Reproductive interference between *Aedes aegypti* and *Aedes albopictus*, and its relevance to vector control techniques

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For my mum and dad.

Abstract

Reproductive interference is where heterospecifics engage in mating activities which do not produce viable offspring and cause a fitness cost to at least one of the species involved. Reproductive interference occurs across a wide range of taxa, including between the mosquito species *Aedes aegypti* and *Aedes albopictus*. These species are primary vectors of dengue, a flavivirus with no vaccine or specific treatment: thus, vector control is key to reducing its public health burden. Pioneering control methods rely on the release of modified mosquitoes, which deplete the wild population or replace it with mosquitoes that cannot transmit dengue. During the development of novel techniques, population dynamic frameworks are required to evaluate their efficacy. However, these models frequently omit important ecological interactions, including reproductive interference. In this thesis, I use a theoretical model to examine the combined effect of the release of modified *Ae. aegypti*, and reproductive interference, on coexistence between *Ae. aegypti* and *Ae. albopictus*. I find that the release ratio of self-limiting *Ae. aegypti* and the strength of reproductive interference act together to determine the population size of each species, and whether stable coexistence can occur. This highlights the necessity to further our understanding of reproductive interference between *Ae. aegypti* and *Ae. albopictus*. In the subsequent chapters, I do this by conducting laboratory studies examining heterospecific insemination rates and mating behaviour. In these experiments I observe that *Ae. albopictus* males do not inseminate *Ae. aegypti* females, even those from strains with no prior contact with *Ae. albopictus*. This contradicts the findings in previous literature, where sympatric *Ae. aegypti* females have evolved resistance to mating by *Ae. albopictus* males, and allopatric *Ae. aegypti* females are susceptible. Behavioural analysis of these strains suggests that *Ae.*

albopictus males may not attempt to mate with these females. To understand this discrepancy further, and general heterogeneity in reproductive interference, I conduct a systematic literature review of reproductive interference between *Aedes spp*. While I find clear predictors of heterospecific insemination rates, there is also considerable unexplained variation, and key differences in experimental methods. This thesis highlights that the strength of reproductive interference impacts the efficacy of vector control techniques and varies greatly between populations of *Ae. aegypti* and *Ae. albopictus*. I suggest that standardised methods to determine the rate of heterospecific insemination should be developed, so that the validity of reproductive interference can be readily parametrised.

Declaration of Originality

I declare that all work in this thesis is my own, under the **supervision** of **Professor Mike Bonsall** and **Dr Simon Gubbins** and with guidance from **Dr Lauren Cator,** except where stated. I wrote the entirety of this thesis, which was edited to incorporate comments from Professor Mike Bonsall, Dr Simon Gubbins, and Dr Lauren Cator. Furthermore, I designed all experiments and conducted all data processing and analysis. However some students aided data collection, as detailed below.

Chenrui Zhang, a master's student supervised by Dr Lauren Cator, aided me with the collection of behavioural data for ***Chapter 4*** of this thesis.

Helen Micheal Youssef Moussa, an intern supervised by Professor Mike Bonsall and me, aided me with the literature search in ***Chapter 5*** of this thesis.

Further details of the contributions of Helen and Chenrui are detailed in the *Methods* sections of the relevant chapters.

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Contents

Chapter 1: Introduction

1. Overview

Due to the diseases they transmit, mosquitoes are the most dangerous animal in the world (CDC, 2023). Most notably, *Anopheles* mosquitoes transmit Malaria, a parasitic infection which causes 219 million cases and 400,000 deaths per year (World Health Organization, 2023), and *Aedes* mosquitoes transmit dengue, a flavivirus that infects approximately 100-390 million people per year (Medlock et al., 2012) and causes 40,000 deaths per year (World Health Organization, 2023). Mosquitoes, therefore, pose a substantial public health threat, currently concentrated in tropical and subtropical areas (World Health Organization, 2023).

The spatial range of mosquitoes, however, is predicted to increase due to international travel and urbanisation (Colón-González et al., 2021). International travel can unintentionally introduce mosquitoes into new areas, giving them the potential to establish. In *Aedes* mosquitos, this often occurs by the transportation of eggs during the international trade of used tires (Reiter & Sprenger, 1987). Urbanisation causes this on a smaller scale, by increased local movement of people and goods (Colón-González et al., 2021).

Furthermore, rises in the global mean temperature are predicted to increase the incidence of many mosquito-borne diseases (Mordecai et al., 2019). As mosquitoes are small poikilotherms, they are sensitive to ambient temperature throughout their lifecycle (Beck-Johnson et al., 2017). Because of this, mosquitoes have lower and upper thermal limits in

which they can survive, and their development rate and reproduction depends upon ambient temperature (Reinhold, Lazzari & Lahondère, 2018). It is predicted that rises in the mean global temperature will allow mosquitoes to establish at higher altitudes and in more temperate regions, but reduce their ability to persist in warmer regions (Colón-González et al., 2021; Ryan et al., 2019).

This increase in global temperature is also predicted to increase pathogen spread, by increasing vector competence and altering pathogen life-history. Increased environmental temperatures can decrease the extrinsic incubation time, the length of time from the vector ingesting the pathogen to being infectious and able to transmit the pathogen, which increases the rate of spread of infection (Trejo et al., 2023; Biggerstaff et al., 2010). Additionally, increases in average global temperatures can increase vector survival, reproduction, biting rate and the length of transmission seasons; this is true for *Ae. aegypti* and *Ae. albopictus* (Messina et al., 2019; Gubler, 2011; Wilder-Smith, Murray & Quam, 2013). In *Ae. aegypti* and *Ae. albopictus*, the changes in *Aedes* spatial distribution, and increases in their competency to spread disease are predicted to cause a net increase in the transmission of *Aedes-borne* viruses (Ryan et al., 2019).

In this thesis, I will focus on *Aedes aegypti* and *Aedes albopictus*, the two primary vectors of dengue and thus mosquito species of high public health concern. Both species are invasive, with *Ae. albopictus* identified as (one of) the most invasive mosquito species in the world (Benedict et al., 2007). Their geographic ranges are expanding rapidly, and both species are already present on all continents, except the Antarctica (Kraemer et al., 2015a, 2015b), resulting in an overlap of their spatial distributions (Lounibos & Juliano, 2018). One trait that

contributes to their invasiveness is their ability to form highly desiccation resistant eggs (Urbanski *et al.*, 2010; Diniz *et al.*, 2017; Mayilsamy, 2019), which can survive dry for over 2 months, and retain an 80% hatch rate (Zheng et al., 2015). This allows eggs to be transported worldwide, and for populations to establish in new locations.

Ae. aegypti and *Ae. albopictus* populations benefit from global increases in urbanisation (Government Office for Science, 2021), as both species can oviposit in artificial containers (as summarised in Egid *et al.*, 2022), and are anthropophilic. Thus, urbanisation increases the number of oviposition sites they can exploit (Li et al., 2014), and the likelihood they will interact with humans to take a blood meal (Colón-González et al., 2021), resulting in an increase in *Aedes* population size and disease transmission (Kolimenakis et al., 2021).

Dengue is currently a global public health and economic burden (Shepard et al., 2016), and the magnitude of this is likely to increase. Messina *et al.* (2019) predicted that 2.25 billion more people will be at risk of dengue by 2080 compared to 2015. As there is no general vaccine for dengue or specific treatment, preventative methods are relied upon to decrease its spread. This includes pioneering methods to control the population sizes of *Ae. aegypti* and *Ae. albopictus*.

2. Methods to Control *Ae. aegypti* and *Ae. albopictus* Populations

2.1. Insecticides

Insecticides are a long-standing method for reducing *Aedes* populations. They are often used to target and kill adult *Aedes* through three key processes: coating internal walls and ceilings with long-lasting insecticide (indoor residual spraying), mass fogging, and use of household insecticides (Gan et al., 2021). However, *Aedes* have evolved to resist insecticides, either by directly detoxifying them (Bariami et al., 2012), or evading their effects, via mutations in the insecticide target protein (Liu, 2015; Hemingway et al., 1989).

Resistance to insecticides is now a global problem, with *Aedes* populations in North America (Kandel et al., 2019), South America (Maciel-de-Freitas et al., 2014; Yakob & Walker, 2016), Africa (Sene et al., 2021; Toé et al., 2022) and Asia (Jangir & Prasad, 2022) having insecticide resistance. Therefore, while insecticides are still extensively used (Gan et al., 2021; Garcia et al., 2018), widespread resistance may prevent them from being a long-term solution to controlling *Aedes* populations.

As well as this, there has been increased knowledge of the negative impacts of insecticides on wider biodiversity (Boyce et al., 2007; Ristyadi, Andrew & Waugh, 2013; Weston et al., 2005; Hua & Relyea, 2019) and human health (Koureas et al., 2012; Radwan et al., 2015), and increased concern of the general public (as summarised in United Nations Environment

Programme, 2021). Together with insecticide resistance, this has resulted in a growing imperative to develop new control techniques.

2.2. Novel Techniques

Pioneering methods of mosquito control are based on modifying mosquitoes and releasing them into the wild, to mate with wild mosquitoes. These techniques can either be based on reducing the size of the mosquito population (population suppression), or reducing the ability of mosquitoes to spread disease (population replacement), and can have self-limiting or self-sustaining dynamics (summarised in Alphey, 2014). Following release, in self-limiting strategies insects decrease in abundance through time, whereas in self-sustaining strategies insects persist over time and in some instances increase in frequency over generations and establish in other populations (Alphey, 2014).

There are multiple examples of self-limiting and self-sustaining techniques that have been proposed to control *Ae. aegypti* populations (as summarised in Alphey *et al.*, 2013). Self-limiting systems include the Sterile Insect Technique (SIT), and the Release of Insects with a Dominant Lethal (RIDL^{TM}) (Toé et al., 2022). Both are population suppression techniques that rely on the release of modified males, that mate with wild females to produce non-viable offspring. In SIT, modification occurs by the irradiation of males, and in RIDL by the genetic modification of males to be homozygous for a dominant lethal genetic construct (Toé et al., 2022; Alphey et al., 2013). Self-sustaining techniques include synthetic *gene-drive systems* (Frieß et al., 2023), and infecting *Aedes* with *Wolbachia* bacteria (Hancock, Sinkins & Godfray, 2011). Synthetic gene-drive systems can either cause population suppression or

population replacement (Min et al., 2018), while infection with *Wolbachia* causes population replacement (Lu et al., 2012; Moreira et al., 2009).

During the development of pioneering vector control methods, mathematical models are formed to evaluate their efficacy. Specifically, theoretical models are formed that predict the dynamic spread of the modified mosquitoes (for example, Atkinson *et al.*, 2007; Beaghton, Beaghton and Burt, 2016), which allow comparisons to be made between techniques (for example, Phuc *et al.*, 2007; Seirin Lee *et al.*, 2013). These models generally focus on the specific details of the control technique (for instance, details of the genetic mechanism), and only include basic life-history details (for instance, birth rate) and intraspecific interactions (for instance, intraspecific competition) of the target vector. The omission of interspecific interactions could result in inaccurate predictions of the efficacy of vector control methods, particularly where the population dynamics of multiple species are strongly coupled.

2.3. Interspecific Interactions between *Ae. aegypti* and *Ae. albopictus*

While *Ae. aegypti* originates in North Africa (Powell & Tabachnick, 2013) and *Ae. albopictus* in the tropical forests of South East Asia (Gratz, 2004), range expansion has resulted in frequent overlap of their spatial distribution, especially in tropical and mild temperate regions (Lounibos & Juliano, 2018). *Ae. aegypti* and *Ae. albopictus* have overlapping realised ecological niches as they are both anthropophilic, thrive in urban and suburban settings, oviposit their eggs above the waterline in natural and artificial containers (as summarised in Egid *et al.*, 2022) and mate in swarms around hosts (Nelson, 1986; Yuval, 2006). Because of this, where the two species come into contact their population dynamics are strongly

coupled, causing strong interspecific interactions (Lounibos and Juliano, 2018). This results in either competitive exclusion, or stable coexistence (Lounibos & Juliano, 2018).

2.3.1. Competition between *Ae. aegypti* and *Ae. albopictus*

A key interspecific interaction mediating coexistence between *Ae. aegypti* and *Ae. albopictus*, is resource competition at the larval stage. This occurs as both species lay their eggs in natural and artificial containers, resulting in larvae sharing resources.

In a meta-analysis, Juliano (2010) found that the relative effects of interspecific larval resource competition are context dependent. When low quality larval food (detritus from deciduous or coniferous tree leaves) was supplied, *Ae. albopictus* larvae survived significantly longer than *Ae. aegypti* larvae. Meanwhile when high quality food (animal material, yeast or grass) was supplied, both species of larvae were competitively equivalent. These results were consistent with field observations in Florida; Murrell *et al.* (2011) found that *Ae. aegypti* populations persist where the nitrogen content of detritus is high but are not present in areas with a lower nitrogen content. Therefore, the food available determines the outcome of larval competition, with *Ae. albopictus* having a competitive advantage in less favourable environments.

2.3.2. Reproductive interference

Reproductive interference is another important interspecific interaction that occurs between *Ae. aegypti* and *Ae. albopictus*, and has been reported in both field (for example, Frederic Tripet *et al.*, 2011; Bargielowski *et al.*, 2015) and laboratory (for example, Bargielowski, Lounibos and Carrasquilla, 2013; Marcela *et al.*, 2015) studies. Reproductive interference is

where individuals from different species engage in mating activities, which do not result in the production of viable offspring and cause a fitness cost to one or both of the species involved (Gröning & Hochkirch, 2008; Shuker & Burdfield-Steel, 2017). As *Ae. aegypti* and *Ae. albopictus* are exophagic, mate in swarms around (preferentially) human hosts (Nelson, 1986; Yuval, 2006; Egid et al., 2022), and have similar peak mating activity times (Manzambi et al., 2023), *Ae. aegypti* and *Ae. albopictus* come into contact during mating, which allows reproductive interference to occur.

In *Ae. aegypti* and *Ae. albopictus* the costs associated with reproductive interference are incurred directly by female harassment, reduction in fertility and gamete wastage and, indirectly, by the wasted time and energy from mismating (Gröning & Hochkirch, 2008; Paton & Bonsall, 2019). Thus, reproductive interference differs from competition as the fitness costs do not result from shared, finite resources (Paton & Bonsall, 2019).

Reproductive interference has a significant impact on population dynamics: theoretical studies have shown that where two species coexist, reproductive interference is more likely to cause exclusion than interspecific resource competition (Kuno, 1992; Kishi & Nakazawa, 2013). I further explore the significance of reproductive interference in *Chapter 2*, where I assess the importance of including reproductive interference into models of vector control techniques.

Reproductive interference has an asymmetric effect on *Ae. aegypti* and *Ae. albopictus* populations. During conspecific mating, males transfer male accessory proteins (Acps) to females, making them refractory to further mating (Robbins et al., 2011). However, Acps

from male *Ae. albopictus* also cause *Ae. aegypti* females to become refractory to further mating (Leahy & Craig Jr., 1965; Robbins et al., 2011). However, Acps from male *Ae. aegypti* do not induce refractory behaviour in *Ae. albopictus* females and *Ae. albopictus* females can re-mate following insemination by *Ae. aegypti* males (Leahy & Craig Jr., 1965; Robbins et al., 2011). Furthermore, reproductive interference occurs at a higher rate in *Ae. aegypti* females than *Ae. albopictus* females (Zhou et al., 2022). Therefore, the frequency of heterospecific mating and the associated direct fitness costs are much greater in *Ae. aegypti* than *Ae. albopictus*.

However, *Ae. aegypti* females can evolve to avoid these substantial fitness costs, following exposure to *Ae. albopictus* males. Bargielowski, Lounibos and Carrasquilla (2013) found that *Ae. aegypti* from populations sympatric with *Ae. albopictus* were significantly less likely than nearby allopatric populations to mate with heterospecific males, suggesting that *Ae. aegypti* females can alter their behaviour to resist heterospecific insemination. In this context, and throughout this thesis, resistance refers to any type of behaviour or signalling that results in a decrease in the rate of heterospecific insemination. Behavioural resistance includes active (for example, kicking a male away), and more passive (for example, avoiding contact with a male) behaviours. I further explore this in *Chapter 3*, where I use strains of *Ae. aegypti* that have not previously been characterised in the laboratory and determine if there is resistance to heterospecific mating in sympatric strains of *Ae. aegypti*, but not allopatric strains of *Ae. aegypti*.

In a later experiment, Bargielowski and Lounibous (2014) confirmed the evolution of resistance to heterospecific mating in *Ae. aegypti* females. In this experiment, strains of *Ae.*

aegypti females with no prior contact with *Ae. albopictus* were exposed to *Ae. albopictus* males for multiple generations. Following 1-3 generations of exposure to *Ae. albopictus* males, the rate of heterospecific insemination of *Ae. aegypti* females decreased, suggesting that females can rapidly evolve to evade heterospecific mating. In *Chapter 4*, I characterise the mating behaviour preventing heterospecific insemination, using *Ae. aegypti* females from resistant strains.

Alongside differences in heterospecific insemination rate due to female species (Leahy & Craig Jr., 1965; Robbins et al., 2011; Zhou et al., 2022), and prior exposure of females to heterospecifics (Bargielowski et al., 2015; Bargielowski & Lounibos, 2014), male origin can affect the rate of heterospecific insemination. Honório *et al.* (2018) found that males from Brazilian strains of *Ae. albopictus* were less successful at inseminating female *Ae. aegypti* than *Ae. albopictus* males from peninsular Florida. This suggests that males from some strains of *Ae. albopictus* are more competent at inseminating heterospecific, *Ae. aegypti* females. In *Chapter 5*, I conduct a meta-analysis to further examine all three explanations for heterogeneity in the rates of heterospecific insemination in *Ae. aegypti*.

3. Thesis Roadmap

The overarching aim of this thesis is to develop an increased understanding of reproductive interference between *Ae. aegypti* and *Ae. albopictus*, and its importance to vector control techniques. Specifically, I focus on the following questions:

(i) How important is it to include reproductive interference into models evaluating the efficacy vector control programmes, in areas where *Ae. aegypti* and *Ae. albopictus* coexist?

(ii) What resistance behaviours do female *Ae. aegypti* females use to prevent insemination by heterospecific, *Ae. albopictus* males?

(iii) Are there consistent rules that explain variation in the strength of reproductive interference?

In ***Chapter 2***, I explore question (i) by examining the combined effect of reproductive interference and the release of self-limiting *Ae. aegypti* on the stability of coexistence between *Ae. aegypti* and *Ae. albopictus*, and population size of each species. To do this, I develop theoretical models of *Ae. aegypti* and *Ae. albopictus* populations, using coupled ordinary differential equations.

I address question (ii) in my experimental chapters, ***Chapters 3 and 4***. In ***Chapter 3***, I conduct laboratory experiments to examine differences in heterospecific insemination rates between strains of *Ae. aegypti* females that are sympatric with *Ae. albopictus*, and strains allopatric

with *Ae. albopictus*. As these strains have not previously been used in reproductive interference experiments, this chapter characterises the strains.

In ***Chapter 4***, some of these strains are used in behavioural assays, to determine the mating behaviours that result in resistance to heterospecific mating. I conduct both real-time cage observations of multiple males and females, and slow-motion recordings of a tethered female and a single free-flying male. This is the first study to examine the behavioural traits governing resistance to heterospecific insemination.

Our results from *Chapters 3 and 4* seemingly contradict conclusions from previous studies. This made me question whether there is consistent support for many of the widely cited explanations for variation in heterospecific insemination. Thus, I conducted a meta-analysis in ***Chapter 5*** to address question (iii). I determined whether across the literature, there is consistent evidence for: higher rates of heterospecific insemination in *Ae. aegypti* females than *Ae. albopictus* females, higher rates of heterospecific insemination in allopatric *Ae. aegypti* females than sympatric *Ae. aegypti* females and differences in heterospecific insemination rates dependent on male geographic origin.

4. Publication Status

Chapter 2 is published in *Proceedings of the Royal Society B: Biological Sciences*.

Vollans, M & Bonsall, MB (2021) The concomitant effects of self-limiting insect releases and behavioural interference on patterns of coexistence and exclusion of competing mosquitoes. Proceedings of the Royal Society B: Biological Sciences. 288 (1951), 20210714. doi:10.1098/rspb.2021.0714.

We plan to publish the remaining chapters:

Chapters 3 and 4 *(draft)*

Vollans M, Zhang C, Alfonso-Parra C, Rydeberg S, Gubbins S, Bonsall MB, Cator LJ. A lack of male mating attempts prevents the insemination of *Ae. aegypti* females by *Ae. albopictus* males.

Chapter 5 *(draft)*

Vollans M, Cator LJ, Gubbins S, Bonsall MB, Cator LJ. A meta-analysis of reproductive interference in *Aedes* mosquitoes

.

Chapter 2: The Concomitant Effects of Self-limiting Insect Releases and Behavioural Interference on Patterns of Coexistence and Exclusion of Competing Mosquitoes[1]

Abstract

Aedes aegypti is the dominant vector of dengue, a potentially fatal virus whose incidence has increased 8-fold in the last two decades. As dengue has no widely available vaccine, vector control is key to reducing the burden on global public health. A promising method is the release of self-limiting *Ae. aegypti*, which mate with wild *Ae. aegypti* and produce non-viable offspring. The resultant decrease in *Ae. aegypti* population size may impact coexistence with *Ae. albopictus*, another principal vector of dengue. A behavioural mechanism influencing coexistence between these species is reproductive interference, where incomplete species recognition results in heterospecifics engaging in mating activities. Here, I develop a theoretical framework to investigate the interaction between self-limiting *Ae. aegypti* releases and reproductive interference between *Ae. aegypti* and *Ae. albopictus* on patterns of coexistence. In the absence of self-limiting *Ae. aegypti* release, coexistence can occur when the strength of reproductive interference experienced by both species is low. Results show that substantial overflooding with self-limiting *Ae. aegypti* prevents coexistence. For lower release ratios, as the release ratio increases, coexistence can occur when the strength of reproductive interference is increasingly high for *Ae. albopictus* and

[1] This chapter is adapted from the paper published in *Proceedings B*, https://doi.org/10.1098/rspb.2021.0714

increasingly low for *Ae. aegypti*. This emphasises the importance of including behavioural ecological processes into population models to evaluate the efficacy of vector control.

1. Introduction

Vector-borne diseases account for 17% of all infectious diseases and cause more than 700,000 deaths annually (World Health Organization, 2023). For example, dengue, a potentially fatal virus spread by *Aedes* mosquitoes, has increased 8-fold in incidence over the last two decades (World Health Organization, 2020a). Coupled with the rise in average global temperatures, the total burden on public health caused by vector-borne diseases is likely to further increase: Messina *et al.* (World Health Organization, 2020a) predicted that 2.25 billion more people will be at risk of dengue by 2080, compared to 2015. In areas where vector-borne diseases are already present, pathogen replication, vector survival, reproduction, biting rate and the length of transmission seasons are set to increase with environmental change (Messina et al., 2019; Gubler, 2011; Wilder-Smith, Murray & Quam, 2013). Furthermore, the global distribution of vectors is likely to widen: for instance, the global abundance of *Aedes aegypti* is predicted to increase by 20% or 30% by the end of the century, for a low and high carbon dioxide emission future, respectively (Liu-Helmersson et al., 2019). Thus, it is increasingly important to have robust strategies to manage and control vector-borne diseases. As many vector-borne diseases have no widely available vaccine or disease-specific drugs (e.g. World Health Organization, 2020a,c, 2022), the key is employing methods to control vector population size.

However, the environment and human health can be negatively impacted by conventional, chemical-based vector control methods, such as the mass spraying of insecticide. Even pyrethroids, which have a low toxicity compared to many other insecticides (Yoo et al.,

2016), decrease the diversity of non-target small-bodied arthropods (Boyce et al., 2007; Ristyadi, Andrew & Waugh, 2013), cause aquatic toxicity (Weston et al., 2005; Hua & Relyea, 2019) and negatively impact the human male reproductive system (Koureas et al., 2012; Radwan et al., 2015). Additionally, the efficacy of chemical control is declining as insecticide resistance spreads (Denholm, Devine & Williamson, 2002). Therefore, there is a growing imperative to develop novel approaches for controlling disease vectors. As such there has been a continued focus on the use of self-limiting insects for the control of mosquito-borne diseases (Toé et al., 2022; Alphey et al., 2013, 2010); a technique with substantial benefits (Alphey et al., 2013), and that is less costly than alternative control methods (Alphey et al., 2010).

Self-limiting insects decrease the number of offspring contributing to the next generation by competing with wild insects for mates and subsequently producing non-viable offspring. Self-limiting systems include the Sterile Insect Technique (SIT), where insects are irradiated so that they cannot produce viable offspring (Knipling, 1955), and the Release of Insects Carrying a Dominant Lethal (RIDL^{TM}), where insects are genetically engineered to be homozygous for a dominant lethal genetic construct (Toé et al., 2022; Alphey et al., 2013). These methods target single species that are detrimental to human well-being, and do not require the direct or indirect release of harmful chemicals, and thus are considered relatively environmentally benign (Alphey et al., 2010). Previous use of SIT has been successful: resistance to the technique is infrequent, and it has successfully eliminated and controlled multiple insect pests (for details, see Alphey et al., 2009).

However, by altering the population size of the target species, the release of self-limiting insects may have indirect environmental impacts on wider biodiversity: for instance, by affecting the interspecific interactions of the target species (Bonsall et al., 2010). These indirect effects could be substantial when the population size of the heterospecific species is strongly coupled with the population size of the target species. This is true for species that have a similar ecological niche to the target species, and therefore are likely to compete strongly with it for resources.

Aedes aegypti and *Aedes albopictus*, the two principal disease vectors of dengue, have overlapping realised ecological niches: they are both anthropophilic; have similar diurnal peak activity periods; use hosts to find mates; exist in urban and suburban settings and deposit their eggs above the water line in (often ephemeral) natural and artificial pools of water (Gubler, Bhattacharya & Bhattacharva, 1972; Hartberg, 1971; Ponlawat & Harrington, 2005; Kauffman et al., 2017; Bagny et al., 2009; Nelson, 1986; Yuval, 2006). However, the two species cannot reproduce to form viable offspring (Leahy & Craig, 1967). Despite these species being native to different continents, their range expansion has resulted in frequent overlap of spatial distribution, causing exclusion or coexistence (Lounibos & Juliano, 2018).

Two key mechanisms influencing coexistence patterns between *Ae. aegypti and Ae. albopictus* are interspecific resource competition and reproductive interference (reviewed by Lounibos & Juliano, 2018a). Reproductive interference is where incomplete species recognition results in heterospecifics engaging in mating activities which do not produce viable offspring and cause a fitness cost to one or both of the species involved (Gröning & Hochkirch, 2008; Burdfield-Steel & Shuker, 2011). Thus, reproductive interference is distinct

from competition, as the fitness costs incurred are not due to shared limited resources (Kuno, 1992). Instead, they can occur directly by female harassment and reduction in fertility, and indirectly through wasted courtship/handling time and energy (Gröning & Hochkirch, 2008; Paton & Bonsall, 2019). Reproductive interference occurs across a wide range of taxa, as summarised by Gröning and Hochkirch (2008). Where two species coexist, theoretical studies have shown that reproductive interference is more likely to cause exclusion than interspecific resource competition (Kuno, 1992). When both are considered together, reproductive interference acts synergistically with resource competition to promote exclusion; their combined effect is greater than the sum of their independent effects (Kishi & Nakazawa, 2013).

Reproductive interference occurs between *Ae. aegypti* and *Ae. albopictus* both in the field (e.g. Robbins et al., 2011), and in the laboratory (e.g. Bargielowski et al., 2015; Marcela et al., 2015). As discussed by Paton & Bonsall (2019), at least four types of reproductive interference occur between *Aedes* species: misdirected courtship, heterospecific mating attempts, erroneous female choice and heterospecific mating (Gröning & Hochkirch, 2008; Robbins et al., 2011; Bargielowski et al., 2015; Marcela et al., 2015). While the impact of reproductive interference upon the coupled population dynamics of *Ae. aegypti* and *Ae. albopictus* has previously been analysed (Kishi & Nakazawa, 2013; Paton & Bonsall, 2019), the consequences of this mating disruption and behavioural interference on patterns of coexistence have not been explored in combination with the release of self-limiting *Ae. aegypti*.

Ae. aegypti is a key target for self-limiting techniques, as the dominant vector of dengue, and a vector of chikungunya, yellow fever and Zika viruses (World Health Organization, 2023). In regions where *Ae. aegypti* and *Ae. albopictus* coexist, their population dynamics are strongly coupled. As *Ae. albopictus* is a secondary vector to dengue, and carrier of chikungunya, yellow fever and Zika viruses, it is also epidemiologically relevant: assessments of the impact of the release of self-limiting *Ae. aegypti* need to consider the impact upon *Ae. albopictus* populations.

Here, I form a theoretical framework (based on modified ecological competition equations) to investigate the interaction between the release of self-limiting *Ae. aegypti* and the impact of reproductive interference between *Ae. aegypti* and *Ae. albopictus* populations on patterns of coexistence and exclusion. To disentangle the effects of reproductive interference caused by wild mosquitoes and self-limiting *Ae. aegypti*, I examine scenarios where the self-limiting *Ae. aegypti* reproductively interfere with *Ae. albopictus*, and where they do not. The results of this study inform on the developing pragmatic applications and policy developments around the ecological (and epidemiological) consequences of the release of self-limiting *Ae. aegypti* to manage the dengue disease burden.

2. Methods

I use a set of simple differential equation models to assess how the release of self-limiting *Ae. aegypti* impacts its coexistence with *Ae. albopictus* when there is reproductive interference. Models are summarised in figure 1.1.

I use two baseline models: Kuno's model of reproductive interference ((Kuno, 1992), eqns 1a-b) and a basic model of self-limiting *Ae. aegypti* release (eqns 2a-b). The former examines the impact of reproductive interference in the absence of self-limiting *Ae. aegypti* release, while the latter explores the effect of self-limiting *Ae. aegypti* release without reproductive interference. The further two models build on these baseline models to assess the combined impact of self-limiting *Ae. aegypti* release and reproductive interference: firstly when the self-limiting *Ae. aegypti* do not reproductively interfere with *Ae. albopictus* (eqns 3a-b) and, secondly, when they do (eqns 4a-b). Comparisons between these models allow the impact of self-limiting *Ae. aegypti* release, and reproductive interference on coexistence to be examined separately and in combination. For all models with self-limiting *Ae. aegypti* release, I use a proportional release policy (Atkinson et al., 2007): at each time point, the number of self-limiting *Ae. aegypti* released is proportional to the number to wild *Ae. aegypti*.

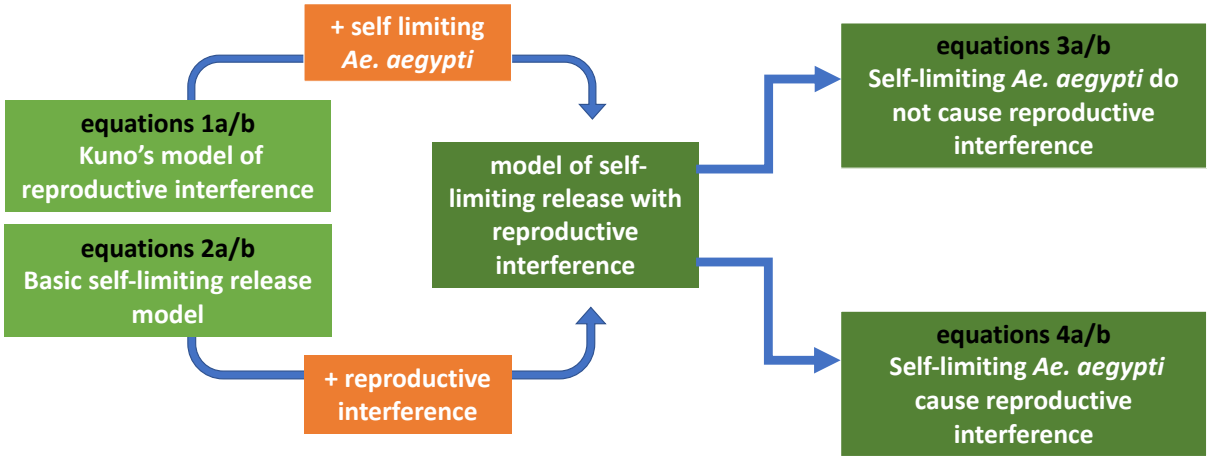

Figure 1.1: Summary of the development of the models of self-limiting Ae. aegypti release with reproductive interference from the baseline models.

As with previous work (Paton & Bonsall, 2019) other demographic processes such as birth and death rate (Southwood et al., 1972; Dye, 1984) were assumed to be equal for both *Aedes* species and parameters were varied to allow the results to be investigated for a variety of environmental contexts (Juliano, 2010; Honório et al., 2018). See table 1 for details.

Parameter	Definition	Value	Reference / Notes
r	Reproductive rate, per capita	1.31	Southwood et al. (1972); Dye (1984)
d	Death rate, per capita	0.12	Southwood et al. (1972); Dye (1984)
α	Strength of density-dependent intraspecific competition, per capita	1	
β_i	Strength of interspecific competition per capita, relative to intraspecific competition (α)	Varied, from 0 to 1	When β_i = 1, the strength of interspecific competition is equal to intraspecific competition. β_i is never greater than 1, as this would make species coexistence impossible.
δ_i	Strength of reproductive interference, per capita	Varied, from 0 to 1	Kishi & Nakazawa (2013)
θ	Ratio of self-limiting *Ae. aegypti*: wild *Ae. aegypti*	Varied, from 0 to 8	Overflooding: θ > 1 Underflooding: θ < 1

Table 1.1: Canonical parameter values for Aedes mosquitoes. The values of subscripted parameters were varied between Ae. aegypti and Ae. albopictus. The relevance of the particular parameter value was evaluated using sensitivity analyses (see section 2.3.2).

