_total
UUIS21_total
fisher.test(Fisher_U21_TR) #P=0.62
#REIvRUI TR 5dpi
Fisher_R5_TR <- matrix(c(REIS5_pos, RUIS5_pos, REIS5_total-REIS5_pos, RUIS5_total-RUIS5_pos), nrow =
2)
Fisher_R5_TR
fisher.test(Fisher_R5_TR) #not sig
#REIvRUI TR 7dpi
Fisher_R7_TR <- matrix(c(REIS7_pos, RUIS7_pos, REIS7_total-REIS7_pos, RUIS7_total-RUIS7_pos), nrow =
2)
```

```
Fisher_R7_TR
fisher.test(Fisher_R7_TR) #not sig
#REIvRUI TR 14dpi
Fisher_R14_TR <- matrix(c(REIS14_pos, RUIS14_pos, REIS14_total-REIS14_pos, RUIS14_total-RUIS14_pos),
nrow = 2)
Fisher_R14_TR
fisher.test(Fisher_R14_TR) #0.203
#no 21dpi


#*********************************************************************************************#
#normality and significance testing of titre data#
#*********************************************************************************************#
SLE_master <- filter(SLE_master, Strain_code == "REI"|Strain_code == "RUI"|Strain_code=="UEI"|
Strain_code=="UUI")
SLE_master
Titre <- SLE_master %>% select("SampleID",
                               "Parent_strain",
                               "Strain_code",
                               "Part",
                               "DPI",
                               "log10pfu",
                               "Treatment")

Titre$log10pfu <- as.numeric(Titre$log10pfu)
###REC U###
#bodies at 5dpi
UB5 <- filter(Titre, Strain_code == "UUI"| Strain_code == "UEI")
UB5 <- filter(UB5, Part =="Body" & DPI =="5")
UB5 <- filter(UB5, log10pfu >0) #removing 0s from this calculation
UEB5 <- filter(UB5, Strain_code =="UEI")
UUB5 <- filter(UB5, Strain_code =="UUI")
shapiro.test(UEB5$log10pfu) # p<0.05
shapiro.test(UUB5$log10pfu) # p<0.05
aov_UB5 <- aov(log10pfu ~ Treatment, data = UB5)
summary(aov_UB5) #
#bodies 7dpi
UB7 <- filter(Titre, Strain_code == "UUI"| Strain_code == "UEI")
UB7 <- filter(UB7, Part =="Body" & DPI =="7")
UB7 <- filter(UB7, log10pfu >0)
UEB7 <- filter(UB7, Strain_code =="UEI")
UUB7 <- filter(UB7, Strain_code =="UUI")
shapiro.test(UEB7$log10pfu) # p>0.05
shapiro.test(UUB7$log10pfu) # p>0.05 need Kruskal
kruskal.test(log10pfu~Treatment, data = UB7) #p=0.72

#bodies 14dpi
UB14 <- filter(Titre, Strain_code == "UUI"| Strain_code == "UEI")
UB14 <- filter(UB14, Part =="Body" & DPI =="14")
UB14 <- filter(UB14, log10pfu >0)
UEB14 <- filter(UB14, Strain_code =="UEI")
UUB14 <- filter(UB14, Strain_code =="UUI")
shapiro.test(UEB14$log10pfu) # p>0.05 kruskal
shapiro.test(UUB14$log10pfu) #p=0.05
kruskal.test(log10pfu~Treatment, data = UB14) #p=0.90
#bodies 21
UB21 <- filter(Titre, Strain_code == "UUI"| Strain_code == "UEI")
UB21 <- filter(UB21, Part =="Body" & DPI =="21")
UB21 <- filter(UB21, log10pfu >0)
UEB21 <- filter(UB21, Strain_code =="UEI")
```

```
UUB21 <- filter(UB21, Strain_code =="UUI")
shapiro.test(UEB21$log10pfu) # p<0.05
shapiro.test(UUB21$log10pfu) #p=>0.05
kruskal.test(log10pfu~Treatment, data = UB21) #p = 0.16