2.1. Baseline Models

2.1.1 Kuno's model of reproductive interference

Kuno (1992) developed a two-species interaction model that described resource competition and reproductive interference. In this model, for the *ith* species an increase in the heterospecific population causes a rise in density dependent resource competition, increasing mortality, and causes a rise in reproductive interference, decreasing recruitment. This model can be described by the following set of differential equations:

$$\frac{\partial A(t)}{\partial t} = r_A A(t)\left(\frac{A(t)}{A(t)+ \delta_A B(t)}\right) - \alpha_A A(t)[A(t) + \beta_A B(t)] - d_A A(t) \quad (1a)$$

$$\frac{\partial B(t)}{\partial t} = r_B B(t)\left(\frac{B(t)}{B(t)+ \delta_B A(t)}\right) - \alpha_B B(t)[B(t) + \beta_B A(t)] - d_B B(t) \quad (1b)$$

where A represents the density of Ae. aegypti and B represents the density of Ae. albopictus. Parameters subscript A correspond to Ae. aegypti, and subscript B to Ae. albopictus. Recruitment is determined by r_i , the reproductive rate, scaled by δ_i, the decrease in reproductive success caused by reproductive interference. Density-dependent adult mortality is determined using α_i , the strength of intraspecific competition, and β_i, the strength of interspecific competition (relative to intraspecific competition), while d_i is the density-independent adult mosquito mortality rate.

2.1.2. Basic self-limiting *Ae. aegypti* release

This model describes the reduction in recruitment of wild *Ae. aegypti* (denoted *A*) by the release of self-limiting *Ae. aegypti.* A proportional release policy (Atkinson et al., 2007) is used: thus, it is assumed that there is a stable proportion of self-limiting *Ae. aegypti* to wild *Ae. aegypti*. Furthermore, it is assumed that the self-limiting males always mate to produce non-viable offspring and are fully competitive with wild mosquitoes. This model can be described by:

$$\frac{\partial A(t)}{\partial t} = r_A A(t)\left(\frac{A(t)}{A(t)+\theta A(t)}\right) - \alpha_A A(t)[A(t) + \beta_A B(t)] - d_A A(t) \quad (2a)$$

$$\frac{\partial B(t)}{\partial t} = r_B B(t) - \alpha_B B(t)[B(t) + \beta_B A(t)] - d_B B(t) \quad (2b)$$

where θ *is the ratio of self-limiting Ae. aegypti to wild Ae. aegypti. All other parameters are given above (for eqns 1a-b).*

2.2. Self-limiting *Ae. aegypti* Release with Reproductive Interference

These models combine Kuno's model (1992) (eqns 1a-1b) and the basic self-limiting *Ae. aegypti* release model (eqns 2a-2b) to assess the impact of reproductive interference (eqns 1a-b) and self-limiting *Ae. aegypti* release (eqns 2a-b) on the densities of *Ae. aegypti* (denoted *A*) and *Ae. albopictus* (denoted *B*). Comparisons between the results from eqns 3a-b and eqns 4a-b allow the impact of reproductive interference caused by self-limiting *Ae. aegypti* and wild mosquitoes to be assessed separately.

2.2.1 Self-limiting *Ae. aegypti* do not cause reproductive interference

Here, the released self-limiting *Ae. aegypti* only act to decrease the recruitment of *Ae. aegypti*:

$$\frac{\partial A(t)}{\partial t} = r_A A(t)\left(\frac{A(t)}{A(t)+\theta A(t)+\delta_A B(t)}\right) - \alpha_A A(t)[A(t) + \beta_A B(t)] - d_A A(t) \quad (3a)$$

$$\frac{\partial B(t)}{\partial t} = r_B B(t)\left(\frac{B(t)}{B(t)+\delta_B A(t)}\right) - \alpha_B B(t)[B(t) + \beta_B A(t)] - d_B B(t) \quad (3b)$$

2.2.2. Self-limiting *Ae. aegypti* cause reproductive interference

This model extends eqns 3a-b. Here, the released self-limiting *Ae. aegypti* reproductively interfere with *Ae. albopictus*:

$$\frac{\partial A(t)}{\partial t} = r_A A(t)\left(\frac{A(t)}{A(t)+ \theta A(t) + \delta_A B(t)}\right) - \alpha_A A(t)[A(t) + \beta_A B(t)] - d_A A(t) \quad (4a)$$

$$\frac{\partial B(t)}{\partial t} = r_B B(t)\left(\frac{B(t)}{B(t)+ \delta_B [A(t)+\theta A(t)]}\right) - \alpha_B B(t)[B(t) + \beta_B A(t)] - d_B B(t) \quad (4b)$$

This model assumes that self-limiting and wild *Ae. aegypti* equally interfere with the reproduction of *Ae. albopictus*.

2.3. Model Analysis

2.3.1. Zero-net-growth isoclines

Zero-net-growth isoclines were used to compare the outcomes of interspecific interactions across models. By definition, the zero-net-growth isoclines for each model were determined by solving d*A(t)*/d*t* = 0 and d*B(t)*/ d*t* = 0 and taking positive solutions. This resulted in a linear (basic self-limiting *Ae. aegypti* release, eqns 2a-b) or quadratic (all other models) equation for each species. Equilibria occur when the population growth rates of both species are equal zero (i.e., d*A(t)*/d*t* = 0 and d*B(t)*/ d*t* = 0); where the zero-net-growth isoclines for each species cross. Equilibria were determined numerically with a multiroot function (Soetaert, 2009), which uses the Newton-Raphson method. Two types of equilibria are possible: exclusion of either species or coexistence of both species. A Jacobian matrix approach was used to determine the stability of equilibrium points, where stable equilibria produce negative dominant eigenvalues and the magnitude of the eigenvalue corresponds to the stability (Vos et al., 2005).

2.3.2. Sensitivity analysis

As in Kuno (1992) and Paton and Bonsall (2019), I examined sensitivity visually. However, I could have numerically evaluated the sensitivity of each model to the parameters of interest (as detailed by Barabás et al., 2014)).

(i) Population Size

I examined the impact of the strength of different parameters on the population size of each species at stable equilibria. While keeping all other parameters constant, the strength of the reproductive interference and interspecific competition experienced by each species, together with the self-limiting *Ae. aegypti* release ratio, were varied. This was conducted separately for exclusion and coexistence equilibria.

As with the isocline analysis, equilibria were determined numerically (Soetaert, 2009). Under certain circumstances, the Newton-Raphson method fails to converge upon a root. For instance, when there is an inflection point at the root that is being approximated, the Newton-Raphson method often fails to converge and instead forms an oscillating sequence. Thus, for each sensitivity analysis, parameter values were selected to prevent these failures occurring. For this reason, the constant parameter values can vary between sensitivity analyses and comparisons were not made between sensitivity analyses.

(ii) Coexistence and Exclusion Boundaries

For each model with a reproductive interference term (eqns 1a-b, 3a-b and 4a-b), further analysis was conducted to determine the parameter space that results in stable coexistence. I examined how the strength of reproductive interference and interspecific competition

influence the potential for coexistence for different self-limiting *Ae. aegypti* release ratios (no self-limiting *Ae. aegypti* release, θ = 0; weak underflooding, θ = 0.4; moderate underflooding, θ = 0.8; weak overflooding, θ = 1.2; substantial overflooding, θ = 8.0). I varied both the strength of reproductive interference and interspecific competition parameters from zero (where they have no effect) to 1 (where heterospecifics are equivalent to conspecifics).

To determine parameter values that result in stable coexistence, cubic expressions (cubic formula, eqn 5) were derived by substituting the solution for *Ae. aegypti* (denoted *A*) into the equation for *Ae. albopictus* (denoted *B*) and vice versa. When both cubic equations in a model have three positive solutions, there is stable coexistence: two unstable coexistence points surrounding one stable coexistence point (Kishi & Nakazawa, 2013). For the coupled cubic equations to have at least three solutions, the discriminants of both equations must be greater than zero (eqn 6) and for all solutions to be positive and, thus, biologically relevant, the coefficients have satisfy certain inequalities (Kishi & Nakazawa, 2013), see eqn 7.

$$ax^3 + bx^2 + cx + d = 0 \quad (5)$$

$$18abcd - 4b^3d + b^2c^2 - 4ac^3 - 27a^2d^2 > 0 \quad (6)$$

$$a \,\&\, c > 0, and\ b \,\&\, d < 0,\ or$$
$$a \,\&\, c < 0, and\ b \,\&\, d > 0 \quad (7)$$

All analyses (simulations, mathematical derivations and graphical analysis) were completed in *R* (version 4.0.4) and *Mathematica* (version 12). Code is available at OSF.

3. Results

3.1. Isocline Analysis

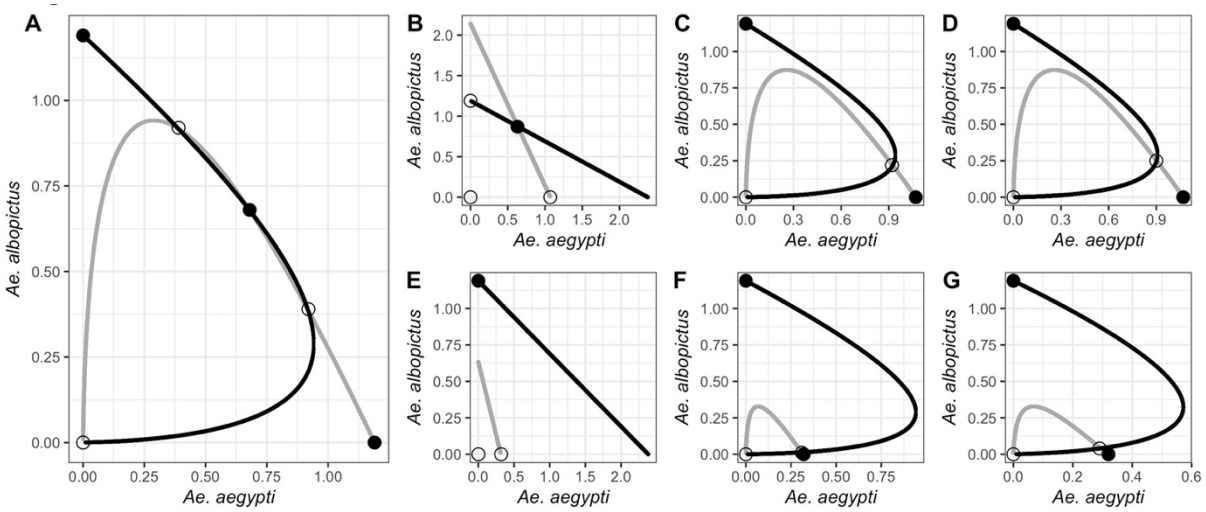

Figure 1.2: Outcomes of interspecific interactions across models. Comparison of zero net growth isocline plots of Ae. aegypti (grey) and Ae. albopictus (black) in an under-flooding (ϑ = 0.1, row 1) and an over-flooding (ϑ = 2, row 2) scenario. Isoclines of two baseline models are shown: Kuno's model of reproductive interference (eqns 1a-b, plot A) and a basic self-limiting Ae. aegypti release model (eqns 2a-b; plots B & E). The remaining plots illustrate models of self-limiting Ae. aegypti release with reproductive interference, where the self-limiting Ae. aegypti do not reproductively interfere with Ae. albopictus (eqns 3a-b; plots C & G) and where they do (eqns 4a-b; plots D & H). Filled circles show stable equilibria, and unfilled circles unstable equilibria. Constant parameter values are given in table 1, β_a and β_b = 0.5, and, where relevant, δ_a and δ_b = 0.15 and ϑ = 0.1 (under-flooding, row 1) or 2 (over-flooding, row 2).

The addition of self-limiting *Ae. aegypti* or reproductive interference to the baseline models can alter the shape or gradient of the zero net growth isocline of one or both species. This

changes where the two isoclines intersect and influences the number of coexistence points, their stability, and the population size of each species at those points.

By comparing the isoclines for Kuno's model of reproductive interference (eqns 1a-b; figure 1.2A) with models of reproductive interference and self-limiting *Ae. aegypti* release, the impact of releasing self-limiting *Ae. aegypti* can be determined. Where there is reproductive interference, but no release of self-limiting *Ae. aegypti* (Kuno's model, eqns 1a-b; figure 1.2A), *Ae. aegypti* and *Ae. albopictus* isoclines cross at three points, one stable coexistence point flanked by two unstable coexistence points (as seen in Shuker & Burdfield-Steel, 2017). However, underflooding (figure 1.2C & D) and overflooding (figure 1.2F & G) with self-limiting *Ae. aegypti* causes vertical and horizontal compression of the *Ae. aegypti* isocline, and minor horizontal compression of the *Ae. albopictus* isocline, causing the isoclines to only cross at a single point. Therefore, following underflooding and overflooding with self-limiting *Ae. aegypti*, coexistence is destabilised, and exclusion are the only stable equilibria.

The influence of reproductive interference on the stable equilibria is assessed by comparing the isoclines of the model of self-limiting *Ae. aegypti* release with no reproductive interference (eqns 2a-b; figure 1.2B & E) to the models with both self-limiting *Ae. aegypti* release and reproductive interference (eqns 3a-b & 4a-b and figure 1.2C & F, 1.2D & G, respectively). In the absence of reproductive interference, the dynamics depend upon the release ratio of self-limiting *Ae. Aegypti*: in an underflooding scenario, there is only stable coexistence (eqns 2a-b; figure 1.2B), however in an overflooding scenario the only stable equilibrium is the exclusion of *Ae. aegypti* by *Ae. albopictus* (eqns 2a-b; figure 1.2E). Thus, where there is no reproductive interference, a high enough release ratio of self-limiting *Ae.*

aegypti promotes the exclusion of *Ae. aegypti* by *Ae. albiopictus*. However, the inclusion of reproductive interference, either where self-limiting *Ae. aegypti* do not reproductively interfere with *Ae. albopictus* (eqns 3a-b; figure 1.2C & F) or where they do (eqns 4a-b; figure 1.2D & G), results in the exclusion of *Ae. aegypti* by *Ae. albopictus* and the exclusion of *Ae. albopictus* by *Ae. aegypti* being stable equilibria. This is true in both an underflooding and overflooding scenario. Thus, in the overflooding scenario, the addition of reproductive interference stabilises the exclusion of *Ae. albopictus* by *Ae. aegypti.* Further, in the underflooding scenario, the addition of reproductive interference destabilises coexistence, and stabilises the exclusion of *Ae. aegypti* by *Ae. albopictus* and the exclusion of *Ae. albopictus* by *Ae. aegypti*.

There is minimal difference between the results of the model where self-limiting *Ae. aegypti* cause reproductive interference (eqns 4a-b; figure 1.2D & G), and the model where only wild mosquitoes cause reproductive interference (eqns 3a-b; figure 1.2C & F). This suggests that the reproductive interference caused by wild *Ae. aegypti* and *Ae. albopictus* has a much greater impact on dynamics than the reproductive interference caused by self-limiting *Ae. aegypti.* This is further explored in the sensitivity analysis (see coexistence and exclusion boundaries, 3.2 (ii)).

3.2. Sensitivity Analysis

Isocline analyses are limited in that they only show stable equilibria for a certain set of parameter values. Thus, I conducted further analyses to examine the sensitivity of the population sizes of *Ae. aegypti* and *Ae. albopictus* to the ratio of self-limiting *Ae. aegypti* (θ; figure 1.3), the strength of reproductive interference (δ_i; figure 1.4), and the strength of interspecific competition (β_i; figure 1.5). I then assessed the sensitivity of the stable coexistence of *Ae. aegypti* and *Ae. albopictus* to the strength of reproductive interference (δ_i; figure 1.6), and interspecific competition (β_i; figure 1.7), for different ratios of self-limiting *Ae. aegypti* (θ).

(i) Population Size

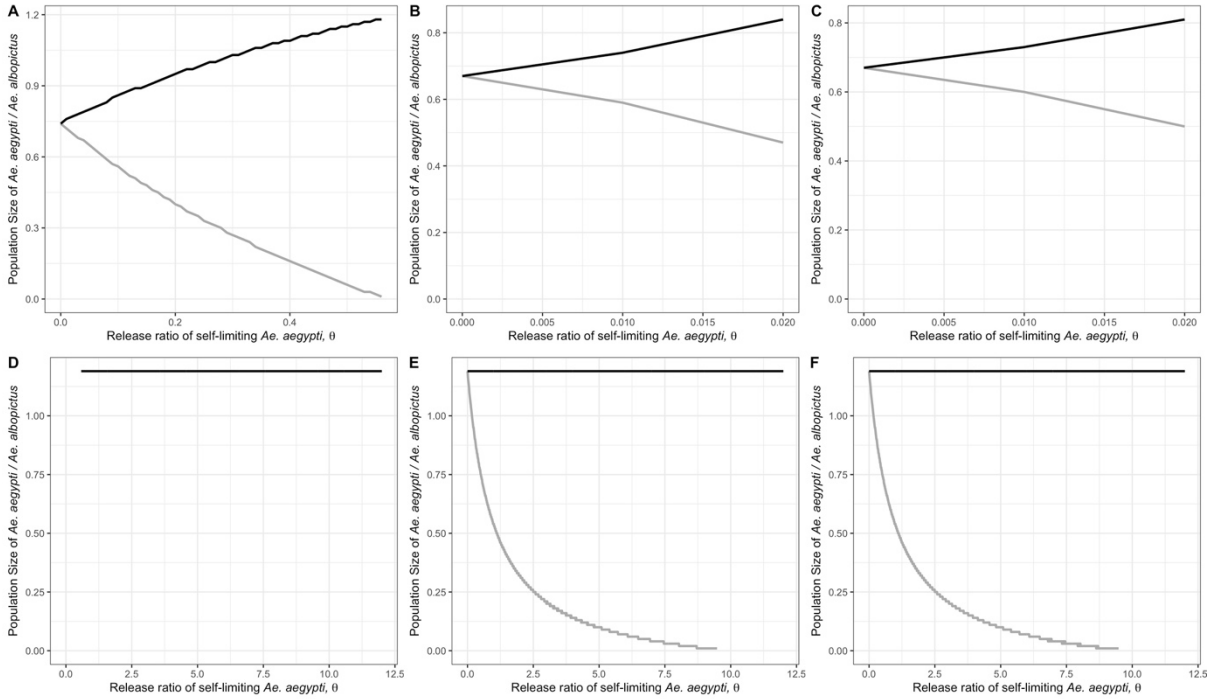

Figure 1.3: The impact of the self-limiting Ae. aegypti release ratio (ϑ) on the population size of Ae. aegypti (grey) and Ae. albopictus (black) at stable coexistence (row one) and competitive exclusion (row two), for the basic self-limiting Ae. aegypti release model (eqns 2a-b; plots A & D), and models of self-limiting Ae. aegypti release with reproductive interference, where the self-limiting Ae. aegypti do not reproductively interfere with Ae. albopictus (eqns 3a-b; plots B & E) and where they do (eqns 4a-b; plots C & F). Plot D only contains a single line, as there is no stable competitive exclusion of Ae. albopictus by Ae. aegypti. Constant parameter values are given in table 1, β_a and $\beta_b = 0.6$ and, where relevant, δ_a and $\delta_b = 0.1$.

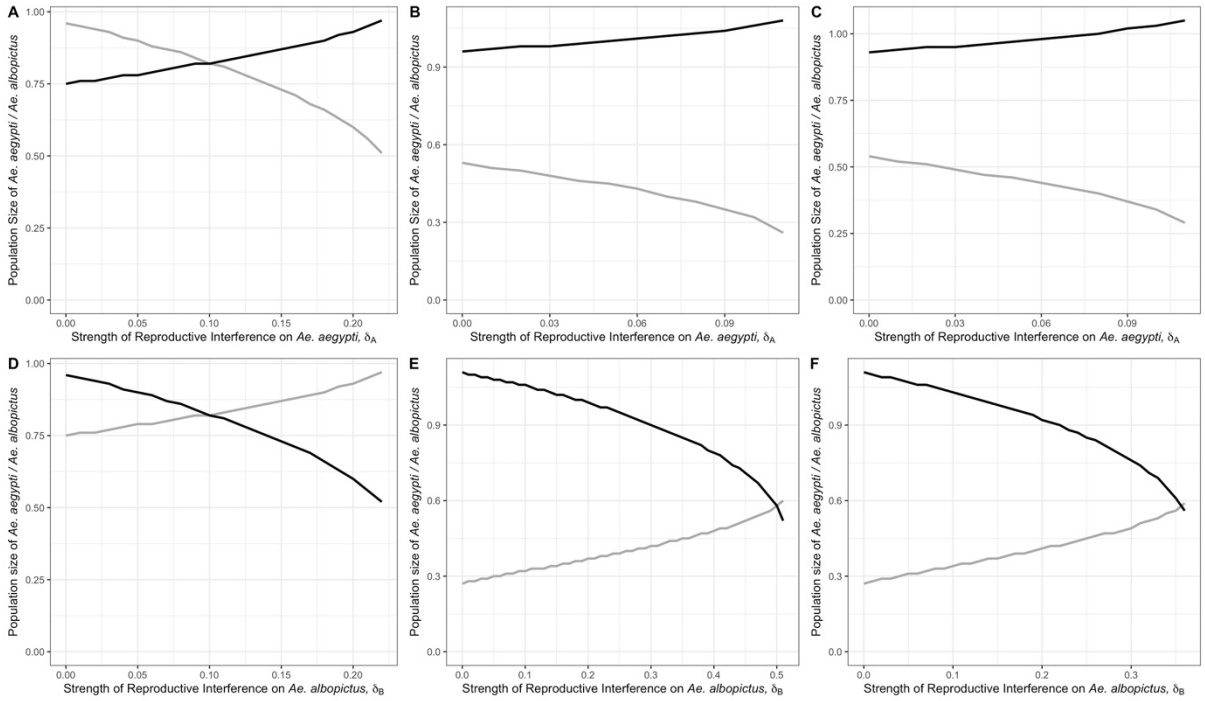

Figure 1.4: The impact of the strength of reproductive interference (δ_i) on the population size of each species (Ae. aegypti, grey; Ae. albopictus, black) at stable coexistence. The strength of reproductive interference experienced by Ae. aegypti is varied in row one, and Ae. albopictus in row two. Analysis was conducted for the basic reproductive interference model (eqns 1a-b; plots A & D), and models of self-limiting Ae. aegypti release with reproductive interference, where the self-limiting Ae. aegypti do not reproductively interfere with Ae. albopictus (eqns 3a-b; plots B & E) and where they do (eqns 4a-b; plots C & F). Constant parameter values are given in table 1, β_a and β_b= 0.301, and, where relevant, ϑ = 0.4 and δ_a and δ_b = 0.1.

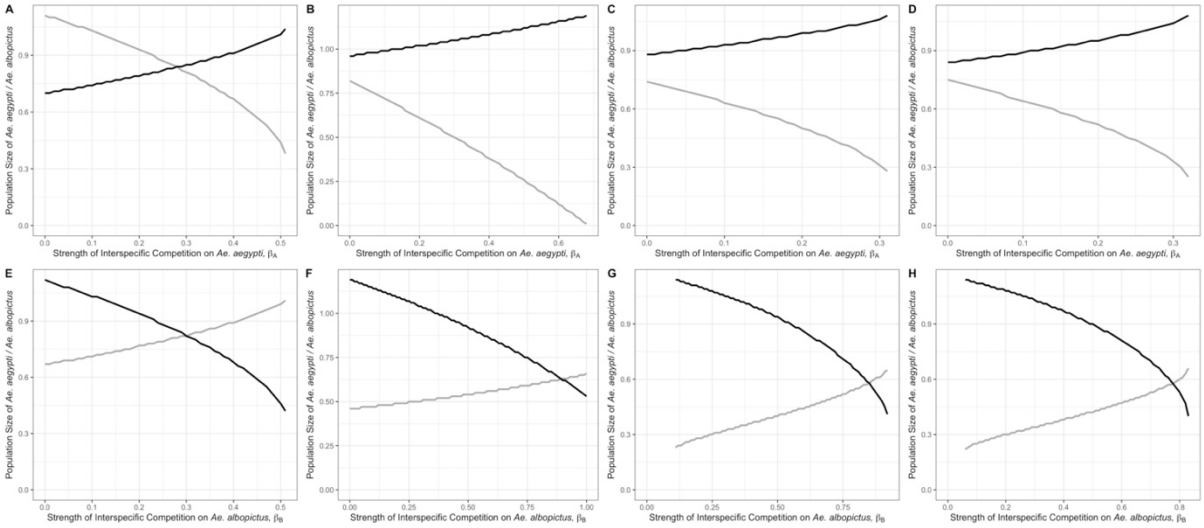

Figure 1.5: The impact of the strength of interspecific competition (β_i) suffered by Ae. aegypti (row one) and Ae. albopictus (row two) on the population size of Ae. aegypti (grey) and Ae. albopictus (black) at stable coexistence. Analysis was conducted for the basic reproductive interference model (eqns 1a-b; plots A & E), the basic self-limiting Ae. aegypti release model (eqns 2a-b; plots B & F), and models of self-limiting Ae. aegypti release with reproductive interference, where the self-limiting Ae. aegypti do not reproductively interfere with Ae. albopictus (eqns 3a-b; plots C & G) and where they do (eqns 4a-b; plots D & H). Constant parameter values are given in table 1, δ_a and δ_b = 0.1 and, where relevant, ϑ = 0.4, β_a = 0.3 and β_b = 0.28.

I explored the impact of different parameter values on the population size of both species at all stable equilibria. The magnitude of the ratio of self-limiting *Ae. aegypti* (θ), reproductive interference (δ_i) and interspecific competition (β_i) all impact the population size of both species at coexistence. In all models with the release of self-limiting *Ae. aegypti*, as the release ratio increases, the population size of *Ae. aegypti* at coexistence decreases, and *Ae. albopictus* increases (figure 1.3A - C). Similarly, an increase in the strength of reproductive interference or interspecific competition (figures 1.4 and 1.5, respectively) causes the

population size of the species experiencing the force to decrease, and the population size of the other species to increase, where the species coexist.

Although the ratio of self-limiting *Ae. aegypti* (θ), strength of reproductive interference (δ_i) and strength of interspecific competition (β_i) influence the population size of both species at coexistence, only the release ratio of self-limiting *Ae. aegypti* (θ) has any impact on population size when there is exclusion (figure 1.3D-F). When there is reproductive interference, as the release ratio of self-limiting *Ae. aegypti* (θ) increases there is a decrease in the population size of *Ae. aegypti* when it excludes *Ae. albopictus* (figure 1.3E & F). These results are sensical: following exclusion of a species, interspecific interactions (through resource competition and reproductive interference) will not occur, and thus will not influence population density. However, following the exclusion of *Ae. albopictus*, self-limiting *Ae. aegypti* will still suppress the population size of *Ae. aegypti*. In the absence of reproductive interference, *Ae. aegypti* cannot stably exclude *Ae. albopictus* (figure 1.3D). For all self-limiting insect release models, the population size of *Ae. albopictus*, when it excludes *Ae. aegypti*, is unaffected by the self-limiting *Ae. aegypti* release ratio (figure 1.3D-F). Again, this is sensical, as there will be no self-limiting *Ae. aegypti* present following the exclusion of *Ae. aegypti*.

(ii) Coexistence and Exclusion Boundaries

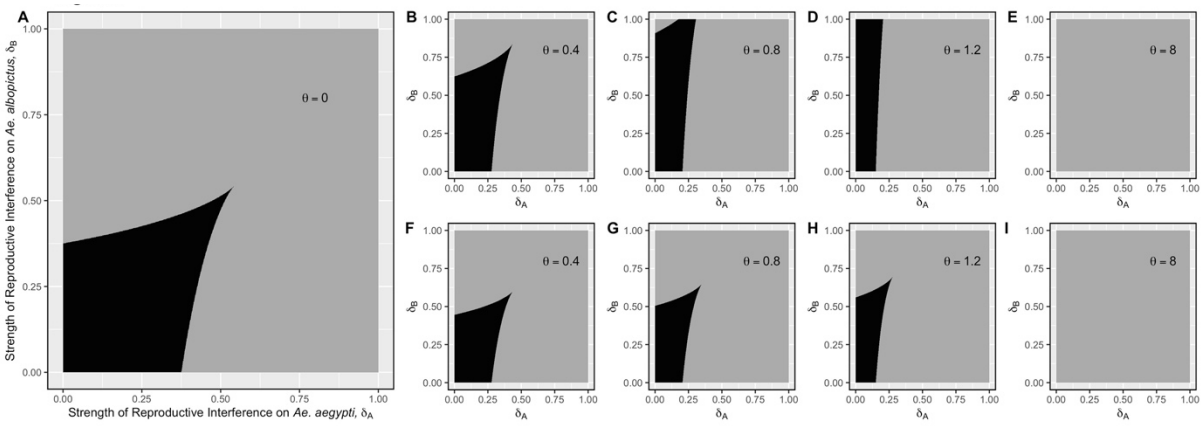

Figure 1.6: The effects of the strength of reproductive interference (δ_i) on stable coexistence in all models with a reproductive interference term (eqns 1a-b; 3a-b & 4a-b). Parameter space where stable coexistence can occur is shaded in black. The ratio of self-limiting Ae. aegypti release (ϑ) is varied across plots, from no self-limiting Ae. aegypti release ($\vartheta = 0$, plot A), to weak underflooding ($\vartheta = 0.4$, plots B & F), moderate underflooding ($\vartheta = 0.8$, plots C & G), weak overflooding ($\vartheta = 1.2$, plots D & H) and substantial overflooding ($\vartheta = 8$, plots E & I). In the first row, self-limiting Ae. aegypti do not reproductively interfere with Ae. albopictus (eqns 3a-b), while in the second they do (eqns 4a-b). Constant parameter values are given in table 1, and β_a and $\beta_b = 0.1$.

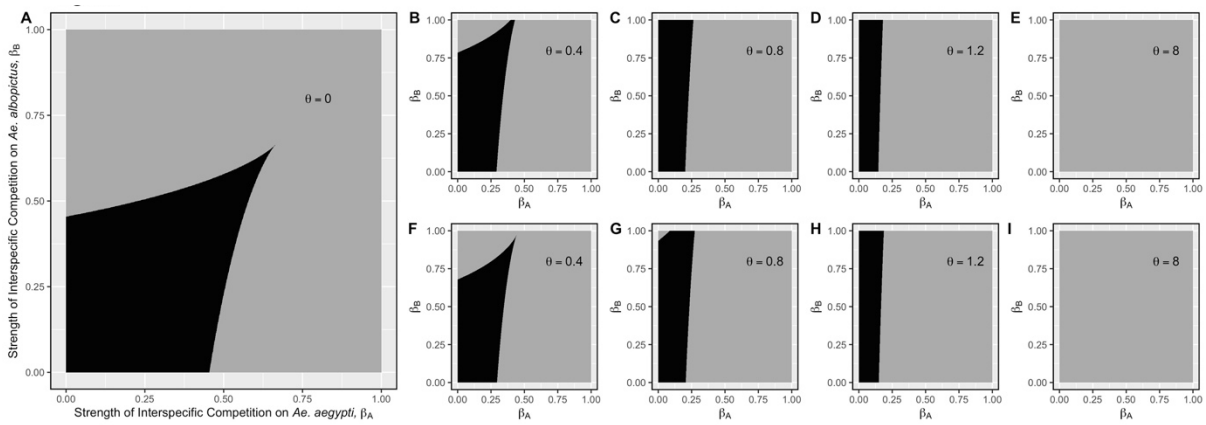

Figure 1.7: The effects of the strength of interspecific competition (β_i) on stable coexistence in all models with a reproductive interference term (eqns 1a-b; 3a-b & 4a-b). Parameter space where stable coexistence can occur is shaded in black. As in figure 1.2, the ratio of self-limiting Ae. aegypti release (ϑ) is varied across plots, from no self-limiting Ae. aegypti release (ϑ = 0, plot A), to weak underflooding (ϑ = 0.4, plots B & F), moderate underflooding (ϑ = 0.8, plots C & G), weak overflooding (ϑ = 1.2, plots D & H) and substantial overflooding (ϑ = 8, plots E & I). In the first row, self-limiting Ae. aegypti do not reproductively interfere with Ae. albopictus (eqns 3a-b), while in the second row, they do (eqns 4a-b). Constant parameter values are given in table 1, and δ_a and δ_b = 0.1.

For all models with the reproductive interference term, analyses explored the ranges of strengths of reproductive interference (δ_i, figure 1.6) and interspecific competition (β_i, figure 1.7) that resulted in stable coexistence, for different ratios of self-limiting *Ae. aegypti* (θ). Similar patterns are observed for both parameters, in Kuno's model of reproductive interference (eqns 1a-b) and the models including self-limiting *Ae. aegypti* release (eqns 3a-b & 4a-b).

In the Kuno's model of reproductive interference model (eqns 1a-b), coexistence regions are biased towards the left corner, where there is low reproductive interference (figure 1.6A), or

low interspecific competition (figure 1.7A) experienced by both species. In models with self-limiting *Ae. aegypti* release, an increase in the release ratio (θ) causes the stable coexistence parameter space to decrease in area and change shape. As the release ratio (θ) increases, coexistence can occur when *Ae. albopictus* suffers increasingly high reproductive interference (figure 1.6) or interspecific competition (figure 1.7), and *Ae. aegypti* suffers increasingly low reproductive interference (figure 1.6) or interspecific competition (figure 1.7).

When self-limiting *Ae. aegypti* do not reproductively interfere with *Ae. albopictus* (figure 1.6B-E and 1.7B-E), coexistence can occur when *Ae. albopictus* suffers greater reproductive interference and interspecific competition, than when *Ae. aegypti* do cause reproductive interference (figure 1.6F-I and 1.7F-I). In the substantial overflooding situation, the self-limiting insect release ratio is high enough (θ = 8) that coexistence cannot occur for any values for reproductive interference (figure 1.6E & I) or interspecific competition (figure 1.7E & I): thus, there is only exclusion.

4. Discussion

This work is the first to investigate the combined role of reproductive interference and self-limiting insect releases on the coexistence of closely related disease vectors, *Ae. aegypti* and *Ae. albopictus*. It is well established, both theoretically (Phuc et al., 2007; Legros et al., 2012; Seirin Lee et al., 2013) and through a limited set of field trials (Carvalho et al., 2015; Harris et al., 2012), that the release of self-limiting mosquitoes can be used to suppress the population size of *Ae. aegypti*, and, potentially, reduce the substantial associated public health burden.

Ae. aegypti shares a similar ecological niche with *Ae. albopictus:* they compete for resources (Ponlawat & Harrington, 2005; Kauffman et al., 2017; Bagny et al., 2009) and interfere with each other's mating attempts (Robbins et al., 2011; Bargielowski et al., 2015; Marcela et al., 2015). Previous studies have separately modelled the impact of reproductive interference (Paton & Bonsall, 2019) and self-limiting *Ae. aegypti* release (Bonsall et al., 2010) upon coexistence between *Ae. aegypti* and *Ae. albopictus*. However, as previously highlighted (Burdfield-Steel & Shuker, 2011), the combined effect has not been investigated. I addressed this by examining interactions between *Ae. aegypti* and *Ae. albopictus,* where there is reproductive interference and self-limiting *Ae. aegypti* release. I explored the potential outcomes following the release of self-limiting *Ae. aegypti*, where *Ae. aegypti* and *Ae. albopictus* coexist. This work remains highly relevant as *Ae. albopictus* and *Ae. aegypti* are undergoing range expansion, making it increasingly likely that these important vectors of disease will come into contact (Lounibos & Juliano, 2018). The results of this study show that

the ratio of self-limiting *Ae. aegypti* and the strength of reproductive interference can act concomitantly to determine whether coexistence is maintained, or exclusion occurs. Self-limiting *Ae. aegypti* releases and reproductive interference also affect the population size of one or both species. Therefore, in locations where the distributions of *Ae. aegypti* and *Ae. albopictus* overlap, both the behavioural ecological and population ecological effects of self-limiting releases have important consequences for the efficacy of vector control programmes.

To investigate the release of self-limiting *Ae. aegypti*, I used a proportional release policy (Atkinson et al., 2007) where, at each time point, the number of self-limiting *Ae. aegypti* is proportional to the number of wild *Ae. aegypti*. To conduct proportional releases in the field requires constant monitoring of mosquito populations. There are well established techniques to monitor *Aedes* populations and entomological surveys are necessary following the release of self-limiting insects, in order to monitor efficacy (Alphey et al., 2010). The proportional release policy has been investigated in previous theoretical studies on self-limiting control (Atkinson et al., 2007; Alphey, Bonsall & Alphey, 2011; Watkinson-Powell & Alphey, 2017). Under this release policy fewer self-limiting mosquitoes need to be released to eradicate the target organism than in a constant release scenario, where the same number of self-limiting mosquitoes are released at each time step (Atkinson et al., 2007). As such, there could be economic benefit of using the proportional release policy, especially in areas where there is already public health monitoring of mosquitoes that is sufficient to estimate mosquito density, so no additional monitoring costs accrue. Economic benefits are an important consideration. In a review by Alphey *et al.* (2010), cost-effectiveness was highlighted as a technical aspect of self-limiting insect release that requires further

investigating. One approach could be to compare different release and control strategies and their associated costs to understand the implications of self-limiting releases on biodiversity patterns such as competition and coexistence. This is important, as results are likely to vary dependent on the release strategy. Atkinson *et al.* (2007) found that compared with a proportional policy, a policy where a constant number of mosquitoes are released requires a minimum of 1.5x more mosquitoes to be released to achieve eradication.

4.1. Population Size

I examined the impact of increasing the ratio of self-limiting *Ae. aegypti* on the population sizes of *Ae. aegypti* and *Ae. albopictus*. Self-limiting *Ae. aegypti* mate with their wild counterparts to produce non-viable offspring, thus these self-limiting insects supress the population density of *Ae. aegypti* by decreasing recruitment. This is shown in my analyses, where increasing the ratio of self-limiting *Ae. aegypti* decreases the population density of *Ae. aegypti* when there is coexistence, or where *Ae. aegypti* excludes *Ae. albopictus*. As there are fewer *Ae. aegypti* to compete with *Ae. albopictus* for resources, or interfere with their mating, the population size of *Ae. albopictus* increases with the self-limiting *Ae. aegypti* release ratio when there is coexistence. This result holds when self-limiting *Ae. aegypti* reproductively interfere with *Ae. albopictus,* and when they do not cause reproductive interference: thus, this outcome is governed by the decrease in the wild *Ae. aegypti* population size, rather than any additional increase in reproductive interference by self-limiting *Ae. aegypti*.

Following the release of self-limiting *Ae. aegypti*, where *Ae. albopictus* persists, it will increase in population size either by excluding *Ae. aegypti* and reaching carrying capacity, or by increasing its population size in coexistence with *Ae. aegypti*. This is an important consideration: as highlighted by Bargielowski *et al.* (2013), *Ae. albopictus* is the principal vector of dengue in regions where *Ae. aegypti* is rare or uncommon (e.g. in China (Gratz, 2004; Wu et al., 2011; Almeida et al., 2005), Bangladesh (Ali et al., 2003) and South India (Denholm, Devine & Williamson, 2002; Ferreira-de-Lima & Lima-Camara, 2018)), and in regions of Africa native to *Ae. aegypti* that *Ae. albopictus* has recently colonised (Paupy et al., 2010). Therefore, in some regions, the decrease in public health burden caused by lowering the *Ae. aegypti* population may be lessened or compensated for by the associated increase in the *Ae. albopictus* population size. This highlights a limitation of using species specific pest control techniques (Alphey et al., 2013). In situations where both species coexist and are significant disease vectors, the release of both self-limiting *Ae. aegypti* and *Ae. albopictus* may be a more appropriate course of action (Alphey et al., 2010).

Additionally, the results of this study show that the strength of reproductive interference and interspecific competition affects the population densities of both species at coexistence. An increase in the strength of reproductive interference or interspecific competition reduces the population size of the species experiencing the behavioural (reproductive interference) or ecological (interspecific competition) effects. For the former, this is due to fewer successful matings, reducing recruitment, and for the latter, more competition with heterospecifics for resources, increasing the number of deaths. The subsequent reduction in population size of the focal species allows the other species to increase in population

density, as there are fewer heterospecifics to compete with for resources, or to interfere with their mating.

However, previous work has shown *Ae. aegypti* may develop some resistance to reproductive interference. Bargielowski *et al.* (2013) showed that female *Ae. aegypti* from populations in allopatry with *Ae. albopictus* mis-mate more frequently than those from populations with a history of sympatry, suggesting that upon contact between *Ae. aegypti* and *Ae. albopictus, Ae. aegypti* females are selected for their ability to evade reproductive interference by *Ae. albopictus.* Thus, where *Ae. aegypti* and *Ae. albopictus* have come into contact more recently, *Ae. aegypti* may experience stronger reproductive interference. The results of this study suggest this will cause a lower population size of *Ae. aegypti*: thus recent contact with *Ae. albopictus* means fewer self-limiting *Ae. aegypti* need to be released to have the equivalent impact upon the *Ae. aegypti* population size. These areas could, therefore, be targeted at a lower economic burden. This is particularly relevant in regions where *Ae. albopictus* is not a disease vector.

4.2. Coexistence and Exclusion Boundaries

The results of this study show that the ratio of self-limiting *Ae. aegypti* released determines whether coexistence is possible. A high enough release ratio of *Ae. aegypti* destabilises coexistence across all strengths of reproductive interference and interspecific competition, thus the only stable outcome is exclusion. However, at lower release ratios, coexistence is still possible. Here, the ratio of self-limiting *Ae. aegypti,* and whether the self-limiting *Ae. aegypti* cause reproductive interference, determines the strength of the reproductive

interference and resource competition that can be experienced by *Ae. aegypti* and *Ae. albopictus*, and coexistence maintained.

When self-limiting *Ae. aegypti* reproductively interfere with *Ae. albopictus*, coexistence can occur when each individual *Ae. aegypti* causes weaker reproductive interference and interspecific competition, than when only wild *Ae. aegypti* reproductively interfere with *Ae. albopictus*. This is because, there are additional individuals (the self-limiting *Ae. aegypti*) that reproductively interfere with *Ae. albopictus*. This increases the total reproductive interference experienced by the *Ae. albopictus* population for the same strength of reproductive interference imposed by each individual *Ae. aegypti*.

As discussed, an increase in the ratio of self-limiting *Ae. aegypti* causes the population size of *Ae. aegypti* to decrease, meaning there are fewer *Ae aegypti* to interfere with the mating of *Ae. albopictus* or compete with them for resources. Thus, as the release ratio increases, each individual *Ae. aegypti* can reproductively interfere and compete for resources increasingly strongly with *Ae. albopictus*, without changing the total ecological effects experienced by the *Ae. albopictus* population. As the population size of *Ae. albopictus* increases with the release ratio of self-limiting *Ae. aegypti*, the reverse occurs to the per capita reproductive interference and competition for resources caused by *Ae. albopictus*. For these reasons, up to a moderate release ratio of *Ae. aegypti*, I found that as the ratio of self-limiting *Ae. aegypti* increases, coexistence can occur when the reproductive interference and resource competition suffered by *Ae. albopictus* is increasingly high and that suffered by *Ae. aegypti* is increasingly low.

However, meta-analyses suggest that *Ae. albopictus* are more likely to experience weaker reproductive interference, and interspecific resource competition than *Ae. aegypti* (Juliano, 2010; Lounibos & Juliano, 2018). A meta-analysis by Juliano (2010) showed *Ae. albopictus* have a competitive advantage over *Ae. aegypti* when there is low food quality, and high food quality results in competitive equivalence. Furthermore, *Ae. aegypti* is more likely to mis-mate than *Ae. albopictus* (Marcela et al., 2015; Bargielowski et al., 2015) and following mis-mating, female *Ae. aegypti* can become refractory to further mating (Robbins et al., 2011). This asymmetry is likely to be due to the greater species recognition abilities of *Ae. albopictus* (Gröning & Hochkirch, 2008; Marcela et al., 2015). This suggests that in the wild, as the release ratio of self-limiting *Ae. aegypti* increases, coexistence will only occur where both species experience an increasingly low strength of reproductive interference and interspecific resource competition, although coexistence is theoretically possible when *Ae. albopictus* suffers more than *Ae. aegypti*.

However, it is plausible that there are regions where the reproductive interference and resource competition experienced by *Ae. aegypti* is sufficiently weak for coexistence to occur, even when self-limiting *Ae. aegypti* are released. For instance, coexistence could be facilitated in areas with high food quality, where the competitive advantage of *Ae. albopictus* is reduced, resulting in lower interspecific competition (Juliano, 2010). Furthermore, *Ae. aegypti* may experience reproductive interference that is weak enough to allow coexistence following the release of self-limiting *Ae. aegypti*. For instance, in a coexistence region in Brazil, *Ae. albopictus* only weakly reproductively interferes with *Ae. aegypti* (Honório et al., 2018). Equally, as discussed, in regions where *Ae. aegypti* initially experiences strong reproductive interference, the *Ae. aegypti* strain could develop resistance to reproductive

interference prior to exclusion (Bargielowski et al., 2015). Additionally, in the wild, habitat partitioning in time or space could allow reproductive interference to be avoided and coexistence to be maintained (Noriyuki & Osawa, 2016). Thus, the release of self-limiting *Ae. aegypti* could initially destabilise coexistence, but subsequent development of resistance to mis-mating in *Ae. aegypti* females, or habitat partitioning, could recover stable coexistence.

4.3. Future Work

I suggest further theoretical work should be conducted. Specifically, these models could be further developed to examine the ability of *Ae. aegypti* and *Ae. albopictus* to invade a patch containing a heterospecific. This invasion model would need to be spatially explicit and include stochasticity. Where *Aedes* coexist, the environment is generally heterogeneous, with only some areas containing suitable oviposition sites (Tedjou et al., 2019; Honório et al., 2009; Bennett, McMillan & Loaiza., 2019) . This, in combination with the short dispersal distance of *Ae. aegypti* (Edman et al., 2005; Hemme et al., 2010) and *Ae. albopictus* adults (Bellini et al., 2010; Marni et al., 2010; Lacroix et al., 2009), results in both species having a patchy distribution, highlighting the necessity for the addition of a spatial component. The inclusion of demographic stochasticity in this model would account for random variation in demographic processes. This is particularly important to include into models examining small population sizes, which is true of invading species, as demographic stochasticity has a greater effect on small populations (Lande, Engen & Saether, 2003).

Furthermore, an epidemiological model could be formed to analyse the combined impact of self-limiting *Ae. aegypti* release and reproductive interference on disease spread. These

models would allow the public health implications of the release of self-limiting *Ae. aegypti* to be evaluated in scenarios where *Ae. albopictus* is also a principal disease vector.

This study highlighted that *Ae. aegypti* and *Ae. albopictus* population dynamics are sensitive to the strength of reproductive interference. Thus, I suggest further investigation into how the strength of reproductive interference varies between different strains of *Ae. aegypti* and *Ae. albopictus*. This would allow reproductive interference to be accurately included in models of vector control. I do this in the following chapter, *Chapter 3*, where I examine how historic exposure to *Ae. albopictus* impacts the rate of heterospecific mating, an important component of reproductive interference, in strains of *Ae. aegypti* females previously uncharacterised in the laboratory.

5. Conclusion

Previous models have examined the impact of reproductive interference (Kuno, 1992; Kishi & Nakazawa, 2013; Paton & Bonsall, 2019) and self-limiting insect release (Bonsall et al., 2010) on the population dynamics between the vectors *Ae. aegypti* and *Ae. albopictus*. However, this study is the first to address the combined impact of self-limiting insect release and reproductive interference upon *Ae. aegypti* and *Ae. albopictus* coexistence. I find that the strength of reproductive interference and the ratio of self-limiting *Ae. aegypti* are important factors that act together to determine the population size of both *Ae. aegypti* and *Ae. albopictus* and whether coexistence can occur. This highlights the importance of including behavioural ecological processes, such as reproductive interference, into population dynamic frameworks to evaluate the efficacy of vector control. I suggest that further theoretical work could be conducted to include stochasticity, a spatial component and epidemiology. Furthermore, future studies could examine how the strength of reproductive interference varies between strains of *Ae. aegypti* and *Ae. albopictus*, so that reproductive interference can be accurately included in models of vector control.

Acknowledgements

I would like to thank two anonymous referees for their detailed suggestions for improving this work.

Chapter 3: Female Resistance to Heterospecific Insemination in *Ae. aegypti* Allopatric and Sympatric with *Ae. albopictus*

Abstract

Heterospecific mating occurs between *Ae. aegypti* and *Ae. albopictus* and does not result in the production of viable offspring. Heterospecific mating is particularly costly to *Ae. aegypti* females: following insemination by *Ae. albopictus* males, *Ae. aegypti* females become refractory to further mating. Previous studies have shown that to evade this fitness cost, *Ae. aegypti* females can evolve to resist heterospecific mating following exposure to *Ae. albopictus*. Thus, *Ae. aegypti* females in sympatry with *Ae. albopictus* are inseminated by *Ae. albopictus* males at a lower rate than allopatric *Ae. aegypti* females. In this study, I aim to determine whether, in strains previously uncharacterised in the laboratory, females from sympatric *Ae. aegypti* strains have evolved to resist heterospecific mating. To do this, I exposed females from sympatric and allopatric *Ae. aegypti* strains to *Ae. albopictus* males, and then determined the heterospecific insemination rate. I predicted that females from sympatric *Ae. aegypti* strains would resist heterospecific mating, and thus have a lower heterospecific insemination rate than the allopatric *Ae. aegypti* strains. However, I found that *Ae. albopictus* males did not inseminate females from any of the strains of *Ae. aegypt*i. Thus, even females from strains of *Ae. aegypti* with no prior exposure to *Ae. albopictus* were not inseminated by *Ae. albopictus* males. I hypothesise that my results differ to previous studies due to strain-specific differences in female *Ae. aegypti* resistance behaviour, or male

Ae. albopictus mating behaviour. This study highlights the necessity to conduct studies to determine the behaviours that result in resistance to heterospecific mating.

1. Introduction

Aedes aegypti and *Aedes albopictus* are two invasive species of mosquito that are principal vectors of flaviviruses, including dengue and chikungunya. Although these species are native to different continents, range expansion has caused frequent overlap in their spatial distribution. Where they co-occur, competitive interaction either results in exclusion or stable coexistence (Lounibos & Juliano, 2018). In most cases, *Ae. albopictus* has a competitive advantage over *Ae. aegypti*, which results in the exclusion of *Ae. aegypti* by *Ae. albopictus* (Juliano, 2010). Previous work has shown that female *Ae. aegypti* can erroneously mate with male *Ae. albopictus*, due to incomplete species recognition. These matings do not result in viable offspring.

Multiple studies have shown female *Ae. aegypti* to be susceptible to heterospecific mating by *Ae. albopictus*. Heterospecific mating occurs in wild, sympatric populations of *Ae. aegypti* and *Ae. albopictus*, but the rates of heterospecific insemination are low. Tripet *et al.* (2011) found that heterospecific mating occurs in 1.6% of wild *Ae. aegypti* in Florida, and Bargielowski *et al.* (2015) found that heterospecific mating occurs in 1.12-3.73% of wild *Ae. aegypti* in populations from Venezuela, Gabon, USA and Singapore. In laboratory studies, the cross-insemination of *Ae. aegypti* can be much higher (Bargielowski & Lounibos, 2014; Bargielowski et al., 2019, 2015; Marcela et al., 2015; Honório et al., 2018).

Heterospecific mating is costly to *Ae. aegypti* females, as the transfer of accessory gland proteins (Acps) from male *Ae. albopictus* causes *Ae. aegypti* females to be refractory to

further mating (Klowden, 1999; Robbins et al., 2011; Leahy & Craig Jr., 1965). Previous comparative and evolutionary studies have shown that to evade this fitness cost, *Ae. aegypti* females can evolve to resist heterospecific mating. In nature, female *Ae. aegypti* from populations sympatric with *Ae. albopictus* are significantly less likely than nearby allopatric populations to mate with heterospecific males (Bargielowski et al., 2015). Furthermore, in cage trials, *Ae. aegypti* previously unexposed to *Ae. albopictus* evolved resistance to reproductive interference within 1-3 generations of cage exposure to *Ae. albopictus* (Bargielowski & Lounibos, 2014).

It has been reported that female resistance to heterospecific mating is not specific to the strain of *Ae. albopictus* that the *Ae. aegypti* females have been exposed to (Maïga et al., 2020). For example, *Ae. aegypti* females from La Réunion Island which have had prolonged contact with *Ae. albopictus* males, exhibit resistance to heterospecific mating with *Ae. albopictus* males from multiple different populations (Maïga et al., 2020). This resistance has been shown to extend to *Ae. albopictus* males from different geographic origins to the *Ae. aegypti* females (Maïga et al., 2020). However, the rate of insemination of *Ae. aegypti* females by *Ae. albopictus* males can vary, depending on the origin of the male *Ae. albopictus*. For example, *Ae. albopictus* males from Brazillian cities inseminated *Ae. aegypti* females at a lower rate than *Ae. albopictus* males from Florida (Honório et al., 2018).

The precise mechanisms of resistance to heterospecific males are unknown. Until relatively recently, most work on mosquito mating systems has focused on scramble competition between males for females (Cator, Wyer & Harrington, 2021). However, recent work on *Ae. aegypti* mosquitoes has highlighted the role of female choice in determining the success of

mating attempts between conspecifics (Aldersley & Cator, 2019; Cator & Harrington, 2011). In all mating attempts, in the first few seconds of male contact, females actively resist mating by either kicking the male away or holding the male away from her body, preventing genital contact (Aldersley & Cator, 2019). Most mating attempts are unsuccessful, and males that successfully mate with female *Ae. aegypti* are kicked at a lower rate (Aldersley & Cator, 2019). Thus, female choice in the early post-contact stages of an interaction is a key determinant of male mating success within this species (Aldersley & Cator, 2019). Whether similar behavioural responses contribute to resistance to mating by *Ae. albopictus* males is unknown.

In addition to the mechanisms females use to resist heterospecific mating attempts, the cues females utilize to distinguish between potential mates remain unclear. Female *Ae. aegypti* may use acoustic mating signals to assess the quality of a conspecific male and determine the extent to resist the potential mate, but this remains an open area of investigation (Cator & Harrington, 2011; Aldersley & Cator, 2019; League et al., 2021).

Resistance to reproductive interference is likely costly. Bargielowski *et al.* (2019) found that when *Ae. aegypti* females are no longer exposed to *Ae. albopictus* males, there was a rapid decrease in resistance to reproductive interference over eight generations. In cage studies, female *Ae. aegypti* that have evolved resistance to reproductive interference are also slower to mate with conspecific males (Bargielowski & Lounibos, 2014). This suggests that females with resistance to reproductive interference substantially modify their general mating behaviour, investing more into mate assessment and selection. Thus, a key cost of evolving resistance to reproductive interference could be the increased latency to mating with

conspecifics, and the associated energetic investment. When heterospecifics are present, there may be a net benefit of the female being 'more choosy': the benefit of not mating with heterospecifics is greater than the cost associated with a decreased rate of conspecific mating. Thus, when there are no heterospecifics, the benefit is removed and the cost remains, leading to the loss of the resistance trait in female *Ae. aegypti*.

This study will examine how historic exposure to *Ae. albopictus* affects the rate of heterospecific insemination in three strains of *Ae. aegypti* that have not previously been characterised. One of these *Ae. aegypti* strains has evolved in the presence of *Ae. albopictus*, while the other two strains of *Ae. aegypti* have no prior exposure to *Ae. albopictus*. I expect the strains previously exposed to *Ae. albopictus* to have evolved resistance to heterospecific mating, and the unexposed strain to be susceptible to heterospecific mating.

Following this, I had planned to conduct an evolution experiment to artificially select susceptible *Ae. aegypti* females to evolve resistance to heterospecific mating. The aim of this was to characterise the behavioural mechanisms underlying resistance to heterospecific insemination, while avoiding any between-strain differences in behaviour. However, the results of the initial strain characterisation meant that this experiment could not be conducted (see supplementary information for the methods of the planned experiment).

Experiment 1

2. Hypotheses, Experiment 1

Hereafter, *Ae. aegypti* that have historically been exposed to *Ae. albopictus*, are discussed as 'exposed' and *Ae. aegypti* that have not historically been exposed to *Ae. albopictus* are discussed as 'unexposed'.

(i) Heterospecific Insemination

I expect that unexposed *Ae. aegypti* females will be inseminated by male *Ae. albopictus*. These *Ae. aegypti* females have no historical exposure to *Ae. albopictus,* therefore I predict they will not have evolved resistance to heterospecific mating.

Furthermore, I predict that exposed *Ae. aegypti* females will have a lower rate of insemination by male *Ae. albopictus* than unexposed *Ae. aegypti*, or will not be inseminated by male *Ae. albopictus* at all. I expect exposed *Ae. aegypti* to have evolved resistance to heterospecific mating, due to their prior exposure to *Ae. albopictus* males.

(ii) Insemination by conspecifics of a different origin

I expect that exposed *Ae. aegypti* females will be inseminated at a lower rate by conspecific males from different origins, than unexposed *Ae. aegypti* females. This is because I predict

that exposed *Ae. aegypti* females will be generally more choosy of mates, due to their resistance to heterospecific mating.

3. Methods

3.1. *Aedes* strain Information

Initially, in experiment 1 (3.1.1.), I used two strains of *Ae. aegypti*, and one strain of *Ae. albopictus*. Following my results from experiment 1, I repeated the experiment using a further strain of *Ae. aegypti* and *Ae. albopictus,* in experiment 2 (3.1.2.) to clarify my findings.

3.1.1. Experiment 1

I established populations from two strains of *Ae. aegypti*: one strain which has not historically overlapped with *Ae. albopictus* ('unexposed') and one strain which has ('exposed').

The unexposed strain of *Ae. aegypti* originated from a field population in Maricopa County, Arizona (AEG_{AZ}). AEG_{AZ} have been reared in The Huijben Lab, Arizona State University since 2019. I was supplied with a mixture of F17 and F18 eggs by Sarah Rydeberg, Center for Evolution and Medicine, Arizona State University.

The exposed strain was collected from ovitraps in Comuna 13 in Medellín, Colombia (AEG_{COL}), a region where *Ae. albopictus* are also present. The eggs used in this experiment are F5.

To conduct heterospecific matings, I used a strain of *Ae. albopictus* from Colombia ($ALBO_{COL}$) collected in the same city as AEG_{COL}: Medellín, Colombia. $ALBO_{COL}$ were collected from a natural site in the Jardín Botánico de Medellín and the eggs used in this experiment are F2.

Both AEG_{COL} and $ALBO_{COL}$ were collected by Catalina Alfonso Parra, Max Planck Tandem Group, Universidad of Antioquia, who also conducted the laboratory colonisation. Following the results of experiment 1, I repeated the experiment using additional mosquito strains.

3.1.2. Experiment 2

In experiment 2, I used the strains used in experiment 1, alongside one further *Ae. aegypti* strain and one further *Ae. albopictus* strain.

The additional *Ae. aegypti* strain is the widely used Liverpool strain (AEG_{LIV}). This strain was originally collected in West Africa (country unknown[2]) and has been maintained in laboratory colony since 1938, and thus has had substantial inbreeding. Due to the prolonged period in the laboratory without contact with *Ae. albopictus*, this strain is 'unexposed'.

The additional *Ae. albopictus* strain was collected from Montpellier, France, an area where *Ae. aegypti* are not present, in 2016. This strain ($ALBO_{MP}$) has since been maintained in the laboratory for more than 20 generations.

[2] Freetown, Sierra Leone is the most likely source of this strain (Kuno, 2010)

Between experiments 1 and 2, AEG_{COL} was put through 2 more generations in the laboratory and $ALBO_{COL}$ was put through 1 more generation in the laboratory, to ensure there were sufficient eggs for the experiment and to maintain a stock.

A summary of the strains used in experiments 1 and 2 is in table 3.1.

Name	Origin	Exposure to *Ae. albopictus*	Generations in laboratory	
			Experiment 1	Experiment 2
AEG_{COL}	Colombia	Exposed	F5	F7
AEG_{AZ}	Arizona	Unexposed	F17/F18	F17/F18
AEG_{LIV}	West Africa	Unexposed	NA	>F100
$ALBO_{COL}$	Colombia	NA	F2	F3
$ALBO_{MP}$	Montpellier	NA	NA	>F20

Table 3.1: summary of the Ae. aegypti strains used in experiments 1 and 2. AEG stands for Ae. aegypti and ALBO stands for Ae. albopictus, and the Ae. albopictus rows are shaded in grey. The subscript refers to their strain name (COL = Columbia, AZ = Arizona, LIV = Liverpool, MP = Montpellier). Exposure refers to previous contact with Ae. albopictus (exposed = evolved in sympatry with Ae. albopictus, unexposed = evolved in allopatry with Ae. albopictus). AEG_{LIV} *and* $ALBO_{MP}$ *were only used in experiment 2.*

3.2. Rearing

Each mosquito strain was reared separately. Dried egg papers that were at least one week old were used for hatching. This allows sufficient time the eggs to fully embryonate, which is required before hatching can occur. Egg papers were placed in a hatch flask labelled with species, date, strain, generation, and the initials of the experimenter. Gloves were used when handling eggs, and new gloves were used for each different egg strain. The egg papers were submerged with just enough water to cover the eggs and were placed under a vacuum for 20 minutes, using a LAX 1 Stage Vacuum Pump. Hatch flasks were given one crushed pellet of *Hikari Tropical Fish Food Cichlid Gold* pellets and placed inside a 27 °C and 70% relative humidity incubator overnight.

The following day, first-instar larvae were put into 500ml plastic tubs in groups of 250 larvae. Until pupal emergence, larval trays were fed 6 medium pellets of *Hikari Tropical Fish Food Cichlid Gold* per day.

Due to the number of virgin males and females required for the experiment, some were sexed at the pupal stage, and some at the adult stage. Pupae were sexed under the dissection microscope, using differences in the genital lobe shape to differentiate between sexes (figure 7,Carvalho *et al.*, 2014). Pupae of the same sex and line were placed into emergence cups in groups of a maximum of 100 pupae. Cups were placed in a flight cage (22 x 8 x 16 cm plastic containers with an 18 x 6 cm mesh window in the lid) and monitored daily for emergence.

Adults were sorted when they were fewer than 24 hours old: thus, prior to reaching sexual maturity. A maximum of 100 pupae of the same line were placed in each emergence cup,

and 2 cups were placed in each cage (thus, a maximum of 200 adults per cage). Upon emergence, sex was determined by morphological differences: females have non-plumose, and males have plumose antennae. A mouth aspirator was used to move adults into cages of the same sex and strain.

Adult mosquitoes were provided with a 10% sucrose solution and held at 27 °C and 70% relative humidity on a D12:N12 (with 30 minutes of dawn/dusk separating each phase) circadian cycle throughout the experiment.

3.3. Colony Cages

When it was necessary to produce more eggs for experiment 2 (AEG_{COL}, $ALBO_{COL}$, $ALBO_{MP}$, AEG_{LIV}) , two colony cages were formed per strain. Larval rearing was conducted as previously detailed; however cages were not sex segregated, thus, colony cages were comprised of two cups of 100 pupae of mixed sex. However, in one instance there was insufficient pupae for this size of colony cage for AEG_{COL}, resulting in one cage of 179 pupae.

Following emergence, adults were left for at least a week to mate before being provided with a blood meal. One day prior to mating, the sucrose solution was removed, to increase the propensity of the mosquitoes to blood feed. Mosquitoes were provided with warmed (~37 °C) equine blood though a Hemotek feeding system (First Link Ltd, UK) for 1 hour. However, $ALBO_{COL}$ struggled to feed using the Hemotek feeding system, so instead Lauren Cator conducted an arm feed for this colony cage (Cator Lab SOP 2, Approved by Imperial College Health and Safety).

Two days later, the mosquitoes were provided with an egg laying substrate (damp filter paper in oviposition cup), which was left in the cage for a minimum of 3 days. Blood meals were repeated twice to increase the number of eggs collected. Prior to hatching, eggs were stored for at least a week to embryonate.

3.4. Determining Insemination Rates

In experiment 1, for each cross I determined the insemination rates for conspecific crosses between males and females from the same strain, conspecific crosses between males and females from different strains, and heterospecific crosses. However, in experiment 2, I did not examine crosses between conspecifics of different origins. Details of the crosses conducted in experiments 1 and 2 can be found in table 3.2.

For each replicate, five 4-11 day old virgin males and five 4-11 day old virgin females were placed in a 16oz cup with a mesh lid and provided with 10% sucrose solution. For each mating combination, 5 replicates were conducted. However, for each cross with $ALBO_{COL}$ males I conducted 3 replicates instead of 5 replicates, due to insufficient males.

After 1 week of exposure to virgin males, all females from each replicate cup were dissected to determine whether insemination had occurred. Females were briefly immobilized on ice, their spermathecae dissected, and the presence/ absence of sperm was determined using a compound microscope.

There is a lot of variation between studies regarding the experimental procedures used to determine the rates of heterospecific insemination (see *Chapter 5* for details). I made informed decisions about the experimental design in order to increase the likelihood of detecting between-group differences in insemination rates, if present.

I conducted more replicates than some key studies (e.g. Bargielowski et al., 2019, 2015; Bargielowski & Lounibos, 2014; Honório et al., 2018), resulting in more power to detect differences in the rate of insemination between groups. Furthermore, I used a great enough number of males to allow swarming behaviour to occur (Facchinelli et al., 2015; Gubler, Bhattacharya & Bhattacharva, 1972) and thus emulate natural mating conditions (Nelson, 1986; Yuval, 2006). Previous studies have shown that a greater male density increases the swarming behaviour of both *Ae. aegypti* and *Ae. albopictus* males (Marcela et al., 2015). In this study, I had a greater density of mosquitoes in each container (0.021 mosquitoes per cm^3) than these same key studies (0.0089-0.011 mosquitoes per cm^3) (Bargielowski et al., 2019, 2015; Bargielowski & Lounibos, 2014; Honório et al., 2018), to further promote the swarming behaviour required for mating.

Female strain	Prior exposure to *Ae. albopictus*	Male strain	Cross Type
AEG_{AZ}	Unexposed	AEG_{AZ}	Conspecific, same strain
	Unexposed	AEG_{COL}	Conspecific, different strain
	Unexposed	$ALBO_{COL}$	Heterospecific
	Unexposed	$ALBO_{MP}$	Heterospecific
AEG_{COL}	Exposed	AEG_{COL}	Conspecific, same strain
	Exposed	AEG_{AZ}	Conspecific, different strain
	Exposed	$ALBO_{COL}$	Heterospecific
	Exposed	$ALBO_{MP}$	Heterospecific
AEG_{LIV}	Unexposed	AEG_{LIV}	Conspecific, same strain
	Unexposed	$ALBO_{COL}$	Heterospecific
	Unexposed	$ALBO_{MP}$	Heterospecific

Table 3.2: summary of the crosses in experiment 1 and experiment 2. AEG and ALBO stand for Ae. aegypti and Ae. albopictus respectively and their subscript refers to their strain (AZ = Arizona, COL = Columbia, LIV = Liverpool, MP = Montpellier). The crosses conducted only in experiment 1 are shaded in blue, the crosses only conducted in experiment 2 are shaded in grey, and crosses that occurred in both experiments are not shaded.

3.5. Analysis

All analyses were conducted in *R* version 4.3.1 (R Core Team, 2023). The package *lme4* (Bates, Mächler & Dai, 2011) was used to fit linear and generalised linear mixed-effects models. The package *MASS* (Venables & Ripley, 2002) was used to conduct likelihood ratio tests, and produce p-values for fixed effects. Data were processed using *dplyr* (Wickham et al., 2023) and *tidyr* (Wickham, Vaughan & Girlich, 2023) and all graphs were formed using *ggplot2* (Wickham, 2016).

3.5.1. Insemination Rate

In experiments 1 and 2, I examined the effects of Mating Type (Conspecific / Heterospecific) on the likelihood of female insemination.

For both experiments, I then conducted further analyses to examine differences between conspecific crosses. In experiment 1, I examined the effect of conspecific origin (whether males and females are from the same strain, or different strains), male strain (AEG_{COL}, AEG_{AZ}) and female strain (AEG_{COL}, AEG_{AZ}) individually on the probability of female insemination in conspecific crosses. However, in experiment 2 conspecific crosses were only conducted between males and females from the same origin, thus, I could not examine the impact of conspecific cross type, female strain or male strain. Instead, I examined the effect of cross (AEG_{COL} x AEG_{COL}; AEG_{AZ} x AEG_{AZ}; AEG_{LIV} x AEG_{LIV}) on the probability of female insemination in conspecific crosses.