#####R bodies####
#bodies 5dpi
RB5 <- filter(Titre, Strain_code == "RUI"| Strain_code == "REI")
RB5 <- filter(RB5, Part =="Body" & DPI =="5")
RB5 <- filter(RB5, log10pfu >0)
REB5 <- filter(RB5, Strain_code =="REI")
RUB5 <- filter(RB5, Strain_code =="RUI")
shapiro.test(REB5$log10pfu) # p>0.05
shapiro.test(RUB5$log10pfu) # p>0.05 Kruskal
kruskal.test(log10pfu~Treatment, data = RB5) #p=0.07

#bodies 7dpi#
RB7 <- filter(Titre, Strain_code == "RUI"| Strain_code == "REI")
RB7 <- filter(RB7, Part =="Body" & DPI =="7")
RB7 <- filter(RB7, log10pfu >0)
REB7 <- filter(RB7, Strain_code =="REI")
RUB7 <- filter(RB7, Strain_code =="RUI")
shapiro.test(REB7$log10pfu) # p>0.05
shapiro.test(RUB7$log10pfu) # p>0.05 Kruskal
kruskal.test(log10pfu~Treatment, data = RB7) #*p=0.01*****
#bodies 14dpi#
RB14 <- filter(Titre, Strain_code == "RUI"| Strain_code == "REI")
RB14 <- filter(RB14, Part =="Body" & DPI =="14")
RB14 <- filter(RB14, log10pfu >0)
REB14 <- filter(RB14, Strain_code =="REI")
RUB14 <- filter(RB14, Strain_code =="RUI")
shapiro.test(REB14$log10pfu) # p>0.05
shapiro.test(RUB14$log10pfu) # p<0.05 n
kruskal.test(log10pfu~Treatment, data = RB14) #*p=0.81

#can't do 21dpi as no RU
######
##REC U heads##
#5dpi#
UH5 <- filter(Titre, Strain_code == "UUI"| Strain_code == "UEI")
UH5 <- filter(UH5, Part =="Head" & DPI =="5")
UH5 <- filter(UH5, log10pfu >0)
UEH5 <- filter(UH5, Strain_code =="UEI")
UUH5 <- filter(UH5, Strain_code =="UUI")
shapiro.test(UEH5$log10pfu) # p=0.05
shapiro.test(UUH5$log10pfu) # p<0.05
aov_UH5 <- aov(log10pfu ~ Treatment, data = UH5)
summary(aov_UH5) #p=0.97
#7dpi#
UH7 <- filter(Titre, Strain_code == "UUI"| Strain_code == "UEI")
UH7 <- filter(UH7, Part =="Head" & DPI =="7")
UH7 <- filter(UH7, log10pfu >0)
UEH7 <- filter(UH7, Strain_code =="UEI")
UUH7 <- filter(UH7, Strain_code =="UUI")
shapiro.test(UEH7$log10pfu) # p<0.05
shapiro.test(UUH7$log10pfu) # p<0.05 ANOVA
aov_UH7 <- aov(log10pfu ~ Treatment, data = UH7)
summary(aov_UH7) #p=0.75
#14dpi#
UH14 <- filter(Titre, Strain_code == "UUI"| Strain_code == "UEI")
```

```
UH14<- filter(UH14, Part =="Head" & DPI =="14")
UH14 <- filter(UH14, log10pfu >0)
UEH14 <- filter(UH14, Strain_code =="UEI")
UUH14 <- filter(UH14, Strain_code =="UUI")
shapiro.test(UEH14$log10pfu) # p<0.05
shapiro.test(UUH14$log10pfu) # p<0.05
aov_UH14 <- aov(log10pfu ~ Treatment, data = UH14)
summary(aov_UH14) #p=0.31