Analyses were conducted using generalised linear mixed effects models (GLMMs) with a binomial response variable and logit link function. Cup was included as a random effect.

3.5.2. Convergence of models

For all models, if a group of data being analysed was comprised of all 0s, a GLMM was inappropriate as a distribution cannot be defined from 0s alone, and the model would not converge. In these instances, a simpler statistical test (e.g. a Chi-squared test) was used.

4. Results, Experiment 1

I found that none of females from heterospecific crosses were inseminated, thus both unexposed and exposed females were resistant to heterospecific mating. However, insemination rates for conspecific crosses were high (with means between 62% and 92%, per cross). This is shown in figure 3.1.

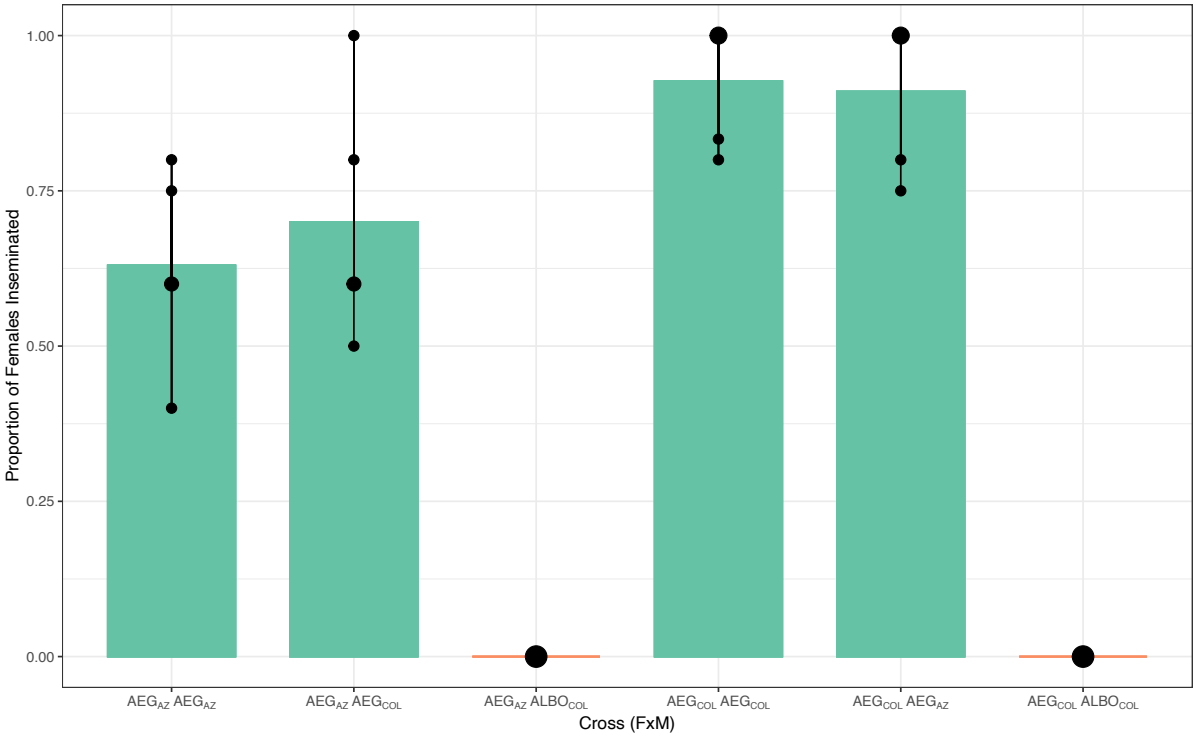

Figure 3.1: Differences in the proportion of females inseminated between crosses. The height of each bar shows the mean proportion of females inseminated, per cross. The colour of the bar corresponds to the cross type (green conspecific, orange heterospecific). Each point represents an individual cup, and the size of the point corresponds to the number of cups with that proportion of females inseminated for that cross. A vertical line connects all the points for a specific cross. The cross is written F/M, such that AEG_{AZ} AEG_{COL} represents AEG_{AZ} females crossed with AEG_{COL} males.

As the heterospecific group is formed of all 0s, I conducted a Chi-square test to assess the impact of mating type (conspecific/heterospecific) on insemination. Mating type has a significant effect on the number of females inseminated (X^2= 75.62, df = 1, $P < 2.20x10^{-16}$), with more females being inseminated in conspecific crosses than heterospecific crosses, as seen in figure 3.1.

The data was then filtered to remove heterospecific crosses, to determine any differences within conspecific crosses. Female strain had a significant impact on insemination rate (P = 0.0017), with AEG_{COL} females being inseminated more frequently than AEG_{AZ} females (coeff = 1.73, SE = 0.30, P = 0.0039). Male strain, however, had no impact on the likelihood of insemination (P = 0.67). Furthermore, I found no difference in the insemination rates of females when crossed with conspecifics from the same strain, and conspecifics from a different strain (P = 0.82).

Our results from experiment 1 show that *Ae. aegypti* females are resistant to heterospecific mating, even when they are from a strain with no prior exposure to *Ae. albopictus* males. These results were unexpected, as previous literature suggest that *Ae. aegypti* females from strains with no previous exposure to *Ae. albopictus* should be susceptible to mating by *Ae. albopictus* males, and those that have been previously exposed to *Ae. albopictus* should have a level of resistance to mating by *Ae. albopictus* males.

Thus, I expected standing levels of resistance to heterospecific mating in AEG_{COL}, due to its previous exposure to *Ae. albopictus*, and I expected AEG_{AZ} females to be susceptible to mating by *Ae. albopictus*, as they had no prior exposure to *Ae. albopictus*. However, my

results show no heterospecific insemination to occur in either AEG_{AZ} or AEG_{COL}, and thus no differences in insemination between the strains.

To confirm my results, I repeated the insemination experiment using additional strains of *Ae. aegypti* and *Ae. albopictus*.

5. Rationale, Experiment 2

Previous research shows that *Ae. aegypti* females with prior exposure to *Ae. albopictus* males are resistant to heterospecific mating, and unexposed females are susceptible to heterospecific mating (Bargielowski et al., 2019; Bargielowski & Lounibos, 2014). My results from experiment 1 contradict these findings, however they need to be clarified in two respects.

Firstly, there is the possibility that AEG_{AZ} females evolved resistance to heterospecific mating following exposure to males from an *Aedes* species other than *Ae. albopictus* or following an unrecorded contact with *Ae. albopictus*. To control for this, I included females from the Liverpool strain of *Ae. aegypti* (AEG_{LIV}) in my second experiment. AEG_{LIV} have been in the laboratory without exposure to other *Aedes* species for hundreds of generations. Thus, I expect AEG_{LIV} to be fully susceptible to heterospecific mating. If this result is observed, it would suggest that AEG_{AZ} evolved resistance to heterospecific mating due to exposure to males from another *Aedes* species.

Secondly, I used an additional strain of *Ae. albopictus* from Montpellier, $ALBO_{MP}$, in my second experiment. A previous study showed that *Ae. albopictus* males from Brazil were significantly worse at mating with *Ae. aegypti* females than *Ae. albopictus* males from Florida (Honório et al., 2018). Thus, I also used $ALBO_{MP}$ males, in case the $ALBO_{COL}$ males used in experiment 1 were particularly bad at mating with heterospecific females. If more

heterospecific females are inseminated when crossed with $ALBO_{MP}$, this would suggest that the results of experiment 1 are due to $ALBO_{COL}$ males being bad mates.

In experiment 2, I examined heterospecific crosses, and conspecific crosses with mates from the same origin. I did not include crosses between conspecifics of different origins as experiment 1 showed no impact of conspecific origin upon the proportion of females inseminated.

6. Results, Experiment 2

Overall, my results from experiment 2 follow the same trends as my results from experiment 1, as seen in figure 3.2. I found that no females were inseminated in heterospecific crosses, apart from the cross between AEG_{AZ} females and $ALBO_{COL}$ males, which resulted in a low proportion of females inseminated. Here, one AEG_{AZ} female in two separate cups was inseminated, giving a total of 2 inseminations out of 117 heterospecific crosses. Furthermore, a high proportion of females were inseminated from conspecific crosses.

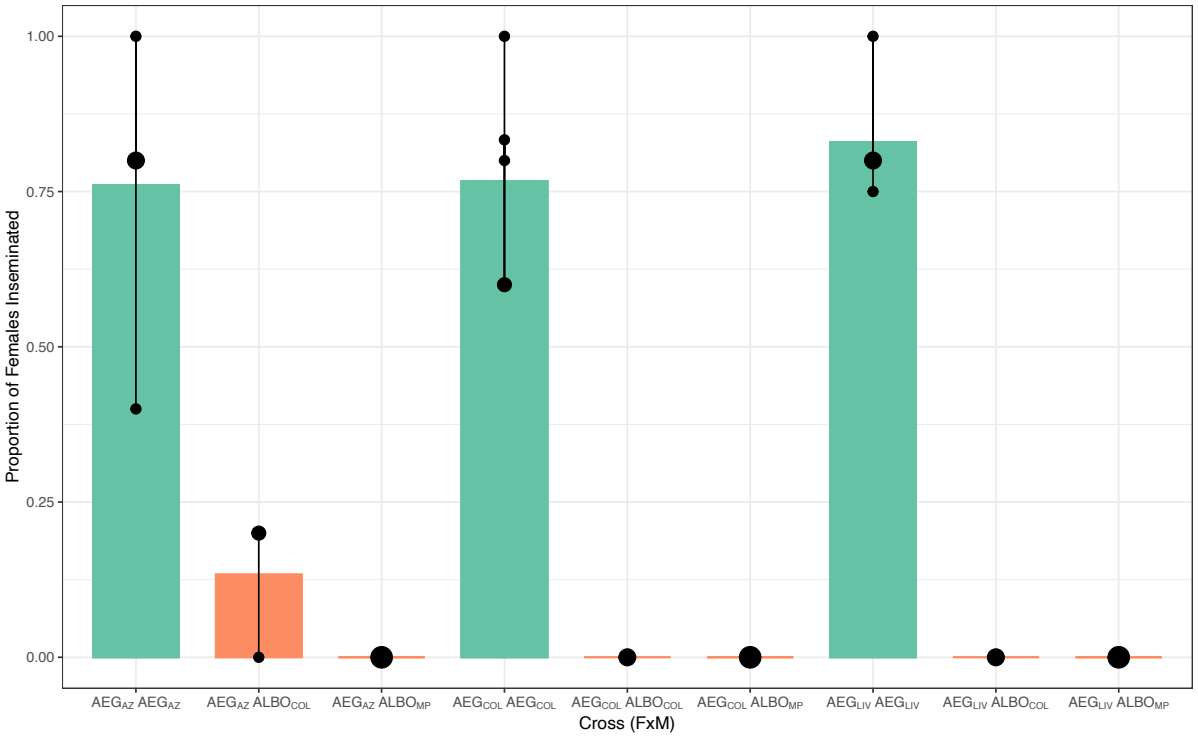

Figure 3.2: Differences in the proportion of females inseminated per cross. The height of each bar shows the mean proportion of females inseminated, per cross. The colour of the bar corresponds to the cross type (green conspecific, orange heterospecific). Each point represents an individual cup, where the size of the point corresponds to the number of cups with that proportion of females inseminated, for that cross. A vertical line connects all the

points for a specific cross. The cross is written F/M, such that AEG_{AZ} AEG_{COL} is AEG_{AZ} females crossed with AEG_{COL} males.

I conducted a logistic regression to determine if more females were inseminated from heterospecific crosses than conspecific crosses. As in experiment one, mating type had a significant effect on the number of females inseminated ($P = 1.16 \times 10^{-15}$): significantly fewer females were inseminated from heterospecific crosses, than conspecific crosses (coeff = -5.34, SE = 0.77, $P = 3.39 \times 10^{-12}$).

$ALBO_{MP}$ males inseminated no heterospecific females. This suggests that the lack of females inseminated by heterospecific ($ALBO_{COL}$) males in experiment 1 is not due to the $ALBO_{COL}$ strain being particularly bad at mating heterospecific females. Furthermore, as AEG_{LIV} has not been exposed to heterospecifics for hundreds of generations, I expected AEG_{LIV} females to be susceptible to heterospecific mating. However, no AEG_{LIV} females were inseminated by heterospecific males. In combination, these results suggest that without any previous exposure to heterospecifics, *Ae. aegypti* females can resist heterospecific males.

Within conspecific crosses, I determined the impact of cross on the proportion of females inseminated. I found no differences in the proportion of females inseminated between different crosses ($P = 0.69$).

7. Discussion

7.1. Overview

Previous studies have shown that *Ae. aegypti* from multiple sites in the USA that are allopatric with *Ae. albopictus* are susceptible to heterospecific mating (Bargielowski & Lounibos, 2014; Bargielowski et al., 2015). Prior to exposure to *Ae. albopictus*, the average heterospecific mating rate was 54.13%, 35.63% and 35.97% in Key West, Miami and Tucson *Ae. aegypti* strains, respectively, and these dropped to 14.68%, 11.81% and 9.43% after 5 generations of exposure to *Ae. albopictus* (Bargielowski & Lounibos, 2014).

This aim of this study was to examine how historic exposure to *Ae. albopictus* affects the rate of heterospecific insemination in strains of *Ae. aegypti* that have not previously been characterised in the laboratory. Initially, I used two strains of *Ae. aegypti*: a strain from Colombia that had previously been exposed to *Ae. albopictus* and a strain from Arizona with no prior exposure to *Ae. albopictus*. To conduct the heterospecific cross, I used a strain of *Ae. albopictus* sympatric with the Colombian *Ae. aegypti* strain. I expected that the unexposed line would have resistance to heterospecific mating, and the exposed line would be susceptible to heterospecific mating, and act as a positive control.

However, I had unexpected results: I found that the *Ae. albopictus* males did not inseminate females from the unexposed *Ae. aegypti* strain. This lack of heterospecific mating was not due to a general lack of mating: there were still high rates of conspecific insemination in this

strain of *Ae. aegypti*. This contradicts previous literature which showed unexposed *Ae. aegypti* to be susceptible to heterospecific mating (Bargielowski et al., 2015; Bargielowski & Lounibos, 2014).

Our results showed that *Ae. aegypti* females with no prior contact to *Ae. albopictus* are not inseminated by *Ae. albopictus* males. This suggests that unexposed *Ae. aegypti* females can be resistant to heterospecific mating. However, to show this more conclusively, I needed to rule out two alternative explanations.

Firstly, it is possible that the strain of *Ae. albopictus* males I used was particularly bad at heterospecific mating. Differences in interspecific mating competency was previously reported by Honório *et al.* (2018), who found that *Ae. albopictus* from two Brazilian cities were much less successful at inseminating *Ae. aegypti* females than *Ae. albopictus* males from peninsular Florida. Secondly, the unexposed *Ae. aegypti* strain may have been exposed to a heterospecific *Aedes* species other than *Ae. albopictus*, or there may have been unreported contact with *Ae. albopictus.* This exposure could have resulted in the evolution of resistance to heterospecific mating.

To address these alternative explanations, I repeated the insemination experiment using one further strain of *Ae. aegypti* and *Ae. albopictus*. The additional strain of *Ae. albopictus*, a laboratory strain originating in Montpellier, France, allowed us to determine whether the *Ae. albopictus* strain used in my initial experiment had particularly uncompetitive males. Furthermore, I used the Liverpool strain of *Ae. aegypti* that had been in the laboratory since

1938, without exposure to *Ae. albopictus* or any other *Aedes* species. Thus, this *Ae. aegypti* strain acted as an unexposed control.

I found that no *Ae. aegypti* females were inseminated by the additional strain of *Ae. albopictus* males, suggesting that the *Ae. albopictus* strains used in my initial experiment are not particularly uncompetitive mates. Furthermore, I found that the unexposed control *Ae. aegypti* strain was not inseminated by *Ae. albopictus*, suggesting that females from this unexposed strain also had resistance to heterospecific mating. As with the *Ae. aegypti* in my initial experiment, there were high rates of conspecific mating in the unexposed control *Ae. aegypti* strain. Furthermore, I found no insemination in the sympatric *Ae. aegypti* strain from my initial experiment, and a very low rate of interspecific insemination in the allopatric strain (1.7% heterospecific insemination).

Thus, the results of both my first and second experiment suggest that unexposed *Ae. aegypti* females can have resistance to heterospecific mating. I expected resistance to have evolved in the exposed *Ae. aegypti* females as heterospecific mating by *Ae. albopictus* males causes *Ae. aegypti* females to be refractory to further mating, and thus poses a substantial fitness cost (Klowden, 1999; Robbins et al., 2011; Leahy & Craig Jr., 1965). However, I only expected this resistance trait to be present in *Ae. aegypti* previously exposed to *Ae. albopictus*; resistance is costly, as it slows the rate of conspecific mating (Bargielowski & Lounibos, 2014).

Previous studies have shown that unexposed *Ae. aegypti* are susceptible to heterospecific mating, yet rapidly evolve resistance to heterospecific mating upon exposure to *Ae.*

albopictus (Bargielowski et al., 2015). However, they lose this resistance when no longer exposed to *Ae. albopictus* (Bargielowski et al., 2019). My results are, therefore, inconsistent with previous findings, and question the theory that resistance to heterospecific mating is a ubiquitously costly trait. I have multiple hypotheses to explain this discrepancy.

7.2. Possible Explanations

(i) Strain-specific female behaviour

There may be variation in female behaviour between different strains of *Ae. aegypti*, prior to exposure to *Ae. albopictus*. This could explain why Bargielowski and Lounibos (2014) observed susceptibility to heterospecific mating in three strains of *Ae. aegypti* previously unexposed to *Ae. albopictus* (two strains from Florida, and one from Tuscon), while this study observed resistance in two unexposed *Ae. aegypti* strains (one strain from Arizona, and one laboratory strain). As heterospecific mating has been studied in so few strains of unexposed *Ae. aegypti,* we cannot know whether resistance or susceptibility to is the more common mating behaviour, prior to exposure to *Ae. albopictus*.

Variation in female resistance to heterospecific mating questions the assumption that resistance is costly to females from all *Ae. aegypti* strains. When resistant *Ae. aegypti* from Key West, Florida and Tucson, Arizona are no longer exposed to *Ae. albopictus* males, there is a rapid decrease in their resistance to heterospecific mating (Bargielowski et al., 2019), suggesting that resistance to heterospecific mating is costly. However, I found that *Ae. aegypti* females previously unexposed to *Ae. albopictus* have resistance to heterospecific mating. This suggests that resistance to heterospecific mating can evolve and persist in the absence of the selection pressure of *Ae. albopictus* males, and thus that resistance may only be costly to some *Ae. aegypti* strains.

This is also supported by my findings that *Ae. aegypti* females resistant to heterospecific mating are equally likely to mate with conspecifics from the same strain, and a different strain. Thus, although Bargielowski and Lounibos (2014) found that the evolution of resistance to heterospecific mating resulted in a delay in conspecific mating in *Ae. aegypti* females, it is possible that in the *Ae. aegypti* strains I used, resistance to heterospecific mating does not result in greater general choosiness of mates and associated delay in mating.

However, even if resistance to heterospecific mating is costly in the *Ae. aegypti* strains I used, there are possible explanations for the persistence of this trait. One possibility is that there was an evolutionary trade-off during local adaptation of these strains that resulted in the evolution of resistance to heterospecific mating. Another option is that resistance to heterospecific mating could have reached fixation via genetic drift during a population bottleneck. However, it seems unlikely that drift would have resulted in the same outcome in both strains of unexposed *Ae. aegypti* in this study.

(ii) Strain specific male behaviour

So far, I have assumed that female choice determines the success of male *Ae. albopictus* during heterospecific mating, as female choice is key in determining mating success in conspecific *Ae. aegypti* matings (Aldersley & Cator, 2019; Cator & Harrington, 2011), and previous studies have shown that female *Ae. aegypti* can evolve to resistance to heterospecific mating (Bargielowski & Lounibos, 2014).

However, my results may indicate strain-specific differences in the behaviour of *Ae. albopictus* males. Previous studies have shown there is variation in the ability of *Ae. albopictus* males to mate with *Ae. aegypti* females (Honório et al., 2018). Thus, it is possible that only males from specific strains of *Ae. albopictus* attempt to mate with *Ae. aegypti* females. This could be due to differences in general mating aggressiveness or choosiness of male *Ae. albopictus*.

There is great variation in heterospecific insemination rates between different strains of *Ae. albopictus* males. For instance, there are high levels of heterospecific mating in *Ae. albopictus* males from two locations in the USA: East St. Louis, Illinois (Bargielowski & Lounibos, 2014; Bargielowski et al., 2019, 2015) and Vero Beach, Florida (Bargielowski, Blosser & Lounibos, 2015; Honório et al., 2018). Meanwhile, there are much lower rates of heterospecific mating in *Ae. albopictus* from two locations in Brazil: Rio de Janeiro and Manaus (Honório et al., 2018). Furthermore, in a study using laboratory strains of *Ae. aegypti* and *Ae. albopictus* from the Universiti Sains Malaysia (USM), *Ae. albopictus* were shown to inseminate *Ae. aegypti* females, but at a lower rate than the *Ae. albopictus* from the USA strains (Marcela et al., 2015). Thus, local selection pressures experienced by male *Ae. albopictus* may influence the competency of males to mate with females from another species, though there is little previous literature about this.

Male competency at heterospecific mating is unlikely depend on whether *Ae. albopictus* males are allopatric or sympatric with *Ae. aegypti*. Previous studies have shown both sympatric (Honório et al., 2018; Bargielowski, Blosser & Lounibos, 2015) and allopatric (Bargielowski & Lounibos, 2014; Bargielowski et al., 2015, 2019) *Ae. albopictus* males to be

competent at mating with *Ae. aegypti* females in laboratory trials. While one study found sympatric males to inseminate more heterospecific females in one group (Bargielowski et al., 2015), only one sympatric and one allopatric male strain was tested. Thus, it is unclear whether previous contact with *Ae. aegypti* females influences the mating behaviour of *Ae. albopictus* to heterospecific males.

The absence of heterospecific mating in my results could be due to a lack of mating attempts by males from the strains of *Ae. albopictus* that I used: one strain from Colombia that is sympatric with *Ae. aegypti*, and one laboratory strain originally collected from Montpellier in 2016, where it was allopatric with *Ae. aegypti*.

(iii) Effect of density or number of mosquitoes

Ae. albopictus and *Ae. aegypti* mate in swarms around hosts (Nelson, 1986; Yuval, 2006). Swarms can form with as few as 2 males (Facchinelli et al., 2015; Gubler, Bhattacharya & Bhattacharva, 1972), so to emulate swarm mating dynamics in the laboratory, 2 or more males are required. However, a greater male density increases the swarming behaviour of both *Ae. aegypti* and *Ae. albopictus* males (Marcela et al., 2015). Thus, the swarming behaviour of mosquitoes in this study may be different to previous studies, due to differences in mosquito density.

A few key studies (Bargielowski et al., 2019, 2015; Bargielowski & Lounibos, 2014; Honório et al., 2018) used very similar methods to assess the rates of heterospecific mating as I did in this study, though they used a much greater number of mosquitoes per replicate. While I

used 5 males and 5 females per replicate, these studies used between 240 and 300 mosquitoes, also with a 1:1 ratio of males and females. Although the number of mosquitoes is much greater in these studies, the density was lower due to the use of a much larger container. In these studies, a 30cm x30cm x30cm cage containing between 240 and 300 mosquitoes was used, which resulted in a density of between 0.0089-0.011 mosquitoes per cm^3. However, I used 473.18cm^3 cups, resulting in a higher density of 0.021 mosquitoes per cm^3. Therefore, if it is density, rather than number of mosquitoes that causes more swarming behaviour, this study is likely to have more swarming behaviour.

Marcela *et al.* (2015a) examined the rates of heterospecific mating when there were a range of different numbers (10, 20 and 30) of male *Ae. aegypti* or *Ae. albopictus* and a fixed number (20) of *Ae. aegypti* or *Ae. albopictus* females (Marcela et al., 2015). When examining crosses between *Ae. albopictus* males and *Ae. aegypti* females, they found that although the number of males increased the swarming behaviour there was no difference in insemination rates between when there were 20 (1:1 ratio of males to females) or 30 males (3:2 ratio of males to females), but there was consistently lowest insemination when there were only 10 males (1:2 ratio of males to females). However, it is unclear whether the lower density of males results in less insemination, or the lower proportion of males (Marcela et al., 2015). If it is the former, this would suggest that there should be a higher insemination rate in this study, than the previous studies with a lower male density.

However, there is the possibility that the number, rather than the density, of males impacts the rates of interspecific insemination. *Ae. aegypti* males and females interact acoustically during mating (Leahy and Craig 1967, Cator et al. 2009) and, due to their similar mating

ecology, *Ae. albopictus* may use similar techniques. Thus, the higher number of heterospecifics in a swarm may result in the masking of auditory signals, preventing accurate mate detection and resulting in a higher proportion of erroneous mating occurrences, and thus a higher rate of heterospecific mating. However, even in studies with a high number of males there is variation in the heterospecific mating rate (Honório et al., 2018), suggesting that mosquito density cannot explain all of the variation observed.

(iv) Effect of length of insemination period

In this study, to examine the rate of interspecific mating I exposed *Ae. aegypti* females to *Ae. albopictus* males for 1 week. Multiple previous studies had a much longer exposure period of 3 weeks (Bargielowski et al., 2019, 2015; Bargielowski & Lounibos, 2014; Honório et al., 2018), which could help explain why the rates of heterospecific mating are higher in these studies. However, a study using laboratory strains of *Ae. aegypti* and *Ae. albopictus* from the Universiti Sains Malaysian (USM) exposed *Ae. aegypti* females to males for 5 days and found much higher heterospecific mating rates than I did in this study, suggesting that the exposure length I chose in this study is not preventing heterospecific mating.

8. Conclusion

Our results show that *Ae. aegypti* females from different strains, previously exposed or unexposed to *Ae. albopictus* males, are not inseminated by *Ae. albopictus* males. This contradicts previous results, which showed that unexposed females are susceptible to insemination by heterospecifics, but exposed females have resistance to heterospecific mating. I hypothesise that my results differ to those previously found due to strain-specific differences in female *Ae. aegypti* rejection behaviour, or male *Ae. albopictus* mating behaviour.

To disentangle the behaviour leading to the lack of heterospecific insemination in the strains I have used, behavioural experiments are required. I suggest two behavioural experiments: one observed in real-time by an experimenter with free flying females and multiple males, and one with a single tethered female and one male, videoed in slow motion. The first allows more natural conditions to be emulated, while the second allows more fine-scale movements to be recorded. These experiments are conducted in the following chapter, *Chapter 4*. Additionally, to further understand differences in the results of studies of heterospecific insemination in *Aedes spp*, I suggest that a systematic literature review should be conducted. Thus, I conduct a systematic literature review in my final chapter, *Chapter 5*.

Supplementary Information

Description: details of the experiment I planned to conduct, prior to the results of strain characterisation.

The results from the initial strain characterisation (experiment 1) showed that the unexposed *Ae. aegypti* strain was resistant to heterospecific mating. As the planned experiment required a strain of *Ae. aegypti* that was susceptible to heterospecific mating, the experiment could not go ahead.

1. Aim

I plan to conduct tests on free-flying mosquitoes to examine mating interactions between *Ae. aegypti* females and *Ae. albopictus* males. I will artificially select susceptible female *Ae. aegypti* to evolve resistance to reproductive interference by exposing *Ae. aegypti* females to *Ae. albopictus* males. By evolving resistant lines, I will be able to make comparisons between the resistant and control lines, while avoiding any between-strain differences in behaviour.

Once the resistant lines have evolved, I plan to conduct 2 experiments:

(i) Compare the rates of conspecific insemination in resistant *Ae. aegypti* and control (susceptible) *Ae. aegypti* females to determine whether selection to avoid heterospecific mating affected conspecific mating rates.

(ii) Observe mating interactions, to determine the behavioural mechanisms underlying resistance to heterospecific insemination.

2. Hypotheses

Hypotheses (i - vi) are strongly expected as these outcomes have been shown multiple previous studies. Thus, I am confirming these findings using different strains of *Ae. aegypti* and *Ae. albopictus*. Hypotheses (vii - xiii) are more uncertain, as experiments to test these hypotheses have not previously been conducted.

Key strain information

The *Ae. aegypti* from Colombia (hereafter denoted AEG_{COL}) have historically been exposed to *Ae. albopictus*, while the *Ae. aegypti* from Arizona (hereafter denoted AEG_{AZ}) have not historically been exposed to *Ae. albopictus*.

2.1. Heterospecific Insemination

2.1.2. At generation 0, prior to exposure to *Ae. albopictus*

(i) AEG_{AZ} females will be inseminated by male *Ae. albopictus.* I expect AEG_{AZ} to be susceptible to heterospecific mating, as they have no historical exposure to *Ae. albopictus*.

(ii) AEG_{COL} females will have a lower rate of insemination by male *Ae. albopictus* than AEG_{AZ} or will not be inseminated by male *Ae. albopictus* at all. I expect AEG_{COL} to have evolved resistance to heterospecific mating due to their historical exposure to *Ae. albopictus* males.

2.1.3. At generation 3, following 3 generations of exposure to *Ae. albopictus*

(iii) At generation 3, AEG_{AZ} females will have a lower rate of insemination by *Ae. albopictus* males, compared to generation 0. This is because AEG_{AZ} will have evolved resistance to heterospecific mating, following exposure to *Ae. albopictus*.

(iv) In the control group of AEG_{AZ} (which has not been exposed to *Ae. albopictus* but has been through the same number of generations in the laboratory) there will be no change in the rate of insemination by *Ae. albopictus* males between generations 0 and 3.

(v) At generation 3, AEG_{COL} females will have no change in the rate of insemination by *Ae. albopictus* compared to generation 0. This is because AEG_{COL} have had historical exposure to *Ae. albopictus*, thus already have resistance to heterospecific mating.

(vi) In the control group of AEG_{COL} (which has not been exposed to *Ae. albopictus* but has been through the same number of generations in the laboratory), there will be an increase in the rate of insemination by *Ae. albopictus* males as there is no selection pressure to maintain resistance to heterospecific mating which is costly.

2.2. Insemination by conspecifics of a different origin

2.2.2. At generation 0, prior to exposure to *Ae. albopictus*

(vii) AEG_{COL} females will be inseminated at a lower rate by conspecific males from different origin, than AEG_{AZ} females. This is because, I predict AEG_{COL} females will be generally more choosy of mates, due to their resistance to heterospecific mating.

2.2.3. At generation 3, following 3 generations of exposure to *Ae. albopictus*

(viii) AEG_{AZ} females will be more resistant to insemination by conspecific males from different origins than they were at generation 0. This is because, AEG_{AZ} females will be generally more choosy of mates, following the evolution of resistance to heterospecific mating.

(ix) In the AEG_{AZ} control group, there will be no change in the proportion of females inseminated by conspecific males from a different origin. This is because the AEG_{AZ} control group will have had no exposure to *Ae. albopictus* males, thus I predict no change in their resistance behaviour.

(x) AEG_{COL} will have no change in the proportion of females inseminated by conspecific males from a different origin, as AEG_{COL} is already resistant to heterospecific mating, and generally more choosy of mates.

(xi) In the AEG_{COL} control group, there will be an increase in the proportion of females inseminated by conspecific males from a different origin. This is because

this group will have lost its resistance to heterospecific mating and, thus, general choosiness, following no exposure to *Ae. albopictus*.

2.3. Mating Behaviour

(xii) Females who are resistant to heterospecific mating (AEG_{COL} generation 0 and 3, AEG_{AZ} generation 3) will exhibit more rejection behaviour (kicks, and holds, as detailed in (Aldersley & Cator, 2019)) than females who are not resistant to heterospecific mating (AEG_{AZ} generation 0, AEG_{AZ} control group generation 3, AEG_{COL} control group generation 3). I expect resistance to heterospecific insemination to be due to female rejection behaviour, as seen in conspecifics (Aldersley & Cator, 2019).

(xiii) Male behaviour will be consistent, independent of strain or species of female. I expect all differences to be due to female resistance behaviours (Aldersley & Cator, 2019).

3. Methods

3.1. *Aedes* strain information

I established populations from two strains of *Ae. aegypti*: one strain which has not historically overlapped with *Ae. albopictus* ('unexposed') and one strain which has ('exposed').

The unexposed strain of *Ae. aegypti* originated from a field population in Maricopa County, Arizona (AEG_{AZ}). AEG_{AZ} have been reared in the Huijben laboratory since 2019, and at the start of this experiment are a mixture of F17 and F18.

The exposed strain was collected from ovitraps in Comuna 13 in Medellin, Colombia (AEG_{COL}), a region where *Ae. albopictus* are also present. The eggs used in this experiment are F5.

To conduct heterospecific matings, I used a strain of *Ae. albopictus* from Colombia ($ALBO_{COL}$) collected in the same city as AEG_{COL}: Medellin, Colombia. $ALBO_{COL}$ were collected from a natural site in the Jardin Botanico de Medellin and the eggs used in this experiment are F2.

I chose to use an unexposed line of *Ae. aegypti* (AEG_{AZ}) to confirm that you can evolve resistance to heterospecific mating following 3 generations exposure to *Ae. albopictus*. I

used an exposed line of *Ae. aegypti* (AEG_{COL}) to act as a positive control for resistance behaviour.

By using two strains of *Ae. aegypti*, I can show the occurrence of resistance to heterospecific mating in *Ae. aegypti* from multiple genetic backgrounds, thus, demonstrating resistance is not a product of local adaptation, or specific to a particular *Ae. aegypti* strain. Furthermore, I can examine any differences in strength of resistance and resistance behaviours between strains. Neither strains of *Ae. aegypti* have previously been used in experiments examining heterospecific mating, therefore this experiment will broaden our general understanding of resistance to heterospecific mating in *Ae. aegypti*.

Strain information is summarised in Table A.3.1.

Name	Origin	Exposure	Generations in laboratory
AEG_{COL}	Colombia	Exposed	F5
AEG_{AZ}	Arizona	Unexposed	F17/F18
$ALBO_{COL}$	Colombia	NA	F2

Table A.3.1: summary of the Ae. aegypti strains that will be used in experiment 1. AEG stands for Ae. aegypti and ALBO stands for Ae. albopictus, and the Ae. albopictus row is shaded in grey. The subscript refers to their strain name (COL = Columbia, AZ = Arizona). Exposure refers to previous contact with Ae. albopictus (exposed = evolved in sympatry with Ae. albopictus, unexposed = evolved in allopatry with Ae. albopictus).

3.2. Rearing

For both AEG_{AZ} and AEG_{COL}, 6 lines will be established: 3 lines where *Ae. aegypti* females are exposed to *Ae. albopictus* males (resistant lines), and 3 lines where they are not (control lines), as seen in Figure A.3.1.

Each mosquito line will be crossed and reared separately. Dried egg papers, at least one week old (to allow for embryonation), will be used for hatching. Egg papers will be placed in a hatch flask labelled with species, line, date, initials, strain, and treatment. The papers will be submerged with just enough water to cover the eggs and will be placed under a vacuum using a LAX 1 Stage Vacuum Pump for 20 minutes. Hatch flasks will be given one crushed pellet of *Hikari Tropical Fish Food Cichlid Gold* pellets and placed inside a 27 °C and 70% relative humidity incubator overnight. Gloves will be used when handling eggs, and new gloves will be used for each different egg origin.

The following day, first-instar larvae will be put into 500ml plastic tubs in groups of 250 larvae. Until pupal emergence, larval trays will be fed 6 medium pellets of *Hikari Tropical Fish Food Cichlid Gold* per day.

Due to the number of virgin males and females required for the experiment, some will be sexed at the pupal stage, and some at the adult stage. Pupae will be sexed under the dissection microscope, using differences in the genital lobe shape to differentiate between sexes (figure 7,Carvalho *et al.*, 2014). Pupae of the same sex and line will be placed into emergence cups in groups of a maximum of 100 pupae. Cups will be placed in a flight cage

(22 x 8 x 16 cm plastic containers with an 18 x 6 cm mesh window in the lid) and monitored daily for emergence.

Adults will be sorted when they are fewer than 24 hours old, prior to reaching sexual maturity. A maximum of 100 pupae of the same line will be placed in each emergence cup, and 2 cups per cage (thus, a maximum of 200 adults per cage). Upon emergence, sex will be determined by morphological differences: non-plumose antennae are female, plumose antennae are male. A mouth aspirator will be used to move adults into cages of the same sex and line.

Adult mosquitoes will be provided with a 10% sucrose solution and held at 27 °C and 70% relative humidity on a D12:N12 (with 30 minutes of dawn/dusk separating each phase) circadian cycle throughout the experiment.

The experiment will be blocked by line, to make it logistically feasible. For both mosquito origins (Colombia and Arizona) and each treatment group (Resistant and Control), one line will be used per experimental block.

3.3. Evolution of Lines

3.3.1. Resistant Line

I will evolve lines of *Ae. aegypti* that are resistant to reproductive interference, using methods based on Bargielowski et al. (2019). 200 *Ae. aegypti* females will be placed in a cage with 100 *Ae. aegypti* males and 100 *Ae. albopictus* males. The heterospecific males (*Ae. albopictus*) will be present to provide the selection pressure for resistance to reproductive interference to evolve. After 1 week of exposure, the mosquitoes will be provided with a blood meal. Two days later, the mosquitoes will be provided with an egg laying substrate, which will be left in the cage for 3 days. The eggs will then be stored for at least a week to embryonate and then hatched to form the next generation, which will take around 8 days (Joon Yau Leong, Amir S. Patel, 2017).

3.3.2. Control Line

The same methods will be employed as above, but the 200 *Ae. aegypti* females will be exposed to 200 *Ae. aegypti* males of the same strain (and no heterospecifics). The total number of males will be kept the same to control for density dependent effects. The control lines will be put through the same number of generations in the laboratory as the resistant lines, to control for any effects of laboratory rearing.

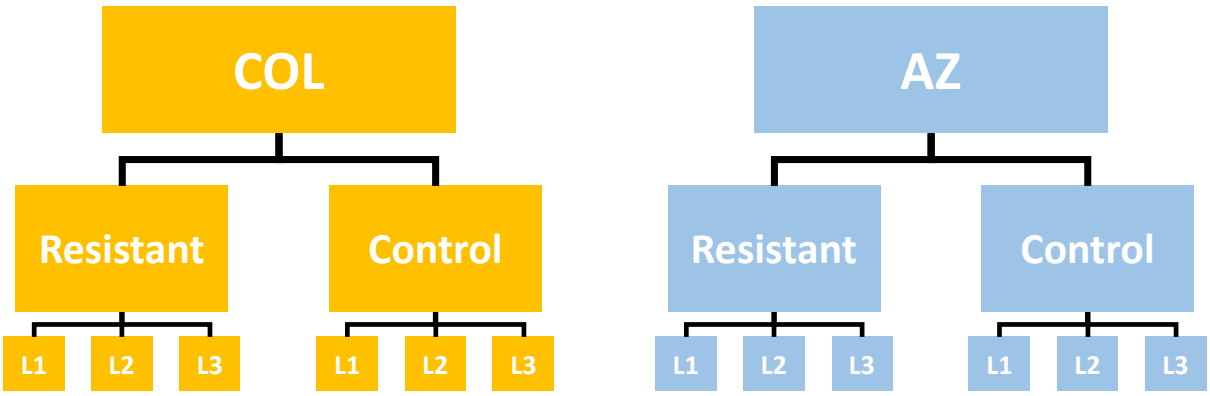

Figure A.3.1: summary of the lines for the two origins of Ae. aegypti (AZ, Arizona and COL, Colombia). For mosquitoes from each origin, I will have two treatment groups (Resistant and Control), each of which will have three lines for each (L1, L2 and L3). Within each line, there will be 5 replicates (not included in this figure).

3.4. Insemination Rates

At generations 0 and 3, I will determine the conspecific (same strain, and different strain) and heterospecific insemination rates to assess whether resistance to heterospecific mating has evolved, and whether it has impacted conspecific mating rates. For each *Ae. aegypti* population I will compare heterospecific and conspecific mating rates between resistant and control lines (Table A.3.2, for details of the crosses). For each replicate, five 3-4 day old virgin males and five 3-4 day old virgin females will be placed in a 16oz cup with a mesh lid and provided with a 10% sucrose solution. For each mating combination, 5 replicates will be conducted. After 1 week of exposure to virgin males all females from each replicate cup will be dissected to determine whether insemination has occurred. Females will be briefly immobilized on ice, their spermathecae will be dissected, and the presence/ absence of sperm will be visualized under a compound microscope.

Female strain	Prior exposure to *Ae. albopictus*	Female Treatment	Male strain	Cross Type
AEG_{AZ}	Unexposed	Resistant	AEG_{AZ}	Conspecific, same strain
	Unexposed	Resistant	AEG_{COL}	Conspecific, different strain
	Unexposed	Resistant	$ALBO_{COL}$	Heterospecific
	Unexposed	Control	AEG_{AZ}	Conspecific, same strain
	Unexposed	Control	AEG_{COL}	Conspecific, different strain
	Unexposed	Control	$ALBO_{COL}$	Heterospecific
AEG_{COL}	Exposed	Resistant	AEG_{COL}	Conspecific, same strain
	Exposed	Resistant	AEG_{AZ}	Conspecific, different strain
	Exposed	Resistant	$ALBO_{COL}$	Heterospecific
	Exposed	Control	AEG_{COL}	Conspecific, same strain
	Exposed	Control	AEG_{AZ}	Conspecific, different strain
	Exposed	Control	$ALBO_{COL}$	Heterospecific

Table A.3.2: summary of the crosses that will be used in experiment 1. AEG and ALBO stand for Ae. aegypti and Ae. albopictus respectively; their subscript refers to their origin (COL = Columbia, AZ = Arizona). Resistant females will evolve in the presence of Ae. albopictus males, and control females will not.

3.5. Female Behaviour

To determine the behavioural mechanisms underlying resistance, I will conduct observed matings of free-flying mosquitoes. I will compare heterospecific and conspecific interactions between resistant and control lines for each population in generations 0 and 3, for each cross detailed in Table A.3.2.

Following the methods of Qureshi *et al.* (2019), four 4-11 day old virgin males from the same treatment group will be placed into a 30cm x30cm x30cm Plexiglas cage. One 4-11 day old virgin female will be released into the cage, marking the beginning of the trial. I will record the total number of mating attempts, the latency from release to the first mating attempt, the time of all later mating attempts (where applicable), whether a copula forms, the start and stop time of the copula, and the type/s of mating resistance behaviour exhibited, as detailed in table A.3.3. Observations will cease after the first copula forms, or after 5 minutes elapses. Resistance behaviours will be classified following Cator and Harrington *et al.* (2011) and Aldersley and Cator (2019). Observations will occur in real time, not via recordings. Females that enter a copula will be dissected to determine the presence of sperm. Females will be immobilized on ice, their spermathecae will be dissected and the presence /absence of sperm will be visualized under a compound microscope.

Variable	Data Type	Variable Type	Definition
Number of Mating Attempts	Count	Fixed Effect	The number of contacts between any of the males and the female. This includes attempts that result in a copula.
Latency to First Mating Attempt	Continuous	Fixed Effect	Length of time from the beginning of the trial until the first mating attempt.
Copula Duration	Continuous	Fixed Effect	Length of time from the beginning of a copula until the separation of the male and female.
Copula	Binary	Fixed Effect	Whether a copula formed, or not.
Kick	Binary	Fixed Effect	Whether the female kicked a male at least once.
Hold	Binary	Fixed Effect	Whether the female held away a male at least once.
Insemination	Binary	Fixed Effect	Whether the female was inseminated, or not.
Timing Block	Categorical	Fixed Effect	Time of day of the specific trial, either AM or PM.

Table A.3.3: Details of the variables of the female behaviour experiment.

3.6. Analysis

All analyses will be run in *R* version 4.3.1 (R Core Team, 2023). The package *lme4* (Bates, Mächler & Dai, 2011) will be used to fit linear and generalised linear mixed-effects models. The package *MASS* (Venables & Ripley, 2002) will be used to conduct likelihood ratio tests, and produce X^2 values and p-values for fixed effects.

3.6.1. Insemination Rate

To determine the effect of cross type, female treatment, male strain and female strain (see table A.2.2 for details) on the probability of female insemination, I will use a generalised linear mixed effects model (GLMM) with a binomial response variable and logit link function. Cup will always be included as a random effect, and line and block will be used as random effects when making comparisons between generations.

If there is a significant effect of an explanatory variable, and there are more than two levels, a Tukey test will be conducted using the *emmeans* package (Lenth, 2023).

3.6.2. Female Behaviour

For all models, I will include line and block as random effects. To determine the effect of cross type, female treatment, male strain and female strain (see table A.3.2 for details) on the probability of all response variables that are binary data, I will use a Binomial GLMM with a logit link function. All count response variables will be analysed using a Poisson GLMM, unless otherwise specified. Continuous behavioural data collected will be analysed

using a linear mixed model (LMM) or a GLMM with a gamma distribution. See table A.3.3 for details of data type.

To choose the appropriate error distribution for each response variable, I will use diagnostic plots. I will use the fitdis() function from the *MASS* package (Venables & Ripley, 2002) to form plots that display how well the error distribution fits the response variable of interest. Following visual inspection, the chosen distribution will be either accepted, or rejected and an alternative distribution trailed (Touchon, 2021).

If there is a significant effect of an explanatory variable, and there are more than two levels, a Tukey test will be conducted using the *emmeans* package (Lenth, 2023).

3.6.3. Convergence of models

For all models, if a group of data being analysed is comprised of all 0s, a G/LMM is inappropriate as a distribution cannot be defined from 0s alone, and the model will not converge. In these instances, a simpler statistical test (e.g. a Chi-squared test) will be used.

Chapter 4 – Characterising the Mating Behaviours that Prevent the Insemination of *Ae. aegypti* Females by *Ae. albopictus* Males

Abstract

While heterospecific insemination is widely reported between *Ae. aegypti* and *Ae. albopictus*, it does not occur between all strains. Previous studies have suggested that female behaviour can prevent heterospecific insemination. Following exposure to *Ae. albopictus*, female *Ae. aegypti* can evolve to resist insemination by *Ae. albopictus* males. However, in *Chapter 3*, I found that females from multiple strains of *Ae. aegypti* with no prior exposure to *Ae. albopictus* were not inseminated by *Ae. albopictus* males. This suggests that in these strains either *Ae. aegypti* females have resistance to heterospecific mating prior to exposure to *Ae. albopictus*, or that the *Ae. albopictus* males do not attempt to mate with the *Ae. aegypti* females. In this chapter, observations of free flying and tethered mosquitoes are conducted to examine the behaviours that prevent heterospecific insemination between these strains. I found that although the male *Ae. albopictus* initially approach the *Ae. aegypti* females, they do not pursue the mating attempt. Therefore, lack of sustained male mating attempts, rather than female behavioural resistance, prevents heterospecific insemination between these strains. Further investigation is required to determine the mechanisms that prevent males from some strains of *Ae. albopictus* from mating with female *Ae. aegypti*.

1. Introduction

Aedes aegypti mate in aerial, male dominated swarms around a blood meal host (Howell & Knols, 2009; South & Catteruccia, 2016; Hartberg, 1971). This occurs as males aggregate around the host (Hartberg, 1971; Cator et al., 2011; Burgess, 2000; Oliva, Damiens & Benedict, 2014) and individual females enter the swarm in search of a blood meal (Hartberg, 1971; Oliva, Damiens & Benedict, 2014; Cator et al., 2011). A male intercepts a female as she approaches the host, or as she leaves the host after blood feeding: the former is more likely when there is high males density, and the latter when there is low male density (Dieng et al., 2019). Where mating is successful, the male grasps the female with his tarsi, orients himself to a venter-to-venter position with the female and inseminates the female (Harrington & Ponlawat, 2009; Cator & Harrington, 2011; Yuval, 2006; Helinski & Harrington, 2011; Cator et al., 2011) as seen in figure 1 in Aldersley and Cator (2019).

During flight, sounds are produced by *Ae. aegypti* wings beating, and these sounds are used as acoustic signals during mating. Male *Ae. aegypti* have highly sensitive hearing (Göpfert, Briegel & Robert, 1999; Göpfert & Robert, 2000), which they use to locate and approach females in ariel swarms (Yuval, 2006; Burgess, 2000). When nearby, male and female pairs engage in an acoustic interaction where each individual dynamically modulates their wingbeat frequency in response to the other (Aldersley et al., 2016). Harmonic convergence occurs where the male and female wingbeat frequencies converge at a shared harmonic frequency (Gibson & Russell, 2006; Cator et al., 2009; Warren, Gibson & Russell, 2009;

Pennetier et al., 2010), which is a whole number multiple of the fundamental wingbeat frequencies of the male and female.

The ability of males to harmonically converge with females may be a heritable signal of male fitness (Cator & Harrington, 2011). Harmonic convergence increases the likelihood of a copula forming (Cator & Zanti, 2016; Cator & Harrington, 2011), and the success of a mating attempt, due to less female rejection behaviour (Aldersley & Cator, 2019).

However, this acoustic signalling does not prevent heterospecific mating occurring. Recent laboratory studies (Bargielowski et al., 2015; Marcela et al., 2015; Honório et al., 2018) and field studies (Robbins et al., 2011) have shown that heterospecific mating occurs between *Ae. aegypti* and *Aedes albopictus*. *Ae. aegypti* females are inseminated by heterospecific males at a greater rate than *Ae. albopictus* females, and *Ae. aegypti* females are much less likely to remate after heterospecific mating than *Ae. albopictus* females (Robbins et al., 2011; Zhou et al., 2022). Due to the greater fitness cost of heterospecific mating to *Ae. aegypti* than *Ae. albopictus*, heterospecific mating is proposed as a key mechanism causing the displacement of *Ae. aegypti* by *Ae. albopictus* (Zhou et al., 2022).

Ae. aegypti females can evolve to evade heterospecific mating by *Ae. albopictus*. Bargiwlowski and Lounibos (2014) found that when *Ae. aegypti* females with no previous contact with *Ae. albopictus* were first exposed to *Ae. albopictus*, they were susceptible to heterospecific insemination. However, following 1-3 generations of exposure to *Ae. albopictus* males, the heterospecific insemination rate decreased (Bargielowski & Lounibos, 2014). Furthermore, female *Ae. aegypti* from populations sympatric with *Ae. albopictus* are

significantly less likely than nearby allopatric populations to mate with heterospecific males (Bargielowski et al., 2015). These results suggest that *Ae. aegypti* females can evolve to resist heterospecific mating attempts by *Ae. albopictus* males and evade the associated fitness cost (Bargielowski & Lounibos, 2014).

Female choice prevents heterospecific insemination in other flying insects, for instance fruit flies (Tomaru, Matsubayashi & Oguma, 1995) and damselflies (Paulson, 1974; Fincke, Fargevieille & Schultz, 2007). Male *Drosophila spp* have a complex array of behaviours that increase their chances of reproductive success, including a species-specific courtship song. Tomaru, Matsubayashi and Oguma (1995) found that female *Drosophila biauraria* performed more resistance behaviour (fluttering and kicking) and less copulation occurred when played an artificial courtship song with heterospecific inter-pulse intervals, than with conspecific inter-pulse intervals. This suggests that *D. biauraria* females discriminate between conspecific and heterospecific males using auditory cues, and then reject heterospecific males (Tomaru, Matsubayashi & Oguma, 1995). Furthermore, female *Enallagma* can identify whether males are conspecific following contact with male cerci (Paulson, 1974; Fincke, Fargevieille & Schultz, 2007), terminal abdominal appendages used to hold females during mating. Cerci morphology varies between species (McPeek et al., 2008; McPeek, Shen & Farid, 2009), and following contact with male cerci, female *Enallagma* reject heterospecific males (Paulson, 1974; Fincke, Fargevieille & Schultz, 2007) and conspecific males with experimentally altered cerci (Robertson & Paterson, 1982). Thus, female *D. biauraria* use auditory cues and female *Enallagma* use tactile cues to identify and reject heterospecific males.

The resistance behaviour of *Ae. aegypti* females towards heterospecific males has not been characterised, however is has been characterised towards conspecific males. Aldersley and Cator (2019) found that in the first few seconds of male contact, *Ae. aegypti* females resist mating by either kicking the male away or holding the male away from their bodies using their hindtarsi, preventing genital contact. Most mating attempts are unsuccessful, and males that successfully mate with female *Ae. aegypti* are kicked at a lower rate (Aldersley & Cator, 2019). Thus, female choice in the early post-contact stages of an interaction is a key determinant of male mating success (Aldersley & Cator, 2019). Female *Ae. aegypti* may evade heterospecific mating by *Ae. albopictus* males using the same resistance behaviours they use in conspecific crosses.

However, Harper and Paulson (1994) found that *Ae. aegypti* females did not resist mating attempts by *Ae. albopictus* by kicking the males away or holding males away from their bodies. They examined the role of female hindtarsi, used in resistance behaviour, in determining the low rate of heterospecific insemination observed between Florida strains of *Ae. aegypti* and *Ae. albopictus*. Upon removing the hindtarsi of female *Ae. aegypti*, there was no increase in the rates of heterospecific insemination. This suggests that in these strains, low heterospecific insemination rates are due to *Ae. albopictus* males not attempting to copulate with *Ae. aegypti* females, which is supported by a previous study demonstrating that male origin determines heterospecific insemination rate (Honório et al., 2018).

However, as previous studies have shown that females can evolve to resist heterospecific mating (Bargielowski et al., 2015; Bargielowski & Lounibos, 2014), in some instances female behaviour must govern the rates of heterospecific insemination. Therefore, in some strains,

lack of insemination by *Ae. albopictus* males may be due to males not attempting to mate with females, and in others it may be due to female resistance behaviour. Equally, in the Florida strains used by Harper and Paulson (1994), there could have been female resistance behaviours that did not require hindtarsi.

It is necessary to further clarify behaviours that result in resistance to heterospecific mating in *Ae. aegypti* females. While Zhou *et al.* (2022) examined mating behaviours in crosses between *Ae. aegypti* and *Ae. albopictus*, they only used susceptible, allopatric strains of *Ae. aegypti* and did not examine female resistance behaviours. Therefore, no previous studies have directly examined the behaviours that prevent heterospecific insemination.

In this study, I aim to characterise the mating behaviours of *Ae. aegypti* females and *Ae. albopictus* males that result in the prevention of heterospecific mating. To do this, I will conduct two experiments using strains of *Ae. aegypti* that I have previously established to be resistant to mating by *Ae. albopictus* males (*Chapter 3*). Firstly, I will observe the mating behaviours of multiple free-flying males and a free-flying female in a cage. By using multiple males, swarming will be able to occur (Facchinelli et al., 2015; Gubler, Bhattacharya & Bhattacharva, 1972) and there will be a male biased sex ratio in the swarm (Howell & Knols, 2009; South & Catteruccia, 2016), both of which will emulate more natural mating conditions. Secondly, I will conduct slow-motion video recordings of a single tethered female mosquito and a single free-flying male mosquito, to gain further understanding of fine-scale mating behaviours. I will conduct both experiments with conspecific and heterospecific crosses so that I can compare the mating behaviours. This is the first study to examine the

mating behaviour that prevents heterospecific mating between *Ae. aegypti* females and heterospecific *Ae. albopictus* males.

2. Aim

I aim to characterise resistance behaviour in *Ae. aegypti* females, in conspecific and heterospecific crosses. Furthermore, I aim to determine if there are any differences in mating behaviour between *Ae. albopictus* and *Ae. aegypti* males when mated with *Ae. aegypti* females.

3. Hypotheses

3.1. Female Resistance Behaviour

I expect female resistance behaviour (kicking males away and holding males away from their abdomens to prevent genital contact) to occur in conspecific crosses, as this has previously been recorded (Aldersley & Cator, 2019).

However, I do not have clear expectations for whether female rejection behaviour will occur in heterospecific crosses. Some previous literature suggests that the rate of heterospecific insemination is determined by female behaviour (Bargielowski et al., 2015), and there are female resistance behaviours that prevent heterospecific mating are present in other flying

insects (Tomaru, Matsubayashi & Oguma, 1995; Paulson, 1974; Fincke, Fargevieille & Schultz, 2007). Meanwhile, other studies suggest that a lack of mating attempts by male *Ae. albopictus* prevent heterospecific insemination (Harper & Paulson, 1994).

3.2. Male Mating Behaviour

I do not have a clear prediction for male behaviour in heterospecific crosses, as resistance to heterospecific mating could either stem from lack of mating attempts by *Ae. albopictus* males (Harper & Paulson, 1994) or resistance behaviour from *Ae. aegypti* females (Bargielowski et al., 2015). If the former is true, I expect males to make more mating attempts towards conspecific females than heterospecific females: thus, the lack of male attempts causes the low heterospecific insemination rate. Conversely, if the latter is true, I do not expect any differences in male behaviour between conspecific crosses, and heterospecific crosses. Instead, differences in insemination success would be to be due to female rejection behaviour.

3.3. Mating Success

(i) Insemination

I expect to observe, as was shown in *Chapter 3*, that no or very few *Ae. aegypti* females will be inseminated by *Ae. albopictus* males and a high proportion of *Ae. aegypti* females will be inseminated by *Ae. aegypti* males.

(ii) Copula formation

I expect most copulas to result in successful insemination, as previous literature has shown a high correlation between copula occurrence and insemination in *Ae. aegypti* (Aldersley &

Cator, 2019). Therefore, I expect either no or a low proportion of heterospecific crosses to result in a copula, and a much higher proportion of conspecific crosses to result in a copula.

4. Methods

All experiments were designed and analysed by me (MV). Rearing and data collection were conducted by MV, and Chenrui Zhang (CZ), a Master's student at Imperial College London. For the *Cage Trials of Free-flying Mosquitoes* experiment, MV collected the data for the first 3 experimental blocks, and CZ collected the data for the latter 3. For the *Slow-Motion Video Recordings of a Tethered Female*, CZ, or Dr Lauren Cator (LJC) set up the camera and took the video recordings, while MV watched and extracted data from the videos. In instances where the mosquitoes would not feed from the Hemotek feeding system, LJC conducted arm feeds (Cator Lab SOP 2, Approved by Imperial College Health and Safety). CZ was supervised and trained in laboratory techniques by LJC.

4.1. *Aedes* Strain Information

We established populations from three strains of *Ae. aegypti*: two strains that have not historically overlapped with *Ae. albopictus* ('unexposed') and one strain which has ('exposed'). All strains were established to be resistant to heterospecific mating by *Ae. albopictus* in *Chapter 3*.

One of the unexposed strains of *Ae. aegypti* originated from a field population in Maricopa County, Arizona (AEG_{AZ}). AEG_{AZ} have been reared in the Huijben laboratory, Arizona State

University since 2019. I was supplied with a mixture of F17 and F18 eggs by Sarah Rydeberg, Center for Evolution and Medicine, Arizona State University.

The second unexposed strain of *Ae. aegypti* is the widely used Liverpool strain (AEG_{LIV}). This strain originated in West Africa[3] and has been maintained in laboratory colony since 1938, thus there has been substantial inbreeding. Due to the prolonged period in the laboratory without contact with *Ae. albopictus*, this strain is 'unexposed'.

The exposed strain of *Ae. aegypti* was collected from ovitraps in Comuna 13 in Medellin, Colombia (AEG_{COL}), a region where *Ae. albopictus* are also present. Both AEG_{COL} were collected, and laboratory colonisation was conducted by Catalina Alfonso-Parra, Max Planck Tandem Group, Universidad of Antioquia. I was supplied with F5 eggs, which went through two further generations in the laboratory before being used in this experiment.

To conduct heterospecific matings, we used a strain of *Ae. albopictus* originally collected in Montpellier, France, an area where *Ae. aegypti* are not present, in 2016. This strain ($ALBO_{MP}$) has since been in maintained in the laboratory for more than 20 generations.

Between 3 of the experimental blocks, all lines (AEG_{COL}, AEG_{AZ}, AEG_{LIV} and $ALBO_{MP}$) were put through an additional generation in the laboratory. Strain information is summarised in Table 4.1.

[3] Freetown, Sierra Leone is the most likely source of this strain (Kuno, 2010).

Name	Origin	Exposure to *Ae. albopictus*	Generations in laboratory
AEG_{COL}	Colombia	Exposed	F7-10
AEG_{AZ}	Arizona	Unexposed	F17-F21
AEG_{LIV}	West Africa	Unexposed	>F100
$ALBO_{MP}$	Montpellier	NA	>F20

Table 4.1: Summary of the Ae. aegypti and Ae. albopictus strains used in this experiment. AEG stands for Ae. aegypti and ALBO stands for Ae. albopictus, and the Ae. albopictus row is shaded in grey. The subscript refers to their strain name (COL = Columbia, AZ = Arizona, LIV = Liverpool, MP = Montpellier). Exposure refers to the previous contact of Ae. aegypti with Ae. albopictus (exposed = evolved in sympatry with Ae. albopictus, unexposed = evolved in allopatry with Ae. albopictus). Each strain has a range of generations in the laboratory, as lines were put through additional generations between some experimental blocks.

4.2. Rearing

Each mosquito line was crossed and reared separately. To allow for embryonation, we used dried egg papers that were at least one week old for hatching. Egg papers were placed in a hatch flask labelled with species, date, generation, strain, and experimenter initials. The papers were then submerged with just enough water to cover the eggs and placed under a vacuum using a LAX 1 Stage Vacuum Pump for 20 minutes. Hatch flasks were given one crushed pellet of *Hikari Tropical Fish Food Cichlid Gold* pellets and placed inside a 27 °C and 70% relative humidity incubator overnight. Gloves were used when handling eggs, and different gloves were to handle eggs from different strains.

The following day, the first-instar larvae were put into 500ml plastic tubs in groups of 250 larvae. Until pupal emergence, larval trays were fed 6 medium pellets of *Hikari Tropical Fish Food Cichlid Gold* per day.

As virgin males and females were required for this experiment, we needed to sex the mosquitoes before they reached sexual maturity. We sexed mosquitoes at the pupal stage under a dissection microscope, using differences in the genital lobe shape to differentiate between sexes (for details, see figure 7 in Carvalho *et al.*, 2014). Pupae of the same sex and strain were placed into emergence cups in groups of a maximum of 50 pupae. Cups were placed into flight cages (22cm x 8cm x 16 cm plastic containers with an 18cm x 6 cm mesh window in the lid) and were monitored daily for emergence.

Adult mosquitoes were provided with a 10% sucrose solution and held at 27 °C and 70% relative humidity on a D12:N12 (with 30 minutes of dawn/dusk separating each phase) circadian cycle throughout the experiment.

4.3. Colony Cages

When necessary to produce more eggs, two colony cages were formed per strain. Rearing was conducted as previously detailed though cages were not sex segregated, thus colony cages were comprised of two cups of 100 pupae of mixed sex. However, there were insufficient pupae for that size of the colony cage in two instances, resulting in smaller colony cages (one cage of AEG_{COL} had 179 pupae, and one cage of $ALBO_{MP}$ had 155 pupae).

Following emergence, adults were left for at least a week to mate before being provided with a blood meal. One day prior to the blood meal, the sucrose solution was removed from the cage to increase the propensity of the mosquitoes to blood feed. *Ae. aegypti* were provided with warmed (~37 °C) equine blood though a Hemotek feeding system for 1 hour. However, *Ae. albopictus* struggled to feed using the Hemotek feeding system, so instead conducted arm feeds for these colony cage (Cator Lab SOP 2, Approved by Imperial College Health and Safety).

Two days after blood feeding, mosquitoes were provided with an egg laying substrate (damp filter paper in oviposition cup), which was left in the cage for a minimum of 3 days. Blood meals were repeated a total of two times per colony cage, to increase the number of eggs collected.

4.4. Cage Trials of Free-flying Mosquitoes

4.4.1. Crosses and Blocking

This experiment aims to determine differences in mating behaviour between conspecific and heterospecific crosses. Thus, *Ae. aegypti* females from each strain were mated with conspecifics of the same strain and *Ae. albopictus* males. Data were collected by two experimenters, MV and CZ. MV examined the conspecific and heterospecific crosses for AEG_{AZ} and AEG_{COL} females. CZ repeated these crosses, and examined the conspecific and heterospecific crosses for AEG_{LIV}.

A total of 6 experimental blocks were conducted, the first 3 blocks were conducted by MV and the latter 3 by CZ. For the first 3 blocks, there were 30 replicates of each cross per block, where a replicate is defined as a cage observation. In the following 3 blocks, there were 10 replicates for each cross per block. Thus, there were fewer observations for AEG_{LIV} than the other *Ae. aegypti* females examined. Details on the crosses examined and total number of replicates for each cross are shown in table 4.3.

In some blocks, there were too few adults to carry out the planned number of replicates, as detailed in the supplementary information (see Table S.4.1).

Female Strain	Male Strain	Mating Type	Total # Replicates	Experimenter/s
AEG_{AZ}	AEG_{AZ}	Conspecific	90	MV and CZ
AEG_{AZ}	$ALBO_{MP}$	Heterospecific	76	MV and CZ
AEG_{COL}	AEG_{COL}	Conspecific	71	MV and CZ
AEG_{COL}	$ALBO_{MP}$	Heterospecific	75	MV and CZ
AEG_{LIV}	AEG_{LIV}	Conspecific	30	CZ
AEG_{LIV}	$ALBO_{MP}$	Heterospecific	30	CZ

Table 4.3: Summary of the crosses examined in the cage trials of free-flying mosquitoes, and the experimenter who conducted the cross.

4.4.2. Cage observations

(i) All blocks

To determine the behavioural mechanisms underlying resistance, we conducted observed matings of free-flying mosquitoes. Observations were conducted in real time (not via recordings).

Our methods were based strongly on those by Qureshi *et al* (2019). We released four 7-10 day old virgin males from the same treatment group into a 30cm x 30cm x 30cm Plexiglas cage. We used multiple males to emulate more natural conditions, as swarms can form with as few as 2 males (Facchinelli et al., 2015; Gubler, Bhattacharya & Bhattacharva, 1972). The cage was placed on a dark mounting to prevent the mosquitoes being disorientated. After the males had been in the cage for a minimum of 5 minutes, one 7-10 day old virgin female was released into the cage, marking the beginning of the trial.

We recorded the total number of mating attempts, the timing of all mating attempts, whether a copula formed, the start and stop time of the copula and the type/s of mating resistance behaviour exhibited. Resistance behaviours were classified following Cator and Harrington *et al.* (2011) and Aldersley and Cator (2019). Although we did not anonymise the recordings of mating behaviour with respect to treatment identity, to prevent any inconsistency in recordings we had clear definitions of the recorded behaviours (see table 4.4).

We calculated two parameters to assess differences in the persistence of males in instances where there was more than one mating attempt. We calculated the Attempt Period (defined as the length of time attempts were made over within a trial) and the Attempt Rate (defined as the number of mating attempts scaled by the Attempt Period). Furthermore, we determined whether each attempt was effortful or not: an effortful attempt is defined as an attempt that resulted either in a copula forming or female rejection behaviour occurring.

Observations ceased after the first copula formed, or after 4 minutes elapsed. If a copula was initiated before 4 minutes elapsed but continued past 4 minutes, we continued recordings until the copula broke apart. After the copula had broken apart, the female was immediately removed from the cage to ensure that any insemination was a result of the observed copula.

To determine the insemination status of females that had engaged in a copula, spermathecae dissections were conducted. Females were briefly immobilized on ice and their spermathecae were dissected under a dissection microscope. The spermathecae were

then examined under a compound microscope, and the presence or absence of sperm was determined.

(ii) Blocks 3-6

In the initial 3 blocks of experiment, MV noticed that in some instances females remained still within the cage. This could be a form of rejection behaviour as males use the acoustic signals generated by female flight to locate females (Cator, Wyer & Harrington, 2021). Furthermore, previous research found that copulation was prevented if flight was terminated before the pre-copulatory mating pose was adopted (Aldersley & Cator, 2019). To investigate this, in the latter 3 blocks CZ recorded the length of time females were inactive. Inactivity was recorded over a 5-minute period.