#21dpi#
UH21 <- filter(Titre, Strain_code == "UUI"| Strain_code == "UEI")
UH21<- filter(UH21, Part =="Head" & DPI =="21")
UH21 <- filter(UH21, log10pfu >0)
UEH21 <- filter(UH21, Strain_code =="UEI")
UUH21 <- filter(UH21, Strain_code =="UUI")
shapiro.test(UEH21$log10pfu) # p<0.05
shapiro.test(UUH21$log10pfu) # p>0.05 Kruskal
kruskal.test(log10pfu~Treatment, data = UH21) # p=0.89

##########
##REC R heads##
#5dpi#
RH5 <- filter(Titre, Strain_code == "RUI"| Strain_code == "REI")
RH5 <- filter(RH5, Part =="Head" & DPI =="5")
RH5 <- filter(RH5, log10pfu >0)
REH5 <- filter(RH5, Strain_code =="REI")
RUH5 <- filter(RH5, Strain_code =="RUI")
shapiro.test(REH5$log10pfu) # p>0.05
shapiro.test(RUH5$log10pfu) # p>0.05 need Kruskal
kruskal.test(log10pfu~Treatment, data = RH5) #p=0.288



#7dpi#
RH7 <- filter(Titre, Strain_code == "RUI"| Strain_code == "REI")
RH7 <- filter(RH7, Part =="Head" & DPI =="7")
RH7 <- filter(RH7, log10pfu >0)
REH7 <- filter(RH7, Strain_code =="REI")
RUH7 <- filter(RH7, Strain_code =="RUI")
shapiro.test(REH7$log10pfu) # p>0.05
shapiro.test(RUH7$log10pfu) # p>0.05 need Kruskal
kruskal.test(log10pfu~Treatment, data = RH7) #p=0.74

#14dpi#
RH14 <- filter(Titre, Strain_code == "RUI"| Strain_code == "REI")
RH14 <- filter(RH14, Part =="Head" & DPI =="14")
RH14 <- filter(RH14, log10pfu >0)
REH14 <- filter(RH14, Strain_code =="REI")
RUH14 <- filter(RH14, Strain_code =="RUI")
shapiro.test(REH14$log10pfu) # p>0.05
shapiro.test(RUH14$log10pfu) # p<0.05 need Kruskal
kruskal.test(log10pfu~Treatment, data = RH14) #p=0.22

no 21dpi

#########REC U saliva
##14dpi only one with enough data points
US14 <- filter(Titre, Strain_code == "UUI"| Strain_code == "UEI")
US14 <- filter(US14, Part =="Saliva" & DPI =="14")
US14 <- filter(US14, log10pfu >0)
```

```
UES14 <- filter(US14, Strain_code =="UEI")
UUS14 <- filter(US14, Strain_code =="UUI")
shapiro.test(UES14$log10pfu) # p>0.05
shapiro.test(UUS14$log10pfu) # p<0.05 Kruskal
kruskal.test(log10pfu~Treatment, data = US14) #p=0.051 *

######################Tables of means and CIs##########
install.packages("Hmisc")
library("Hmisc")
Titre$log10pfu <- as.numeric(Titre$log10pfu)
Titre$DPI<- factor(Titre$DPI, levels = c("5", "7", "14", "21"))
Titre$Treatment<- factor(Titre$Treatment, levels = c("Unexposed", "Exposed"))
Titre$Part <- factor(Titre$Part, levels = c("Body", "Head", "Saliva"))
Titre$Parent_strain<-factor(Titre$Parent_strain, levels=c("REC-U", "REC-R"))
Titre <- filter(Titre, log10pfu>0)
Mean_titre <- Titre %>%
  group_by(Strain_code, DPI, Treatment, Part) %>%
  summarise(mean.titre = mean(log10pfu, na.rm = TRUE),
            sd.titre = sd(log10pfu, na.rm = TRUE),
            n.titre = n()) %>%
  mutate(se.titre = sd.titre / sqrt(n.titre),
         lower.ci.titre = mean.titre - qt(1 - (0.05 / 2), n.titre - 1) * se.titre,
         upper.ci.titre = mean.titre + qt(1 - (0.05 / 2), n.titre - 1) * se.titre)
```