(iii) Differences in methods between blocks 1-3 and 3-6

There were two minor differences in the methods employed by the two experimenters. Firstly, MV systematically changed the order that crosses were observed each day, as *Aedes* activity levels change throughout the day (Gentile et al., 2013; Egid et al., 2022; Araripe et al., 2018) and thus this minimises any time-of-day effects on mating behaviour. However, CZ observed crosses in the same order each day. Secondly, MV recorded the trials for 4 minutes, while CZ recorded the trials for 5 minutes. To make the data compatible, the data from CZ was truncated to 4 minutes for all variables collected by both CZ and MV (thus, all variables apart from female inactivity).

Variable	Data Type	Definition
Number of Mating Attempts	Count	The number of contacts between a male and the female. This includes attempts that result in a copula.
Latency to First Mating Attempt	Continuous	Length of time from the beginning of the trial until the first mating attempt.
Copula Duration	Continuous	Length of time from the beginning of a copula until the separation of the male and female.
Copula	Binary	Whether a copula formed, or not.
Kick	Binary	Whether the female kicked a male at least once.
Hold	Binary	Whether the female held away a male at least once.
Insemination	Binary	Whether the female was inseminated or not.
Timing Block	Categorical	Time of day of the cage trial. This was processed into AM / PM for analysis.
Female inactivity	Continuous	Total length of time females are inactive (not in flight) throughout the trial.
Attempt Period	Continuous	Time of the last mating attempt, minus the time of first mating attempt. Only calculated for trials with more than one attempt.
Attempt Rate	Continuous	Number of Mating Attempts divided by the Attempt Period
Effortfulness	Binary	Whether an effortful attempt occurred or not. An effortful attempt is defined as an attempt that resulted either in a copula forming or female rejection behaviour (kick or hold) occurring.

Table 4.4: Details of the mating behaviours recorded in cage trials of free-flying mosquitoes. The variables shaded in grey are calculated from raw recordings.

4.5. Slow-motion Video Recordings of Tethered Females

To examine fine scale mating behaviour, we took slow-motion video recordings of the first mating attempt in heterospecific crosses (*Ae. aegypti* females x *Ae. albopictus* males) and conspecific control crosses (*Ae. aegypti* females x *Ae. aegypti* males and *Ae. albopictus* females x *Ae. albopictus* males). For each cross, we conducted 10 replicates. We only used the Arizona strain of *Ae. aegypti* within this experiment (AEG_{AZ}). The methods are based on those of Aldersley and Cator (2019).

4.5.1. Cage set-up

For these recordings, we tethered a female in the centre of a recording chamber. To do this, an individual 5-10 day old virgin female was extracted from a colony cage, and placed on ice for approximately 4 minutes to immobilise the female. The female was then glued to a short (~20mm) strand of human hair (from CZ) which was attached to a metal pin. This pin was then mounted to a stand, which was placed in the centre of the recording chamber. A raised surface was placed inside the recording chamber, and the female was allowed to rest its tarsi on this surface for a minimum of 3 minutes to recover from tethering before the trial began.

We could not conduct recordings in a controlled environment room, as the lighting and humidity prevent the camera from functioning. Instead, the recording chamber was placed on top of a heated plate to increase the temperature inside the recording chamber, and to keep the temperature approximately constant between trials. To minimise any consistent differences in the behaviours recorded between groups due to daily variation in climate, on

each day we took video recordings for all crosses. Recordings were conducted under ambient lighting conditions.

As *Ae. aegypti* and *Ae. albopictus* mate in swarms around their hosts, we simulated host presence by placing a worn t-shirt between the heating plate and the recording chamber, and by standing <30cm from the recording chamber throughout the experiment.

4.5.2. Camera and software set-up

We used a configurable software trigger to initiate recordings when the male first attempts to make physical contact with the tethered female. This recorded the 5 seconds prior and 23 seconds following the first contact between the male and female, resulting in a total of 28s of video recording. We used a high-speed digital camera (Phantom Miro 310, Vision Research, Wayne, NJ, USA) to record local interactions between a male and female pair (1000fps and frame size of approximately 10 body lengths, 5-7cm).

4.5.3. Recording

Cage trials were conducted within 15 minutes of the female recovering from being tethered. Flight was encouraged by removing the raised surface that the female was previously resting on. Prior to the start of the trial, the female had to be in stable flight: this was confirmed by the adoption of the characteristic airborne position. In instances where the female did not fly, the trial was disregarded.

An aspirator was used to place a single 5-10 day old virgin male into the recording chamber, marking the beginning of the trial. Where males settled on the side of the arena and did not

move, they were encouraged to fly by physical disturbance. Following physical disturbance, if the male did not attempt to make physical contact with the female within the first 3 minutes of the trial, the male was removed from the cage and a new male was placed in the cage. This was conducted a maximum of 3 times per cage trial.

Observations were conducted between 9:30 and 14:30 and up to seven observations were conducted per day. Each cross was conducted on multiple days, to prevent systematic differences due to daily variations in climate.

4.5.4. Mating Behaviours

Following the results of our cage trials of free-flying mosquitoes, a key focus of this experiment was to examine differences in the persistence of conspecific and heterospecific males when mating with *Ae. aegypti* females. Initially, I watched the slow-motion videos to determine which mating behaviours address key differences in persistence behaviour between conspecific and heterospecific crosses. I decided to record the duration of contact between the male and female, occurrence of a copula, copula duration and the occurrence of resistance behaviour (females kicking males away or holding them away from her abdomen). The mating behaviours recorded are detailed in table 4.5.

Behaviours were recorded for each interaction, where an interaction was defined as a period of time where the male mosquito did not leave the video frame for longer than 0.2s. For continuous mating behaviours (duration of contact, duration of copula), the sum of time of the interaction was reported: for instance, if a male and female lost contact and then later regained contact without the male leaving the frame for longer than 0.2s, the sum of

these two lengths of contact would be reported. In some cases, there were multiple interactions within one recording.

In some instances, mosquitoes were in a copula or in contact when the recording began, or finished, so, I was not able to determine the full duration of the contact / copula. In these instances, the length of time observed was recorded, and it was reported as greater than the number of seconds recorded (>Xs).

Variable	Data Type	Definition
Duration of Contact	Continuous	Sum of the length of time the male and female were in physical contact within an interaction.
Copula	Binary	Whether a copula formed or not.
Duration of Copula	Continuous	Sum of the length of time from the beginning of a copula until the separation of the male and female within an interaction.
Occurrence of Resistance Behaviour	Binary	Whether resistance behaviour (females kicking males away, or holding them away from her abdomen) occurred or not.

Table 4.5: Details of the mating behaviours recorded in the slow-motion recordings of tethered females.

5. Analysis

All analyses were run in *R* version 4.3.1 (R Core Team, 2023). Data was processed using *dplyr* (Wickham et al., 2023) and *tidyr* (Wickham, Vaughan & Girlich, 2023) and all graphs were formed using *ggplot2* (Wickham, 2016). The package *lme4* (Bates, Mächler & Dai, 2011) was used to fit linear and generalised linear mixed-effects models.

I used the fitdis() function from the *MASS* package (Venables & Ripley, 2002) to form plots to display how well the error distribution fits the response variable of interest. Following visual inspection, the chosen distribution was either accepted, or rejected and an alternative distribution trialled (Touchon, 2021).

The package *MASS* (Venables & Ripley, 2002) was used to conduct likelihood ratio tests, using a Chi-squared test to produce p-values for the fixed effects. For each fixed effect, the values reported were from the simplest model containing that variable. Explanatory variables with a significant effect ($p \leq 0.05$) were reported in the results section, and those without a significant effect were excluded from models and included in the supplementary information (see Tables S.4.2 - S.4.4). When an explanatory variable with a significant effect on the response variable had two levels, *lmerTest* to was used to produce summary statistics of the directional effect (Kuznetsova, Brockhoff & Christensen, 2017). However, where an explanatory variable with a significant effect had more than two levels, a Tukey test was conducted to examine the pairwise differences between groups, using the *emmeans* package (Lenth, 2023).

I used the package *stats* (R Core Team, 2023) in base *R* to conduct simple statistical tests: Whitney-U test, Kruskal-Wallis Tests and Pearson's Chi-squared tests. The Whitney-U test and Kruskal-Wallis Tests were used for continuous data that did not fit the normal distribution or the gamma distribution. When the explanatory variable had two levels, the Mann-Whitney U test was used and when the explanatory variable had more than two levels, the Kruskal-Wallis rank sum test (an extension of the Mann-Whitney U test) was used. Pearson's Chi-squared tests were used where the group of count data or binary data being analysed was comprised of all 0s. This is because generalised linear mixed effects models are inappropriate in these instances as the Poisson distribution and Binomial distribution cannot be defined from 0s alone: thus, the models would not converge.

The *stats* package (R Core Team, 2023) was also used to determine whether there is a correlation between two behaviours, by calculating the Pearson's product moment correlation coefficient.

The package *AICcmodavg* (Mazerolle, 2023) was used to determine the weighted AIC, which was used in model selection.

5.1. Cage Trials of Free-flying Mosquitoes

5.1.1. Partitioning timing block

Aedes activity levels vary in a predictable way diurnally (Gentile et al., 2013; Egid et al., 2022; Araripe et al., 2018), and thus during experiments we recorded the timing of each cage observation so that we could include timing as a fixed effect in the models. We recorded timing in approximately 1.5 hour blocks. However, partitioning timing so finely resulted in some timing blocks having a very high standard error in some models. For instance, this occurred in models of count data, when there were some timing blocks where all observations were zero.

To solve this problem, I re-partitioned time. To decide on the size of the partitions, I examined the impact of timing block in isolation on all mating behaviours. I examined this separately for *Ae. albopictus* and *Ae. aegypti* males, as there may be differences in how time affects activity between these species.

I only found significant differences between timing blocks in the morning, and timing blocks in the afternoon, so I grouped as such. This grouping of timing block makes theoretical sense, as previous studies have shown differences in the activity levels of *Aedes* between the morning and afternoon (Gentile et al., 2013; Egid et al., 2022; Araripe et al., 2018).

5.1.2. Mating Behaviour

To analyse the mating behaviour data, I formed generalised linear mixed effects models (GLMM). The distribution used depends on the type of data of the response variable (see table 4.4 for details). Details of the distribution used to analyse each response variable is specified in supplementary information (see Tables S.4.2- S.4.4). In each model, I included timing block as a fixed effect, as *Aedes* activity levels vary predictably throughout the day (Gentile et al., 2013; Egid et al., 2022; Araripe et al., 2018).

When analysing data from both conspecific and heterospecific crosses, for each response variable I formed a model examining the impact of female strain, male strain, and their interactions with timing block. Male strain incorporates two pieces of information – strain specific differences and mating type (conspecific/heterospecific), as each female is crossed with a conspecific of the same strain, and a heterospecific. Thus, when I found a significant effect of male strain, I formed another model to examine the impact of mating type (conspecific/heterospecific) and its interaction with timing block, on the response variable. I then used weighted AIC to determine whether the initial model, or the latter model is a better fit to the data. This allowed me to determine whether the observed differences are better explained by male strain or mating type. Only the results of the selected model were reported in the results section. Details of all models formed, and model selection are in the supplementary information (see Tables S.4.2- S.4.5).

When examining data from conspecific crosses, for each response variable I formed a model examining the impact of cross, timing block and their interaction. This is because, in conspecific crosses, mating only occurs between males and females of the same strain.

For all models, I included experimenter, and experimental block as random effects. As each experimenter conducted multiple experimental blocks, I nested experimental block within experimenter.

5.2. Slow-motion Video Recordings of Tethered Females

I conducted simple statistical tests to determine the impact of mating type and cross on the response variables (detailed in table 4.5). For continuous data where the explanatory variable had two levels I used a Mann-Whitney U test and where the explanatory variable had more than two levels, I used a Kruskal-Wallis rank sum test. For binary data, I used a Chi-squared test to compare groups.

In some conspecific interactions, contact between a male and female began, or a copula formed, before the recording started, or after the recording finished. Thus, an incomplete time was recorded. This happened in quite a high proportion of cases (contact – $ALBO_{MP}$ $ALBO_{MP}$ = 64.29%, AEG_{AZ} AEG_{AZ} = 29.41%, copula - $ALBO_{MP}$ $ALBO_{MP}$ = 37.50%, AEG_{AZ} AEG_{AZ} = 33.33%), and thus I did not want to exclude these data from histograms. Instead, I formed a final column of greater than smallest incomplete time recorded. However, I excluded some small incomplete timings (contact – $ALBO_{MP}$ $ALBO_{MP}$, n = 1, AEG_{AZ} AEG_{AZ}, n = 1, copula – $ALBO_{MP}$ $ALBO_{MP}$, n = 1, AEG_{AZ} AEG_{AZ}, n = 2) from the dataset, to prevent the final column being too low, and information being lost from the histogram. However, for statistical analyses I removed the incomplete times.

6. Results

6.1. Cage Trials of Free-flying Mosquitoes

6.1.1. Mating attempts

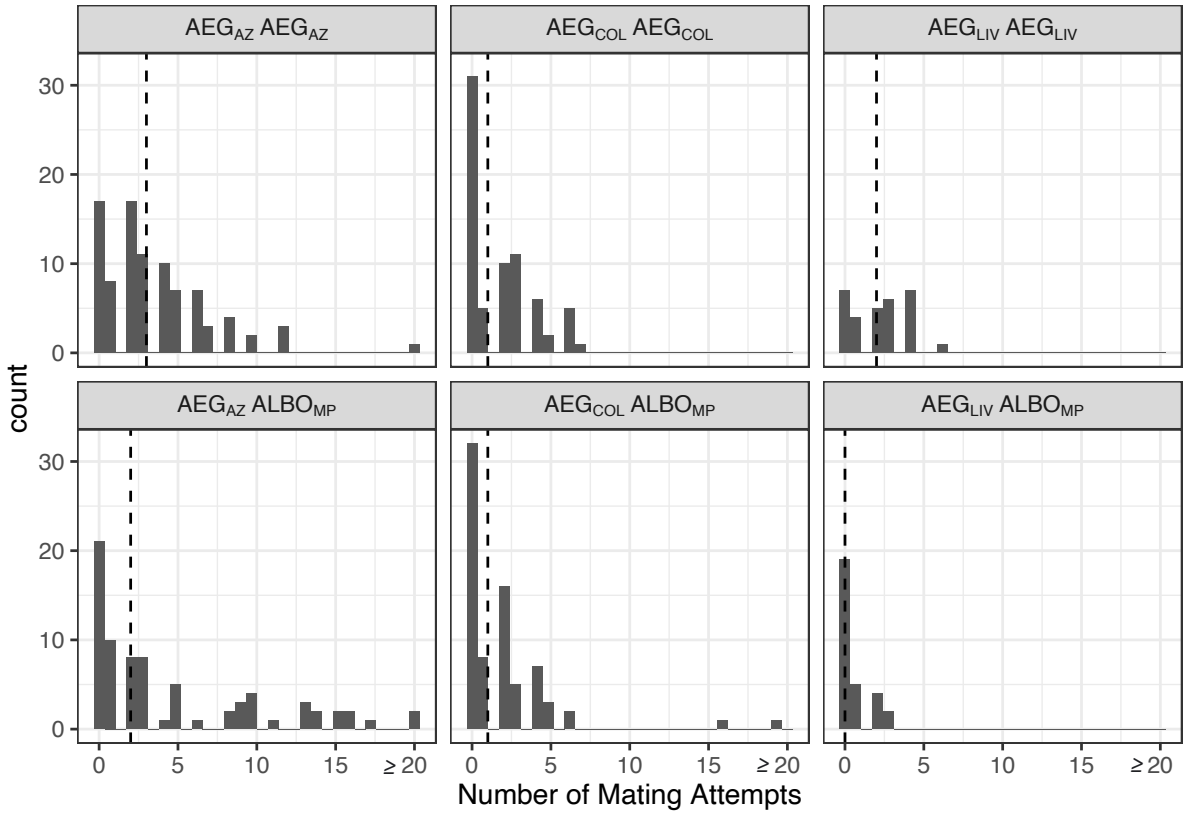

Figure 4.1 – Differences in the number of mating attempts between crosses. Crosses are female-male, such that AEG_{AZ} $ALBO_{MP}$ represents AEG_{AZ} females crossed with $ALBO_{MP}$ males. Each column represents a particular female strain, where the top row examines conspecific crosses and the bottom row represents heterospecific crosses. The vertical dashed lines show the median for that cross. To see the left-hand side of the distribution in more detail, the few trials (n=3) that resulted in >20 mating attempts were grouped as ≥ 20.

In general, I found that there was considerable variation in the number of mating attempts between individual trials, yet not much difference in the median number of mating attempts

between mating types (conspecific / heterospecific), as seen in figure 4.1. Between conspecific crosses (median =2, IQR= 4) and heterospecific crosses (median =1, IQR = 3) there was a small difference in the median number of mating attempts, however within each mating type the interquartile range was at least 2x the median. Thus, both conspecific and heterospecific males attempt to mate with *Ae. aegypti* females, and there is a lot of variation in the number of mating attempts made by individual males.

Similar trends were seen within crosses. There was high variation in the number of mating attempts in conspecific (median = 3, IQR = 4), and even more so in heterospecific (median = 2, IQR= 9) crosses with AEG_{AZ} females and a small difference in the median number of mating attempts. High variation in the number of mating attempts was also seen in conspecific (median = 1, IQR = 3) and heterospecific (median = 1 , IQR = 2.5) crosses with AEG_{COL} females, yet no difference in the median number of mating attempts. For crosses with AEG_{LIV} females, on average 2 more mating attempts occurred in conspecific crosses (median = 2, IQR = 2.75) than in heterospecific crosses (median = 0, IQR = 1); a greater difference than in the other two strains of *Ae. aegypti* female.

I planned to assess the number of mating attempts using generalised linear mixed effects models with Poisson errors. However, the resultant model had very high deviance and standard errors. To address this, I formed three alternative models: a generalised linear mixed effects model with a negative binomial error distribution, a hurdle model with a truncated Poisson distribution and a hurdle model with a truncated negative binomial distribution. I also fitted these models using a truncated dataset, removing three outliers

(where there were greater than or equal to 20 mating attempts). However, all three models for the full and truncated dataset resulted in high deviance and standard errors.

Thus, to conduct statistical analysis I altered the response variable being assessed. I converted the count data to binary data, and examined which explanatory variables influenced the likelihood that at least one mating attempt occurred, rather than the number of mating attempts.

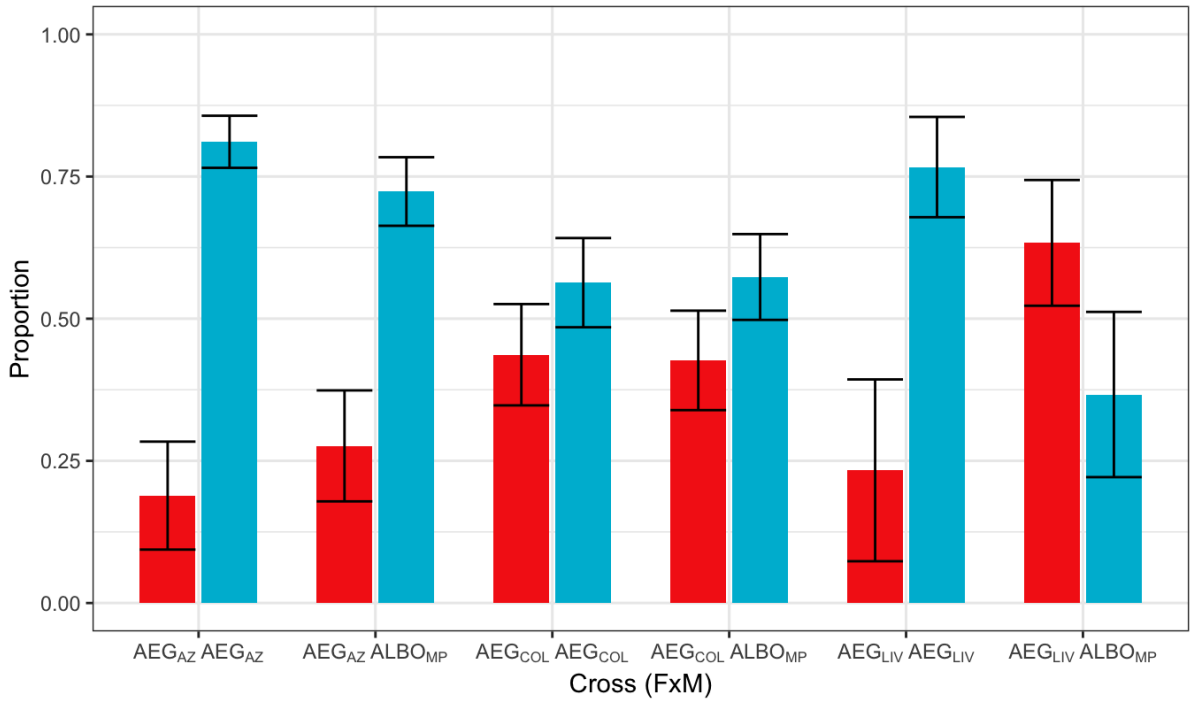

Figure 4.2 – Differences in the proportion of trials where one or more mating attempts were made (blue), or no mating attempts were made (red), between crosses. Bars are ordered by female strain and mating type, such that the conspecific cross for a particular female strain is followed by the heterospecific cross for that strain. Crosses are female-male, such that AEG$_{AZ}$ ALBO$_{MP}$ represents AEG$_{AZ}$ females crossed with ALBO$_{MP}$ males. Error bars represent the standard error of the proportion.

I found that the likelihood of a mating attempt occurring varied between different female strains (*P*= 0.00014), as seen in figure 4.2. Post-hoc analysis showed that males were less likely to attempt to mate with AEG_{LIV} females than both AEG_{AZ} females (coeff = -2.16, SE = 0.54, *P* = 0.00020), and AEG_{COL} females (coeff = -1.36, SE = 0.50, *P* = 0.018). Furthermore, males were marginally more likely to mate with AEG_{AZ} females than AEG_{COL} females (coeff = 0.80, SE = 0.37, *P* = 0.073).

Furthermore, some male strains were more likely to attempt to mate with females than other male strains (*P* = 0.013). Post-hoc analysis showed that AEG_{LIV} males were more likely to make a mating attempt than AEG_{COL} males (coeff = 1.93, SE = 0.68, *P* = 0.025) and $ALBO_{MP}$ males (coeff = 1.78, SE = 0.58, *P* = 0.012).

Timing block had a significant effect on the likelihood of a mating attempt occurring (*P* = 0.029), with mating attempts being more likely to occur in the morning than the afternoon (coeff = 0.62, SE = 0.29, *P* = 0.030).

There was no difference in the likelihood of a mating attempt occurring between conspecific and heterospecific crosses.

6.1.2. Mating Persistence

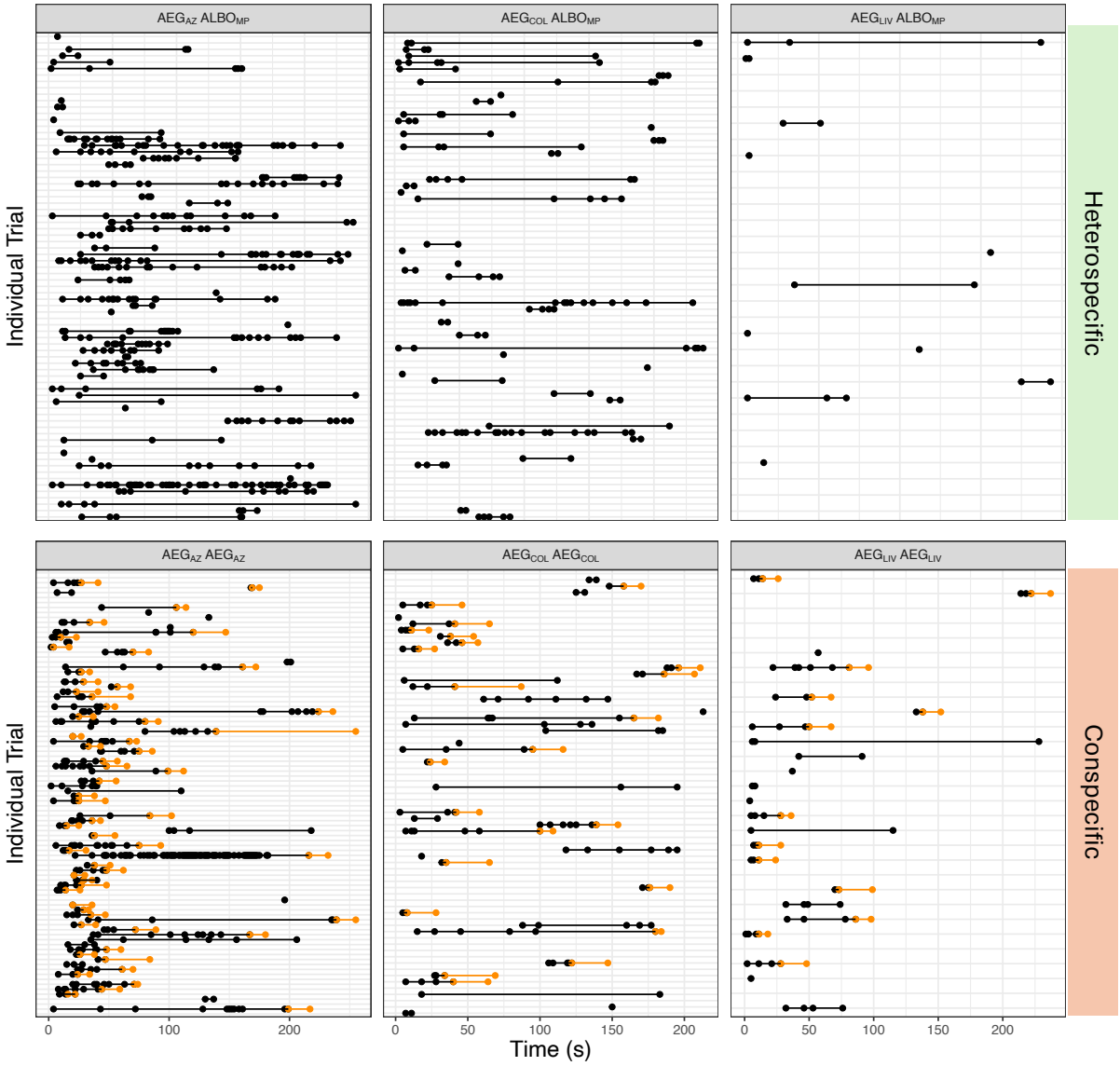

Figure 4.3 – Differences in the timing of mating attempts in each trial between crosses. Each dot represents a mating attempt, and mating attempts in the same trial are joined by a horizontal line. The dots representing the beginning and end of a copula are coloured in orange, as is the line connecting them, and all other mating attempts are coloured in black. Crosses are female-male, such that AEG$_{AZ}$ ALBO$_{MP}$ represents AEG$_{AZ}$ females and ALBO$_{MP}$ males. Each column represents a particular female strain, and the top row has conspecific crosses and the bottom row heterospecific crosses.

I conducted analysis to examine differences in the persistence of male mating attempts between strains and mating types. I calculated two parameters, Attempt Period and Attempt Rate, to examine how persistent males were when attempting to mate with a female. Attempt Period is defined as the length of time mating attempts occur over and was calculated by the time of the last mating attempt minus the time of the first mating attempt. The Attempt Rate is defined as the number of mating attempts divided by the Attempt Period.

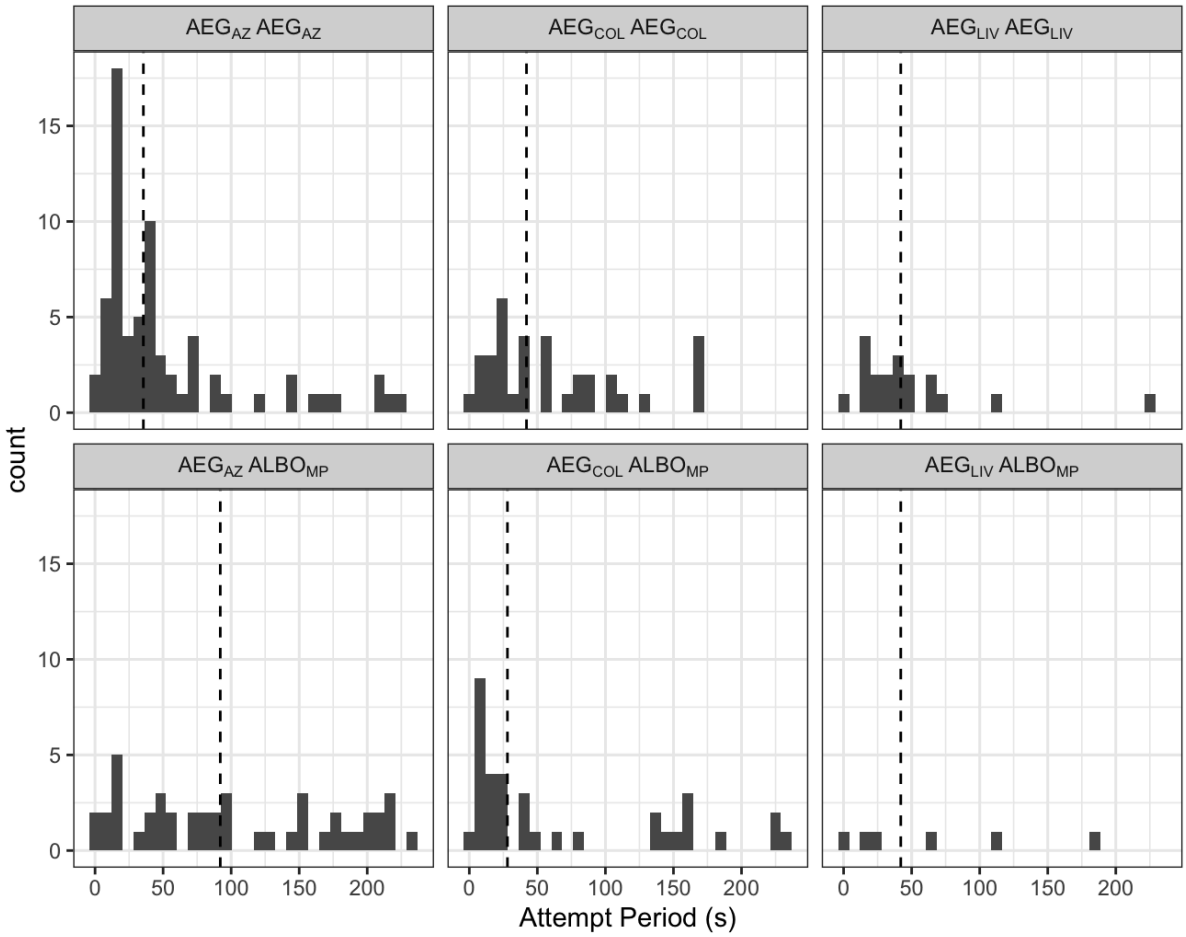

Figure 4.4 – Differences in the length of the Attempt Period between crosses. Crosses are female-male, such that AEG$_{AZ}$ALBO$_{MP}$ means AEG$_{AZ}$ females crossed with ALBO$_{MP}$ males. Each column represents a particular female strain, where the top row examines conspecific crosses, and the bottom row represents heterospecific crosses. The vertical dashed lines show the median for that cross.

I found that mating type had a significant effect on Attempt Period (P = 0.00047). In conspecific crosses, males attempted to mate with the female for longer than in heterospecific crosses (coeff = 0.0068, SE = 0.0020, P = 0.00056), as seen in figures 4.3 and 4.4. However, there was no effect of male strain, female strain, mating type or their interaction with timing block on the Attempt Rate, as seen in figure 4.5.

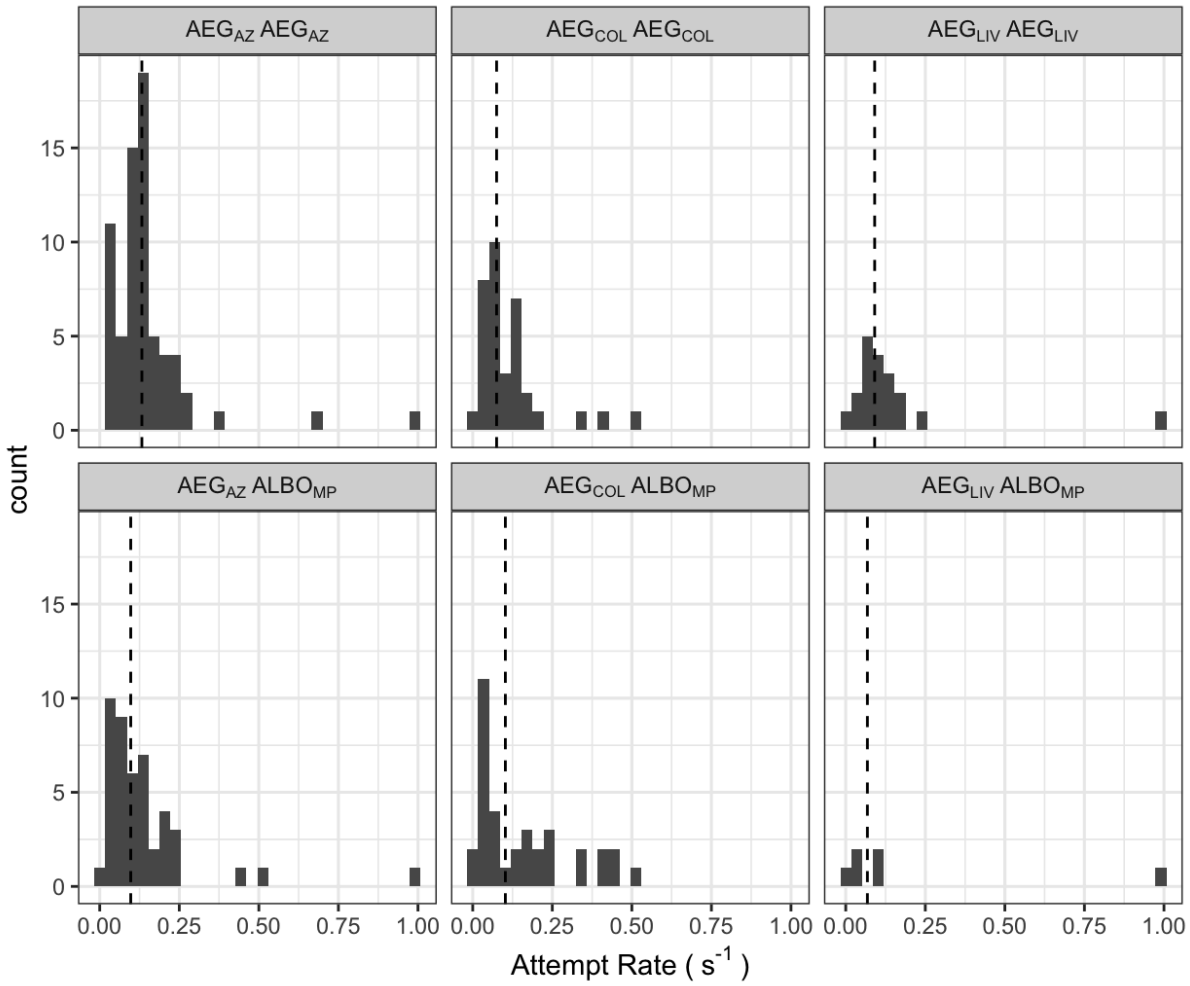

Figure 4.5 – Differences in the Attempt Rate between crosses. Crosses are female-male, such that AEG_{AZ} $ALBO_{MP}$ represents AEG_{AZ} females crossed with $ALBO_{MP}$ males. Each column represents a particular female strain, where the top row examines conspecific crosses and the bottom row represents heterospecific crosses. The vertical dashed lines show the median for that cross.

6.1.3. Latency to first mating attempt

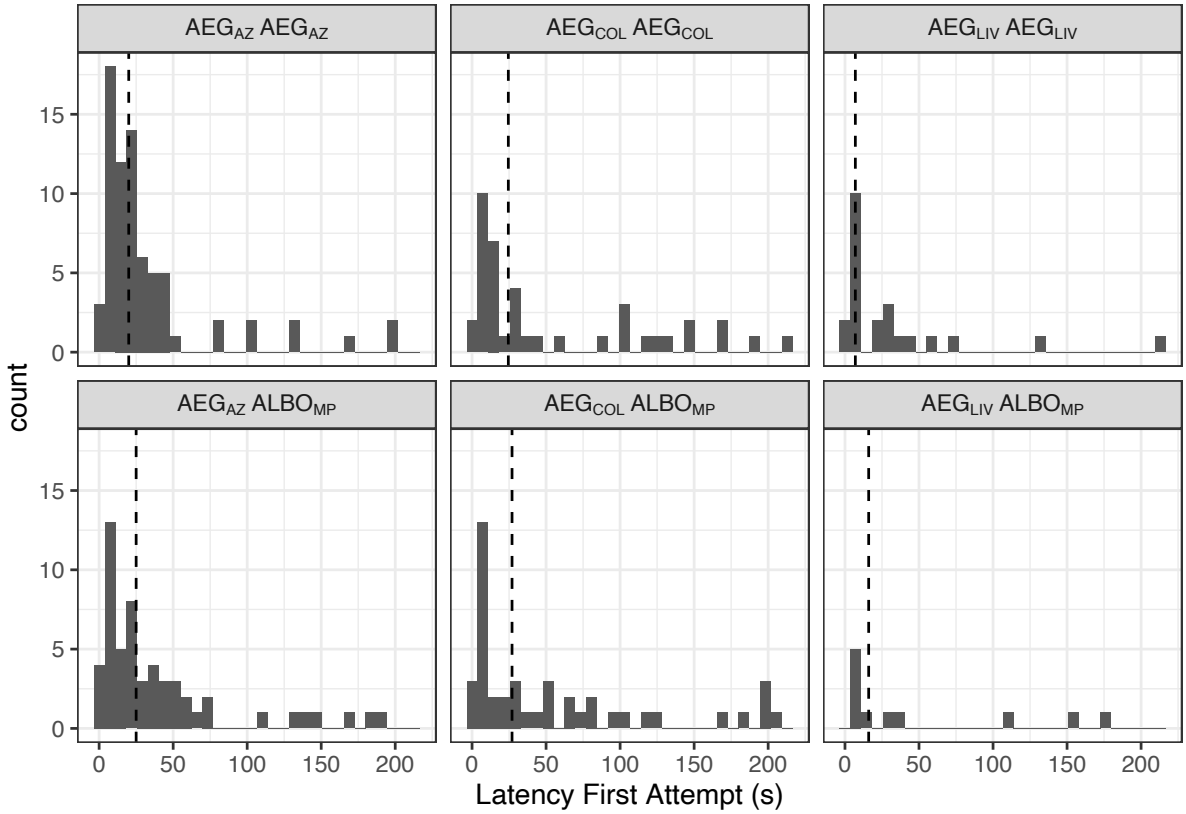

Figure 4.6 – Differences in the latency to first mating attempt between crosses. Crosses are female-male, such that AEG$_{AZ}$ ALBO$_{MP}$ represents AEG$_{AZ}$ females crossed with ALBO$_{MP}$ males. Each column represents a particular female strain, where the top row examines conspecific crosses and the bottom row represents heterospecific crosses. The vertical dashed lines show the median for that cross.

I found a significant interaction between male species and timing block on the latency to first mating attempt (P = 0.00019). The post-hoc analysis shows that AEG$_{AZ}$ males have a long latency to first mating attempt when the cross occurs in the morning. When comparing crosses that occur in the morning, AEG$_{AZ}$ males take longer to make their first mating attempt than both AEG$_{COL}$ (coeff = 0.035, SE = 0.0097, P = 0.0079) and ALBO$_{MP}$ males (coeff = 0.032, SE = 0.0096, P = 0.019). Furthermore, AEG$_{AZ}$ males take longer to make their first

mating attempt in the morning than they do in the afternoon (coeff = 0.029, SE = 0.010, *P* = 0.080), and longer than $ALBO_{MP}$ males take to make their first mating attempt the afternoon (coeff = 0.028, SE = 0.010, *P* = 0.095), both with marginal significance.

6.1.4. Female rejection behaviour

(i) Hold

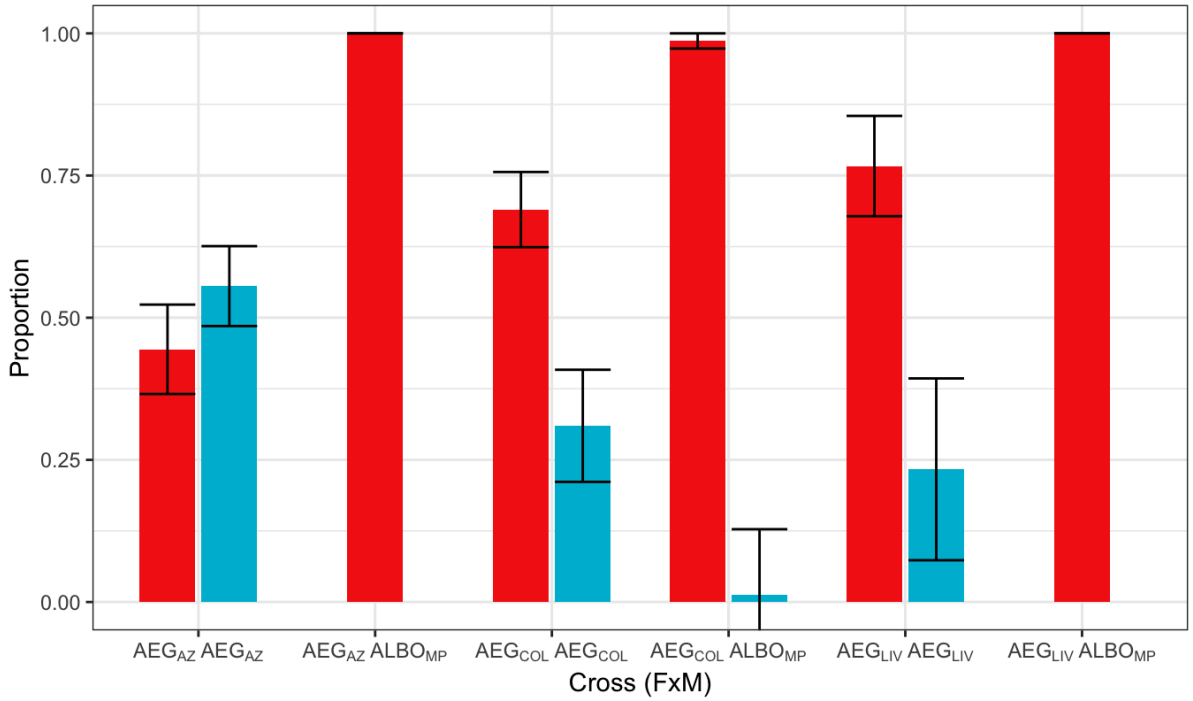

Figure 4.7 – Differences in the proportion of trials where females held males away (blue) as a form of resistance behaviour, or did not (red), between crosses. Bars are ordered by female strain, and mating type, such that the conspecific cross for a particular female strain is followed by the corresponding heterospecific cross. Crosses are female-male, such that AEG_{AZ} $ALBO_{MP}$ represents AEG_{AZ} females crossed with $ALBO_{MP}$ males. Error bars represent the standard error of the proportion.

Females hold males away from their abdomen to prevent insemination, as a form of resistance behaviour. Females almost exclusively perform this rejection behaviour in conspecific crosses; this behaviour was only observed in one heterospecific cross. As the heterospecific category is made of almost all 0s, I could not examine this using a generalised mixed linear model. A Chi-squared test confirmed that females are more likely to hold males away in a conspecific cross, than heterospecific crosses (X^2 = 89.281, df = 1, P < 2.2e-16,). I

could not conduct any further analyses including the heterospecific crosses, as there was such little variation within that category.

When comparing the likelihood of rejection behaviour in conspecific crosses, I found no impact of cross or its interaction with timing block on the likelihood of females holding males away.

(ii) Kick

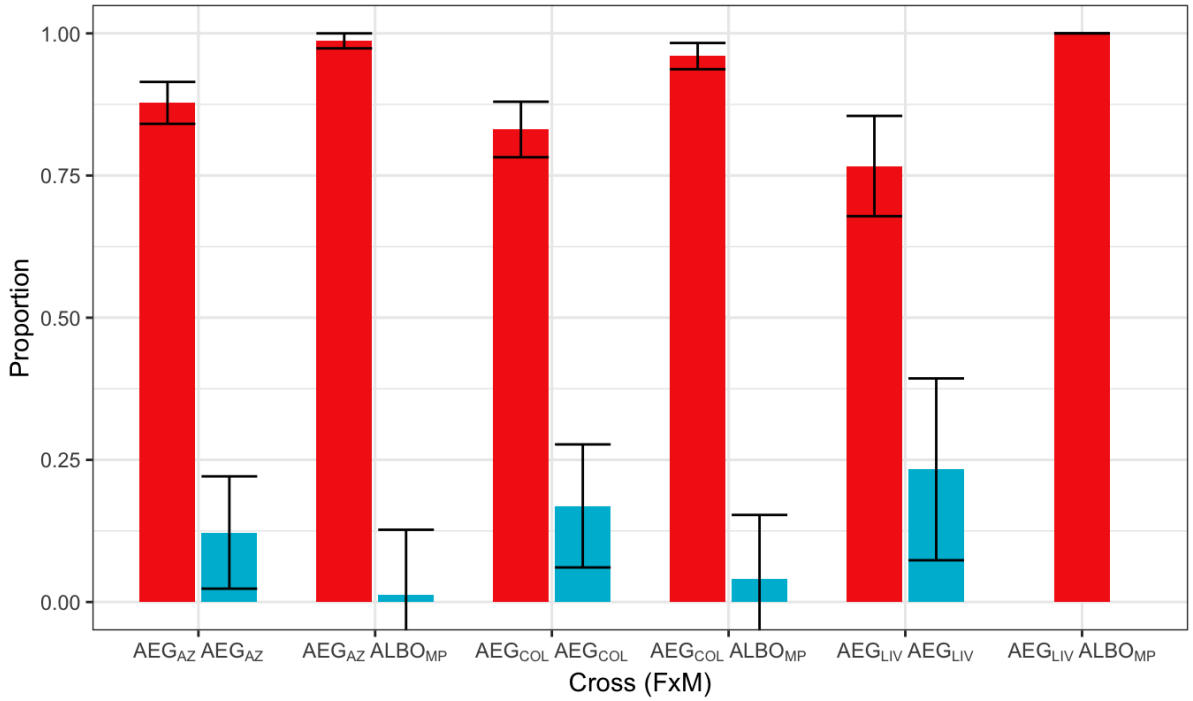

Figure 4.8 – Differences in the proportion of trials where females kicked males away (blue) as a form of resistance behaviour, or did not (red), between crosses. Bars are ordered by female strain, and mating type, such that the conspecific cross for a particular female strain is followed by the corresponding heterospecific cross. Crosses are female-male, such that AEG_{AZ} $ALBO_{MP}$ represents AEG_{AZ} females crossed with $ALBO_{MP}$ males. Error bars represent the standard error of the proportion.

Female mosquitoes kick males away to prevent the male inseminating them, as another form of rejection behaviour. This type of rejection behaviour is very rarely seen in heterospecific crosses; only 4 kicks were observed in heterospecific crosses. Thus, there was a significant effect of mating type (conspecific/heterospecific) on the likelihood of females

kicking males away (P= 3.3 x10^{-6}), and females were significantly more likely to kick away conspecific males than heterospecific males (coeff = -2.090, SE = 0.54, P = 0.00013).

6.1.5. Copula occurrence

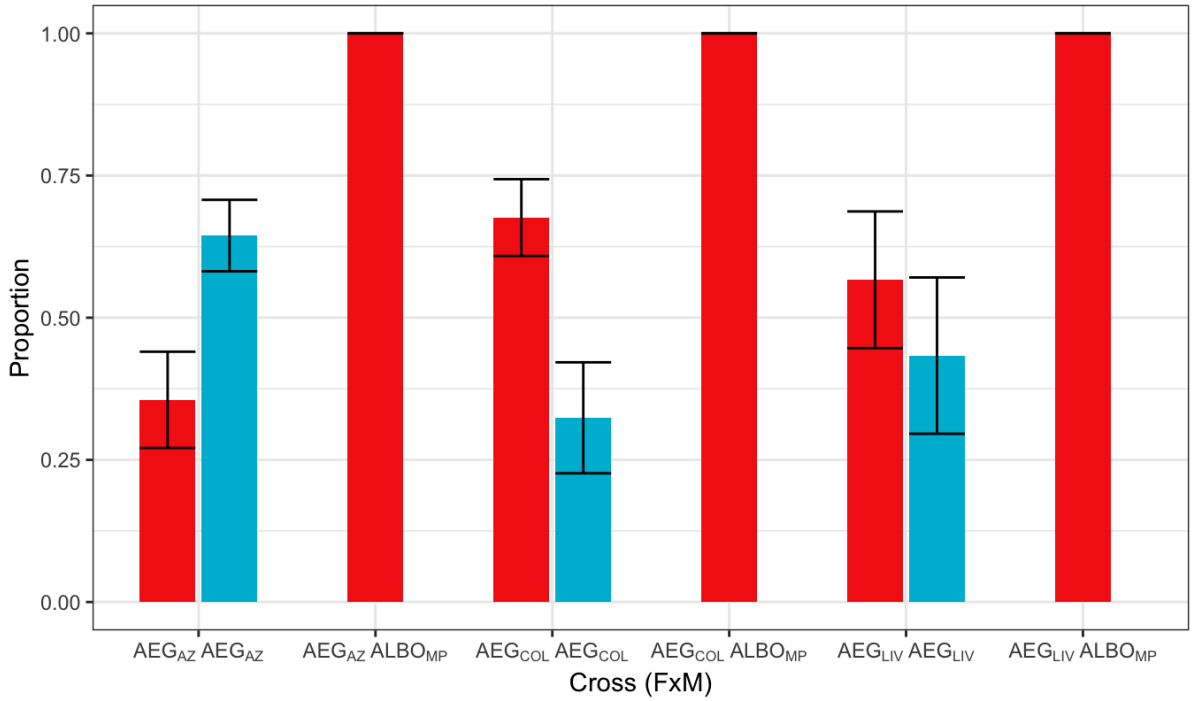

Figure 4.9 – Differences in the proportion of females that formed a copula (blue) or did not form a copula (red), between crosses. Bars are ordered by female strain, and mating type, such that the conspecific cross for a particular female strain is followed by the corresponding heterospecific cross. Crosses are female-male, such that AEG_{AZ} $ALBO_{MP}$ represents AEG_{AZ} females crossed with $ALBO_{MP}$ males. Error bars represent the standard error of the proportion.

Although mating attempts were made in heterospecific crosses, no copulas were formed. However, for all conspecific crosses, a proportion resulted in a copula, as seen in figures 4.3 and 4.9.

I examined differences in the number of copulas formed using a Chi-squared test, as the heterospecific category was made up of all 0s. I found that conspecific crosses are significantly more likely to result in a copula than heterospecific crosses (X^2= 116.61, df = 1, *P*

$< 2.20x10^{-16}$). I could not conduct any further analyses including the heterospecific crosses, as there was no variation within that category.

I conducted further analysis of the conspecific crosses and found that there was a significant impact mating type (*P*= 0.011) on the likelihood of a copula forming. Post-hoc analysis showed that conspecific crosses between AEG_{AZ} males and females resulted in more copulas than conspecific crosses between AEG_{COL} males and females (coeff = 1.39, SE=0.46, *P* = 0.0076).

Furthermore, timing block had a significant effect on the likelihood of a copula forming (*P* = 0.044): copulas were more likely to occur in the morning than in the afternoon (coeff = 0.86, SE = 0.44, *P* = 0.051).

6.1.6. Copula Duration

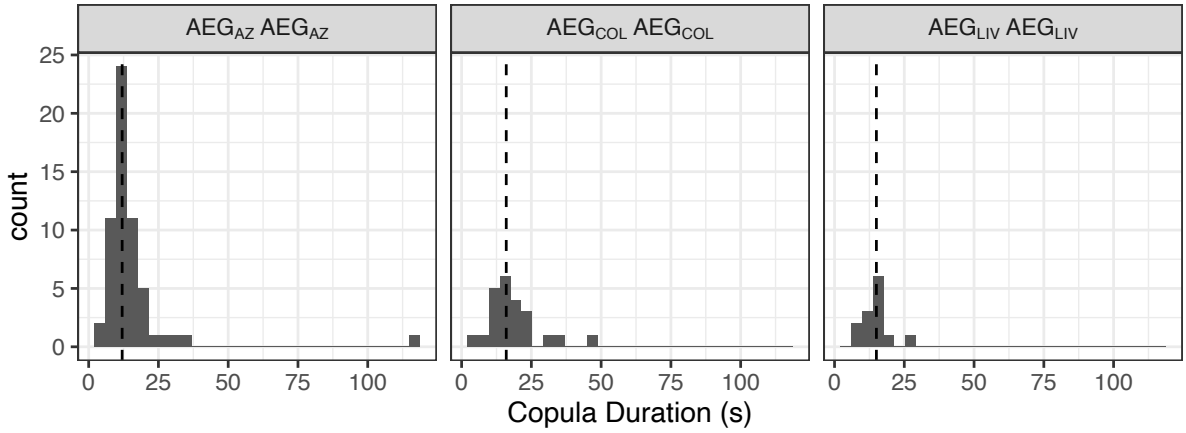

Figure 4.10 – Differences in copula duration, between conspecific crosses. The vertical dashed lines show the median for that cross. Crosses are female-male, such that AEG_{AZ} $ALBO_{MP}$ represents AEG_{AZ} females crossed with $ALBO_{MP}$ males.

Differences in the length of a copula can only be compared between conspecific crosses, as no copulas formed in heterospecific crosses. I found that there was a significant effect of timing block on the length of copula (*P*= 0.0042), and that copulas were shorter in in the morning than in the afternoon (coeff = -0.023, SE = 0.0082, *P* = 0.0042). Furthermore, copulas were longer in some conspecific crosses than others, with marginal significance (*P*=0.061), however post-hoc analysis showed no significant pairwise differences.

6.1.7. Insemination Occurrence

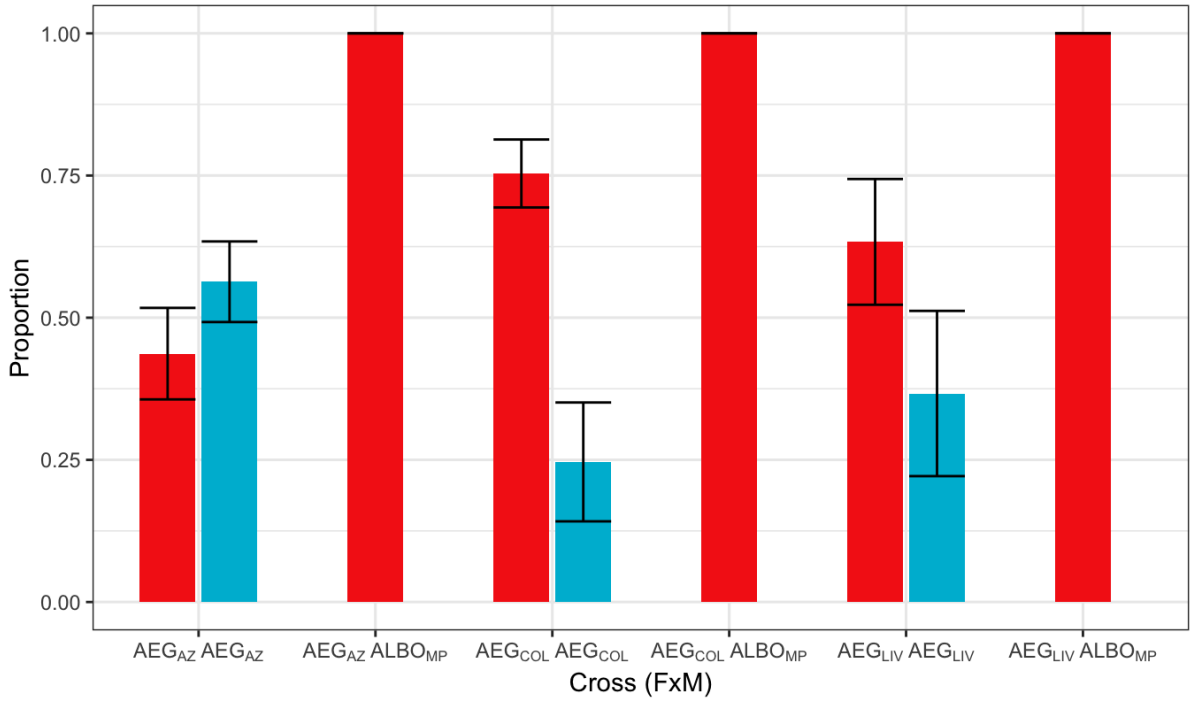

Figure 4.11 – Differences in the proportion of females that were inseminated (blue) or not inseminated (red), between crosses. Bars are ordered by female strain, and mating type, such that the conspecific cross for a particular female strain is followed by the corresponding heterospecific cross. Crosses are female-male, such that AEG_{AZ} $ALBO_{MP}$ represents AEG_{AZ} females crossed with $ALBO_{MP}$ males. Error bars represent the standard error of the proportion.

I found similar results with insemination occurrence as copula occurrence. This is to be expected, as a copula is a prerequisite to insemination. I found that no insemination occurred in heterospecific crosses, however there was a proportion of females inseminated in all conspecific crosses. Again, as the heterospecific category contains only 0s, I used a Chi-squared test to assess any differences between conspecific and heterospecific crosses. This showed that insemination is significantly more likely in conspecific crosses than

heterospecific crosses (X^2= 92.35, df = 1, $P < 2.2x10^{-16}$). I could not conduct any further analyses including the heterospecific crosses, as there was no variation within that category.

I compared the proportion of females inseminated in conspecific crosses, and found that there was a significant effect of timing block (P = 0.038): significantly more conspecific females were inseminated in the morning than in the afternoon (coeff = 0.95, SE= 0.47, P = 0.045). Furthermore, I found that insemination was more likely to occur in some conspecific crosses than others (P =0.042). As with copula formation, I found that insemination was significantly more likely to occur in conspecific crosses between AEG_{AZ} males and females than AEG_{COL} males and females (coeff = 1.25, SE = 0.49, P = 0.030).

6.1.8. Copula – Insemination Correlation

I found a strong, significant correlation between the occurrence of a copula, and insemination (r = 0.91, 95% CI [0.89, 0.93], $P < 2.2x10^{-16}$). The formation of a copula is a prerequisite for insemination, and the significant correlation between the two show that a high proportion of copulas result in successful insemination, as shown in figure 4.12.

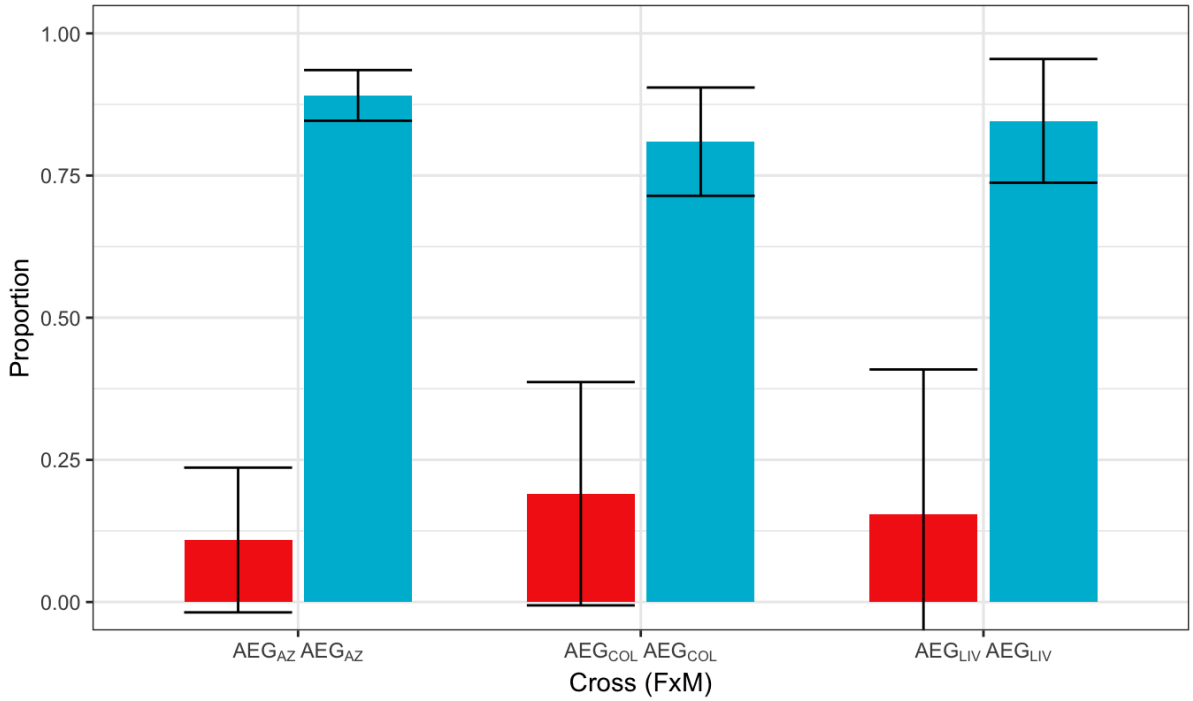

Figure 4.12 – Differences in the proportion of copulas that resulted in a successful insemination (blue) and did not (red), between crosses. Crosses are female-male, such that AEG_{AZ} $ALBO_{MP}$ represents AEG_{AZ} females crossed with $ALBO_{MP}$ males. Error bars represent the standard error of the proportion.

6.1.9. Effortfulness of attempt

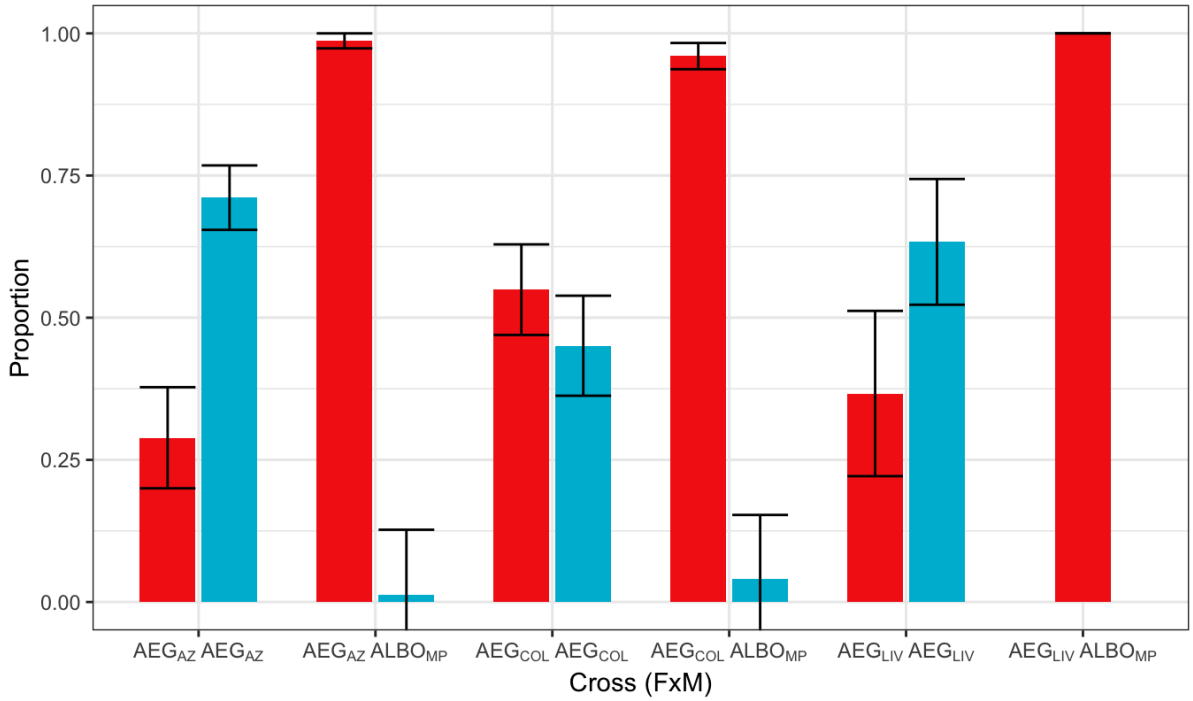

Figure 4.13 – Differences in the proportion of males that made an effortful attempt (blue) or did not (red), between crosses. Bars are ordered by female strain, and mating type, such that the conspecific cross for a particular female strain is followed by the corresponding heterospecific cross. Crosses are female-male, such that AEG_{AZ} $ALBO_{MP}$ represents AEG_{AZ} females crossed with $ALBO_{MP}$ males. Error bars represent the standard error of the proportion.

I examined how effortful an attempt by a male was, where an effortful attempt resulted either in a copula forming or female rejection behaviour (kick or hold) occurring. I found significant differences in the effortfulness attempts between different strains of males ($P < 2.2x10^{-16}$). Post-hoc analysis showed that all conspecific males – AEG_{AZ} (coeff = 4.62, SE = 0.57, $P < 0.00010$), AEG_{COL} (coeff = 3.69, SE = 0.57, $P < 0.00010$) and AEG_{LIV} (coeff = 4.34, SE = 0.65, $P < 0.00010$) made more effortful attempts than the heterospecific male, $ALBO_{MP}$.

Furthermore, AEG_{AZ} made marginally more effortful attempts than AEG_{COL} (coeff = 0.93, SE = 0.39, *P* = 0.078).

6.1.10. Female inactivity

I graphically examined female inactivity across all trials, as seen in figure 4.14. Females that were more active within the trial were more likely to form a copula. In 70.6% of trials that resulted in a copula, the females spent most of their time active, while in 97.5% of trials that did not result in a copula, the female was inactive for most of the trial.

When statistically examining female inactivity, I used data from trials where a copula did not form and thus the trial lasted the full 5 minutes. This allowed us to make direct comparisons of inactivity between trials. Female inactivity data did not fit either the normal or the gamma distribution, so I used non-parametric tests to analyse these data.

I found that some strains of female *Ae. aegypti* were more inactive than others (Kruskal-Wallis $X^2 = 12.63$, df = 2, $P = 0.0018$), and inactivity was not dependent on the strain of male. Post-hoc pairwise Wilcoxon test found that there was a significant difference in female inactivity between AEG_{LIV} and AEG_{COL} females ($P = 0.0012$), where AEG_{LIV} (median inactivity = 291s) was more inactive than AEG_{COL} (median inactivity = 276s).

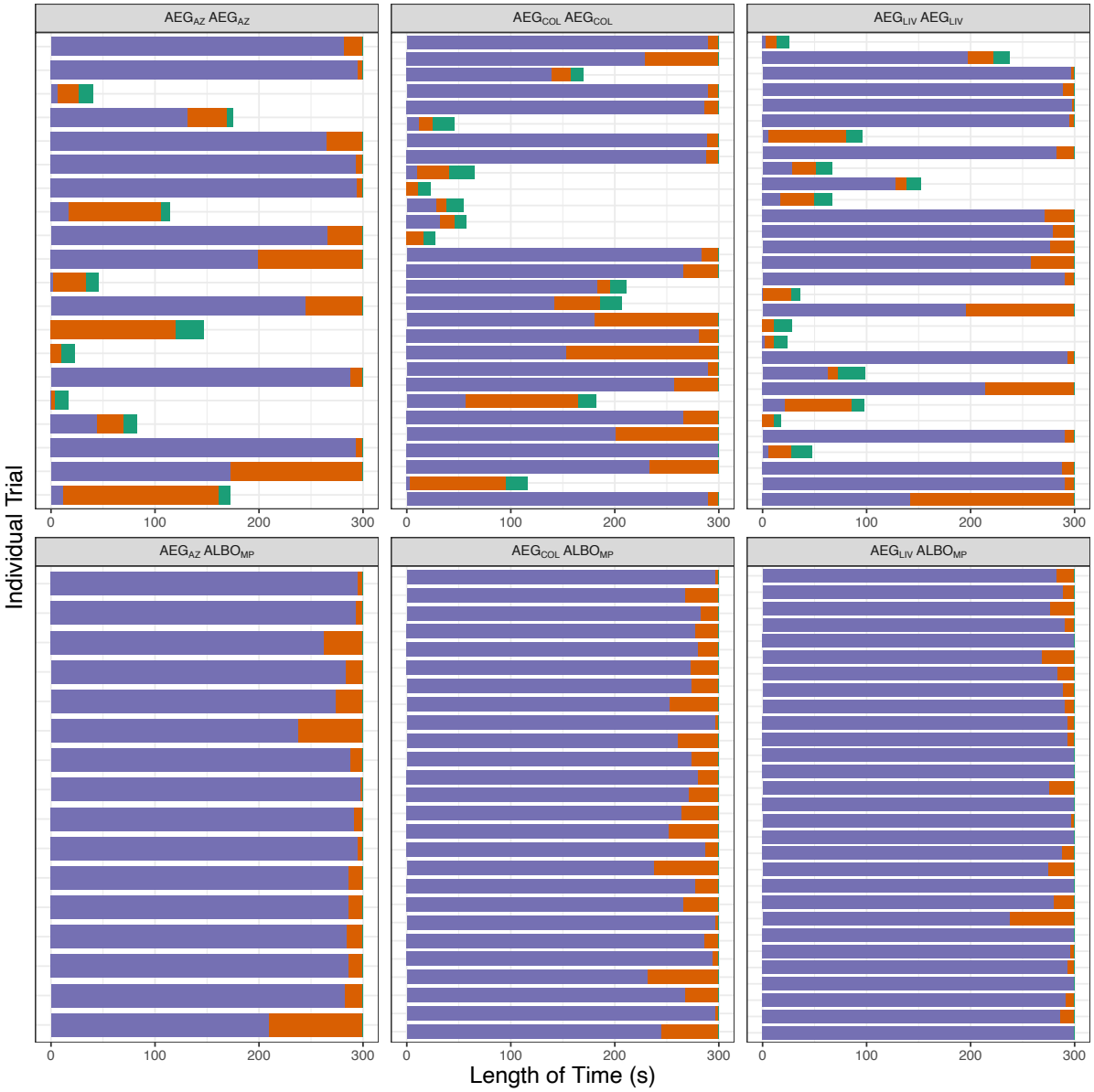

Figure 4.14 – Differences in female activity in each individual trial, between crosses. The purple section of the bar represents the total length of time females are inactive within the trial, and the orange and green sections show the total length of time females are active within the trial. The green section of the bar shows when a female is engaged in a copula, and the orange section shows female activity when not in a copula. Trials ceased when a copula was formed, or when 5 minutes (300s) was completed. Crosses are female-male, such that AEG_{AZ} $ALBO_{MP}$ represents AEG_{AZ} females crossed with $ALBO_{MP}$ males. Each column represents a particular female strain, and the top row has conspecific crosses and the bottom row heterospecific crosses.

6.2. Slow-motion Video Recordings of Tethered Females

6.2.1. Overview

I observed key differences in male mating behaviour between conspecific and heterospecific crosses. Aldersley and Cator (2019) described the key stages of mating as: a male flying towards a female, making contact with the female, moving to the front of the female, securing her tarsi, the pair ventrally aligning and a copula forming. I observed that in heterospecific matings, males made brief initial contact with the female, sometimes followed by further exploratory contact, and then did not to pursue mating, and flew away, as seen in figure 4.15. Thus, heterospecific males never proceeded past the second stage of mating, as described by Aldersley and Cator (2019), and did not trigger female resistance behaviour. However, in conspecific matings, males frequently went through all stages of mating, triggering female resistance behaviour and often resulting in a copula.

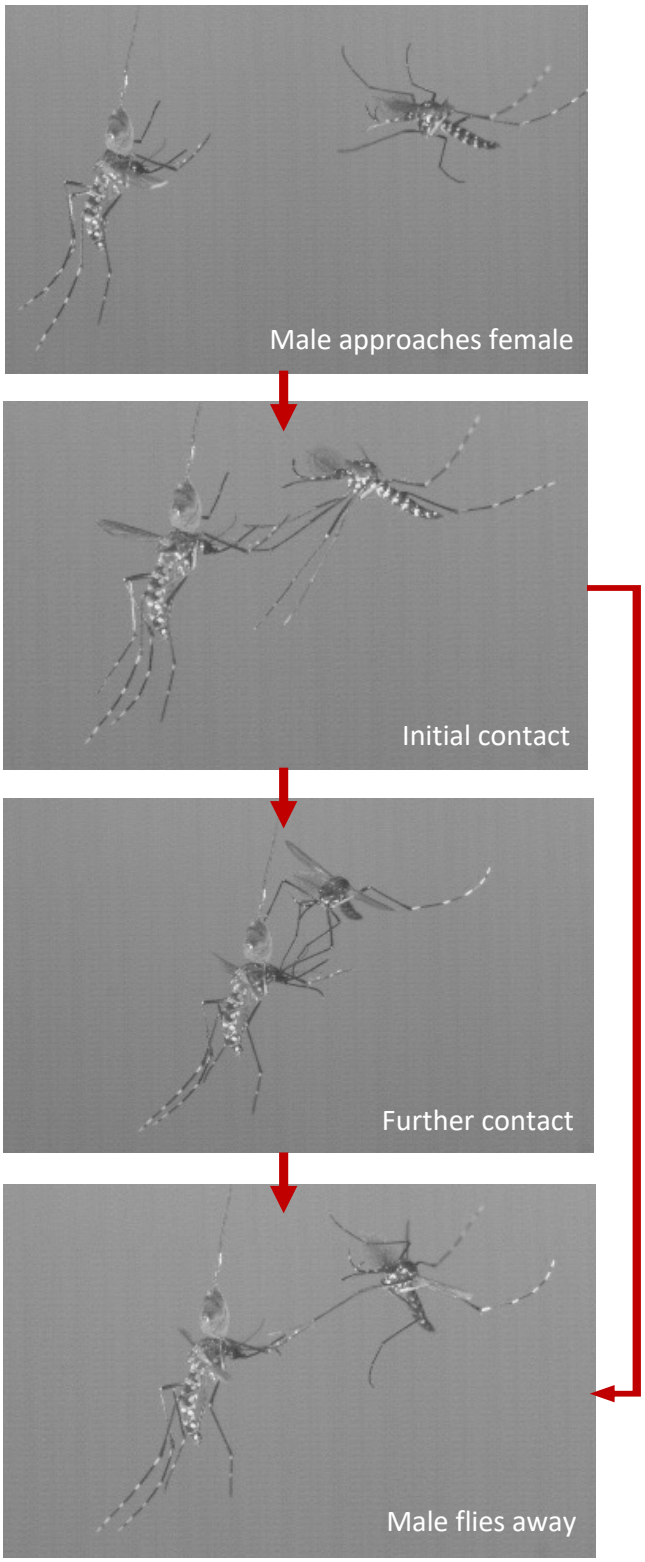

Figure 4.15 – an overview of heterospecific mating behaviour between an Ae. aegypti female and an Ae. albopictus male. I observed that in heterospecific matings, the Ae. albopictus male approached the Ae. aegypti female, made initial contact and, in some cases, the male made further exploratory contact. The male then did not pursue the mating attempt any further and flew away. Images are taken from a recording we took of a tethered Ae. aegypti female and a free-flying Ae. albopictus male.

6.2.2. Duration of Contact

I found that males were much less persistent in heterospecific crosses than conspecific crosses. This is demonstrated by clear differences in the length of male contact – 94.12 % (16/17) of heterospecific interactions were less than 0.5s long, while only 17.64% (3/17) of conspecific interactions were less than 0.5s long, as seen in figure 4.16. Thus, the duration of contact was significantly greater in conspecific crosses than heterospecific crosses (W = 258, P=9.93 x10^{-5}).

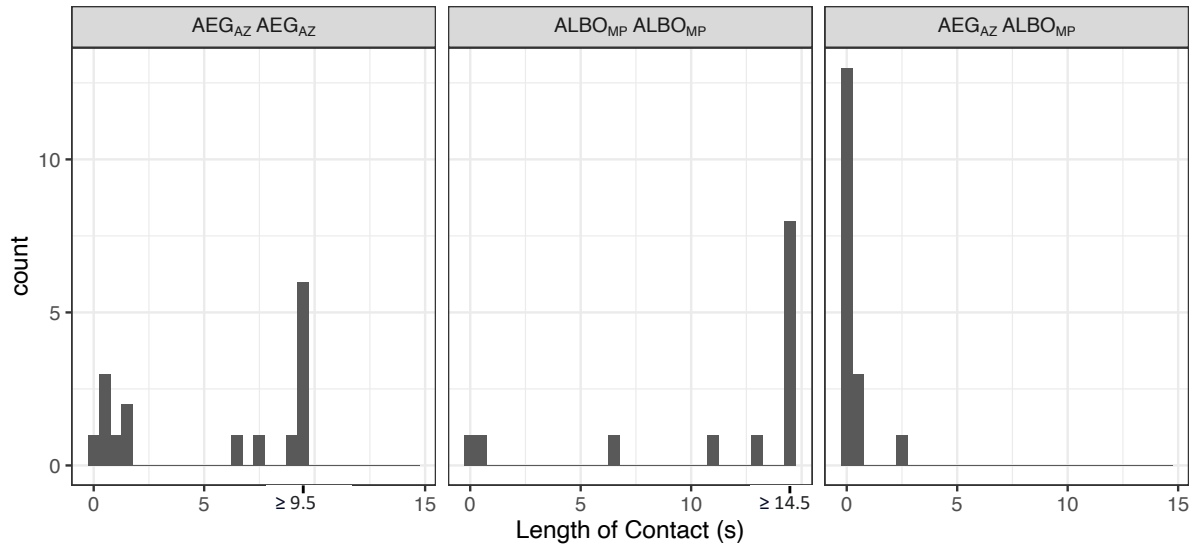

Figure 4.16 – Differences in the length of contact within an interaction between a male and female, between crosses. Crosses are female-male, such that AEG$_{AZ}$ ALBO$_{MP}$ represents AEG$_{AZ}$ females crossed with ALBO$_{MP}$ males. For the conspecific crosses, I had to group some of timings as they began before the recording started or finished after the recording stopped, as detailed in the methods.

6.2.3. Copula

Copulas only formed during conspecific matings, because heterospecific males did not pursue mating following brief contact with the female. Thus, copulas were more likely to form in conspecific than heterospecific crosses (X^2=8.76, df=1, *P* = 0.0031), as seen in figure 4.17. Furthermore, more copulas formed in the conspecific cross between *Ae. albopictus x Ae. albopictus* (57.14%, 8/14) than *Ae. aegypti* x *Ae. aegypti* (35.29%, 6/17), and this difference is significant (X^2= 16.80, df=2, *P* = 0.00022).

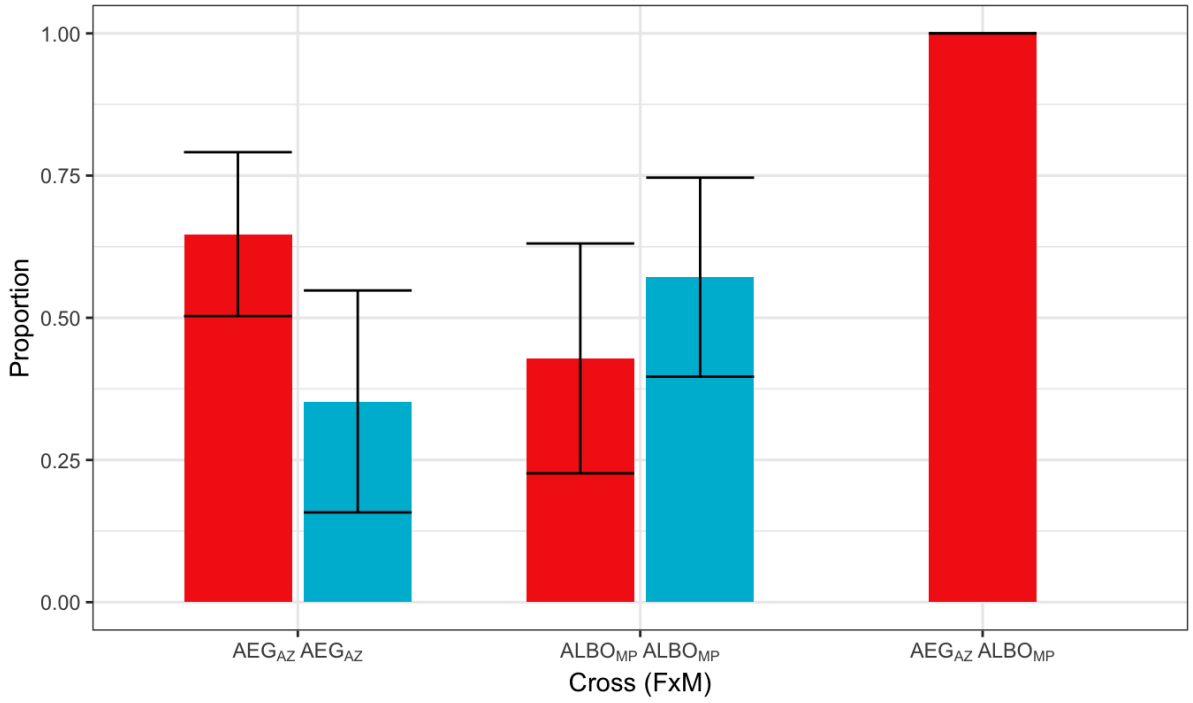

Figure 4.17 – Differences in the proportion of trials where a copula formed (blue), or did not (red), between crosses. Crosses are female-male, such that AEG_{AZ} $ALBO_{MP}$ represents AEG_{AZ} females crossed with $ALBO_{MP}$ males. Error bars represent the standard error of the proportion.

The histogram of length of copula (figure 4.18) shows differences between the conspecific crosses. However due to the small number of copulas for each cross ($ALBO_{MP}$, n=8, AEG_{AZ} AEG_{AZ}, n=6) little can be deduced, and I did not conduct a statistical test.

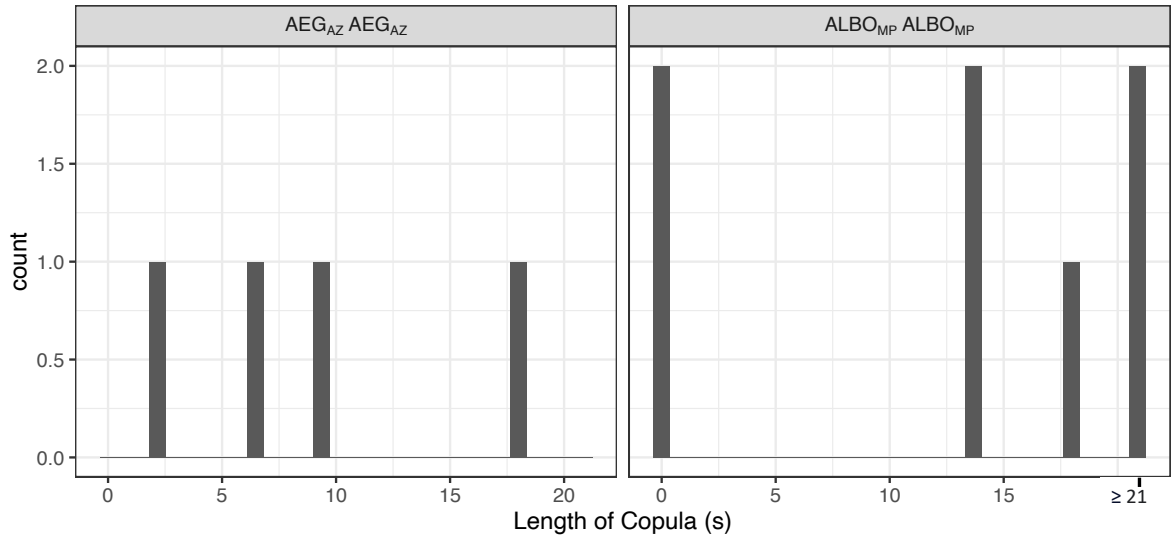

Figure 4.18 – Differences in the length of copula within an interaction between conspecific males and females, between crosses. Crosses are female-male, such that AEG_{AZ} $ALBO_{MP}$ represents AEG_{AZ} females crossed with $ALBO_{MP}$ males. For the $ALBO_{MP}$ $ALBO_{MP}$ cross, I had to group some timings, as they began before the recording started or finished after the recording stopped, as detailed in the methods.

6.2.4. Resistance Behaviour

Resistance behaviour did not occur in heterospecific crosses, but occurred in a high proportion of conspecific crosses: 78.57% of $ALBO_{MP}$ $ALBO_{MP}$ crosses, and 70.59% of AEG_{AZ} AEG_{AZ} crosses, as seen in figure 4.19. Thus, more resistance behaviour occurred in conspecific than heterospecific crosses (X^2 = 6.83, df = 1, *P* = 0.0090).

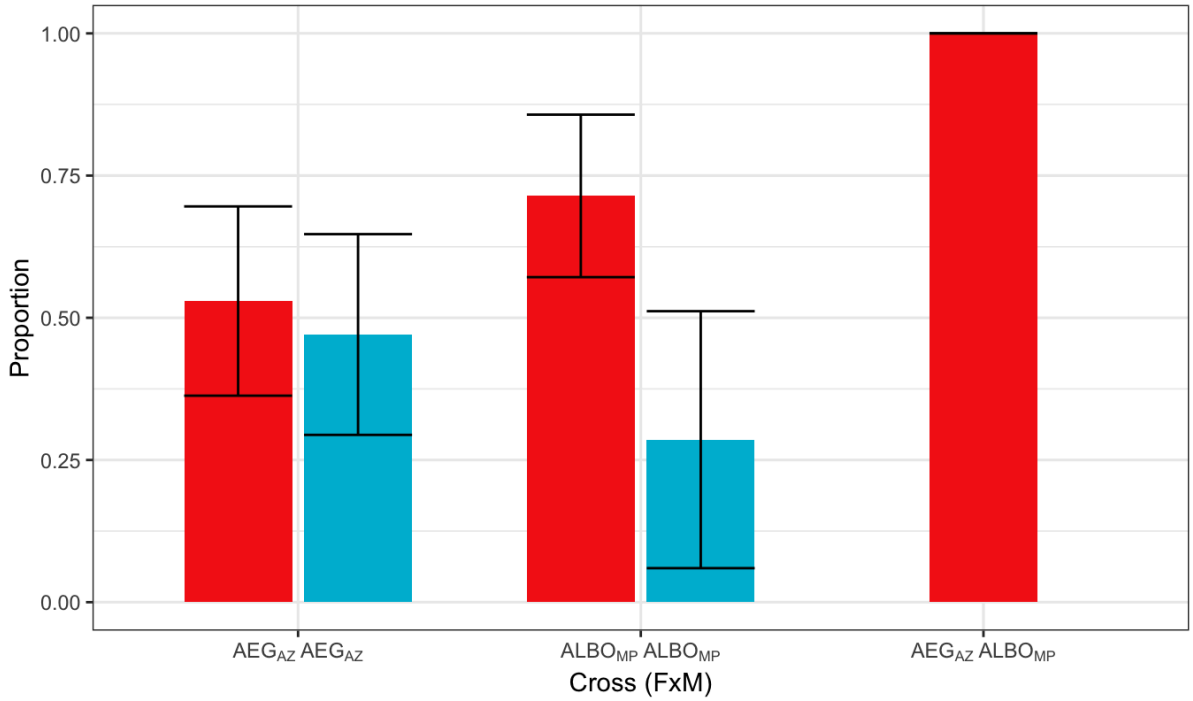

Figure 4.19 – Differences in the proportion of trials where a resistance behaviour occurred (blue), or did not occur (red), between crosses. Crosses are female-male, such that AEG_{AZ} $ALBO_{MP}$ represent AEG_{AZ} females crossed with $ALBO_{MP}$ males. Error bars represent the standard error of the proportion.

7. Discussion

7.1. Overview

While mating behaviour between susceptible *Ae. aegypti* females and *Ae. albopictus* males has previously been examined (Zhou et al., 2022), this is the first study to characterise the mating behaviour that prevents insemination of *Ae. aegypti* females by *Ae. albopictus* males. To examine this, we conducted real-time observations of mating behaviour between a free-flying *Ae. aegypti* female and four free-flying *Ae. albopictus* males and took slow-motion recordings of the first mating interaction between a tethered female, and a free-flying male. The former gave us an overview of mating behaviour in more natural conditions; the mosquitoes were free-flying and there were multiple males, allowing swarming behaviour to occur (Facchinelli et al., 2015; Gubler, Bhattacharya & Bhattacharva, 1972). The latter of these allowed us to examine mating behaviour at a finer scale. In combination, these observations gave us a comprehensive overview of mating behaviour between *Ae. aegypti* females and *Ae. albopictus* males.

As in *Chapter 3*, I found that no copulas formed, and no insemination occurred in heterospecific crosses. The results of our cage trials of free-flying mosquitoes, and slow-motion recordings of tethered females, showed that this was not due to females resisting insemination by heterospecific males, by kicking males away or holding males away from their abdomen, as occurs in conspecific crosses (Roth, 1948; Aldersley & Cator, 2019; Cator & Zanti, 2016; Cator & Harrington, 2011; Jones & Pilitt, 1973).

In our cage trials of free-flying mosquitoes, despite the insemination rate being higher in conspecific crosses, females kicked and held away conspecific males more than heterospecific males. *Ae. aegypti* females only held away a heterospecific male on one occasion (0.552% of the heterospecific crosses), yet held away conspecific males at a much higher rate (n=79, 41.4% of conspecific crosses). *Ae. aegypti* females also kicked away conspecific males (n=30,15.71 %) at a higher rate than heterospecific males (n=4, 2.21%). The same trends were seen in our slow-motion recordings of tethered females: female resistance behaviour only occurred in conspecific crosses, yet no copulas formed in heterospecific interactions, but did occur in 43.75% of conspecific interactions. Therefore, the lack of copulas and insemination in heterospecific crosses was not due to resistance behaviour from *Ae. aegypti* females.

I suggest that the lack of observed female resistance behaviour in heterospecific crosses in this experiment is because male *Ae. albopictus* are not vigorously attempting to mate with the *Ae. aegypti* females, and thus do not trigger resistance behaviour. This is consistent with the results of Harper and Paulson (1994), who found that removing hindtsarsi, which are used in mating resistance behaviour, from *Ae. aegypti* females did not increase heterospecific insemination rates. Therefore, suggesting that the lack of heterospecific insemination was due to male *Ae. albopictus* behaviour. In our cage trials of free flying mosquitoes, I found that male *Ae. albopictus* were equally as likely as male *Ae. aegypti* to attempt to mate with female *Ae. aegypti*, however I found differences in male persistence. *Ae. aegypti* males attempted to mate with *Ae. aegypti* females over a longer period than *Ae. albopictus* males, thus, male *Ae. albopictus* made less sustained mating attempts than *Ae.*

aegypti males. This could suggest that *Ae. albopictus* males are making exploratory contact with the female, rather than a mating attempt that may result in a copula.

The results from our slow-motion recordings of tethered females support this conclusion. I observed that male *Ae. albopictus* approached *Ae. aegypti* females and made brief contact with the female before deciding not to pursue mating and flying away. Thus, male *Ae. albopictus* never proceeded past the second stage of mating, as described by Aldersley and Cator (2019). I observed different behaviour in conspecific crosses: after the male initially made contact with the conspecific female, the male further pursued mating with the female. Thus, males in conspecific crosses made physical contact with females for a significantly longer period than males in heterospecific crosses.

I suggest that signalling occurs when male mosquitoes make physical contact with female mosquitoes. When this contact occurs between *Ae. albopictus* males and *Ae. aegypti* females, the information received seems to prevent *Ae. albopictus* males from attempting to copulate with the *Ae. aegypti* females. However, in conspecific crosses this contact results in males pursuing the mating attempt.

7.2. Possible Mechanisms

I suggest that male *Ae. albopictus* do not pursue mating with *Ae. aegypti* females after initial contact due to the detection of contact pheromones on the tarsi of *Ae. aegypti* females. Contact pheromones require physical contact to be detected; thus, these pheromones will only have an effect after the male has made physical contact with the female mosquito. This is consistent with the results of our slow-motion recordings of a tethered female, where male *Ae. albopictus* initially make physical contact with female *Ae. aegypti* and then do not further pursue attempting to mate.

Early work by Nijhout and Craig (1971) found that treating the last two segments of the front and middle tarsi of *Aedes* males with solvent caused a reduction in sustained contact between conspecific male and female *Aedes* and a complete prevention of sustained contact between heterospecifics (Nijhout & Craig, 1971). As suggested by Nijhout and Craig (1971), this could be because male *Aedes* use the last two segments of the front and middle tarsi to recognise females via contact pheromones, and treatment with a solvent inactivates these chemosensory receptors. Therefore, the transmission of heterospecific contact pheromones from *Ae. aegypti* females to male *Ae. albopictus* may deter the *Ae. albopictus* males from attempting to copulate with the *Ae. aegypti* females. Meanwhile, the transfer of conspecific contact pheromones may encourage the males to pursue mating attempts.

One type of contact pheromone is cuticular hydrocarbons (CHCs), which are waxy molecules derived from fatty acids that cover the cuticle of most insects. CHCs are complex, chemically diverse and have a low volatility, and thus they are used for contact communicative

processes (Howard & Blomquist, 2005; Menzel et al., 2019; Ginzel & Blomquist, 2016) including mate recognition (reviewed by Chung and Carroll, 2015; Würf *et al.*, 2020) across multiple taxa. CHCs are involved in identifying conspecifics in *Timema spp*: Schwander *et al.* (2013) found that hind tarsi cuticular hydrocarbon profiles vary among *Timema* species and interspecific mating is more likely between species with similar hydrocarbon profiles.

Recent research suggests that CHCs are used as a signal in mosquito mating and are under sexual selection. There are differences in the CHCs in mated and unmated mosquitoes: mated *Anopheles coluzzii* males have a higher CHC abundance than unmated controls (Adams et al., 2021) and *Ae. aegypti* and *Anopheles gambiae* females have a different CHC profile following mating (Polerstock, Eigenbrode & Klowden, 2002). Furthermore, *Anopheles stephensi* males treated with the CHC heptacosane inseminate more females than untreated controls (Wang et al., 2021). The role of CHCs in preventing heterospecific mating in *Aedes* mosquitoes has not been investigated. I suggest that *Ae. albopictus* males may be able to distinguish between conspecific and heterospecific CHCs, and only pursue mating with females with conspecific CHCs.

7.3. Possible Evolutionary Contexts

The ability of *Ae. albopictus* males to distinguish between conspecific and heterospecific females may have evolved as a form of male choice, or female choice. *Ae. albopictus* males may have evolved the ability to distinguish between conspecific and heterospecific females, so to prioritise mating efforts towards conspecific females and not waste time and effort attempting to mate with heterospecific females that will produce no progeny. Equally, female *Ae. aegypti* could have evolved to produce contact pheromones that deter heterospecific males to avoid being mated by a heterospecific male and becoming refractory to future matings (Klowden, 1999; Robbins et al., 2011; Leahy & Craig Jr., 1965). Due to the large fitness cost of effective sterilisation, there is a stronger selection pressure for female choice than male choice.

Both explanations rely on there being strain-specific differences, as studies examining insemination rates of different strains of *Ae. aegypti* and *Ae. albopictus* have different results. While in some instances, *Ae. aegypti* females previously unexposed to male *Ae. albopictus* are inseminated by *Ae. albopictus* males at a high rate (Bargielowski & Lounibos, 2014; Bargielowski et al., 2019, 2015; Honório et al., 2018), in *Chapter 3* I found no insemination of unexposed *Ae. aegypti* females by *Ae. albopictus* males. Therefore, there must be strain-specific differences in male *Ae. albopictus* or female *Ae. aegypti* behaviour that cause these discrepancies.

However, neither strain-specific differences in male choice or female choice alone can easily explain all the previous literature. Bargielowski and Lounibos (2014) found that *Ae. aegypti*

females previously unexposed to male *Ae. albopictus* are initially inseminated at a high rate by *Ae. albopictus*, however following exposure to *Ae. albopictus* evolved resistance to this heterospecific mating. These observations cannot be explained by male choice as the male *Ae. albopictus* lines used in the experiment were not evolved in the presence of *Ae. aegypti* females, thus there was no selection pressure for the evolution of male choice. However, other experimental results are difficult to explain by female choice alone. For instance, Honório *et al.* (2018) found differences in the ability strains of *Ae. albopictus* males to inseminate *Ae. aegypti* females. This result could be due to differences in the strength of male choice between *Ae. albopictus* strains, or due to differences in the receptivity of different strains of *Ae. albopictus* males to female choice signals. Alternative considerations, for example local adaptation and genetic drift, may be necessary to explain the variation in rates of heterospecific mating. Further experiments are necessary to clarify the evolutionary context of the mating behaviours observed in this experiment, and the mechanisms behind these behaviours.

7.4. Future Directions

7.4.1. Determining Role of Contact Pheromones

Following the behaviour observed in the slow-motion video recordings, I predict that cuticular hydrocarbons (CHCs) on the surface of female *Ae. aegypti* deter *Ae. albopictus* from attempting to copulate with female *Ae. aegypti*. To determine whether CHCs have a role in mating behaviour, I suggest repeating the behavioural assay, and for each cross to have a treatment group where CHCs are removed from the tarsi of females, and a control group where they are not removed. Any differences in behaviour between the treatment and control groups would suggest CHCs have a role in mating behaviour.

To determine whether CHCs are involved in male *Ae. albopictus* distinguishing between conspecific and heterospecific females, I suggest conducting reciprocal transplants of CHCs between *Ae. aegypti* and *Ae. albopictus* females and repeating the behavioural assay. If male *Ae. albopictus* are more likely to mate with *Ae. aegypti* females when they have CHCs from *Ae. albopictus* females and are less likely to mate with *Ae. albopictus* females when they have the CHCs from *Ae. aegypti* females, this would indicate that CHCs on the surface of females acts as a signal to encourage or deter mating in *Ae. albopictus* males.

7.4.2. Characterise inter-strain differences

This experiment examined mating interactions between heterospecifics, where no insemination occurred. However, previous experiments have shown that males from some strains of *Ae. albopictus* do inseminate some strains of *Ae. aegypti* females, I suggest

repeating the behavioural assay using these strains, as well as the strains in this experiment. I suggest conducting a full-factorial experiment, resulting in a cross between each female strain and each male strain. Comparing the insemination rates of the different crosses would allow us to determine whether differences in heterospecific mating rates are due to the strain of male *Ae. albopictus*, the strain of female *Ae. aegypti*, or their interaction. Additionally, the experiment would allow behavioural differences to be identified between heterospecific crosses that result in insemination, and heterospecific crosses that do not result in insemination.

Furthermore, the CHC experiments could be repeated on these strains. This would allow us to determine whether any of the differences in mating behaviour between heterospecific crosses with different strains, are due to differences in the CHCs on the surface of females.

8. Conclusion

In previous work (*Chapter 3*), I found that *Ae. aegypti* strains previously unexposed to *Ae. albopictus* males were not inseminated by *Ae. albopictus* males. In this study, I aimed to determine the mating behaviour that prevented heterospecific insemination in these strains.

Previous studies showed that *Ae. aegypti* females use resistance behaviour to prevent insemination by conspecific males (Roth, 1948; Aldersley & Cator, 2019; Cator & Zanti, 2016; Cator & Harrington, 2011; Jones & Pilitt, 1973), however no previous studies have examined what prevents insemination in heterospecific crosses between *Aedes sp*. I predicted that resistance behaviour in *Ae. aegypti* females would prevent insemination by *Ae. albopictus* males, or that male *Ae. albopictus* would not attempt to mate with *Ae. aegypti* females. I found that while no copulas formed, and no insemination occurred in heterospecific crosses between *Ae. aegypti* females and *Ae. albopictus* males, very little resistance behaviour was observed in *Ae. aegypti* females. Instead, after *Ae. albopictus* males made initial contact with *Ae. aegypti* females, they did not pursue the mating attempt. I suggest that male *Ae. albopictus* detect contact pheromones on the surface of female mosquitoes, and this deters further mating attempts when the female is heterospecific. However, the role of contact pheromones requires further investigation. Additionally, in combination with the results of *Chapter 3*, this study highlights heterogeneity in reproductive interference between *Ae. aegypti* and *Ae. albopictus*. This is further investigated in the following chapter, where I conduct a systematic literature review and meta-analysis of reproductive interference between *Aedes* species.

Supplementary Information

	Experimental Block						
Cross	1	2	3	4	5	6	Total
AEG_{AZ} AEG_{AZ}	10	30	30	10	-	10	90
$AEG_{AZ}ALBO_{MP}$	30	30	-	6	-	10	76
$AEG_{COL}AEG_{COL}$	30	11	-	10	10	10	71
$AEG_{COL}ALBO_{MP}$	30	19	-	6	10	10	75
$AEG_{LIV}AEG_{LIV}$	-	-	-	10	10	10	30
$AEG_{LIV}ALBO_{MP}$	-	-	-	10	10	10	30

Table S.4.1 – summary of the number of replicates per experimental block, for each cross. One replicate is defined as one cage observation. Crosses are female-male, such that AEG_{AZ} $ALBO_{MP}$ represents AEG_{AZ} females crossed with $ALBO_{MP}$ males.

Response Variable	Distribution	Female Strain			Male Strain			Timing Block			Female Strain* Timing Block			Male Strain * Timing Block		
		LRT	df	*p*	LRT	df	*p*	LRT	df	*p*	LRT	df	*p*	LRT	df	*p*
Mating Attempt Occurrence	Binomial	17.767	2	0.000139	10.802	3	0.0128	4.7962	1	0.0285	0.336	1	0.562	1.192	2	0.551
Latency to First Mating Attempt	Gamma	1.657	2	0.437	-	-	-	-	-	-	1.498	1	0.221	17.145	2	0.000189
Female Kick	Binomial	2.804	2	0.246	23.439	3	3.270e-05	0.1003	1	0.7515	1.301	1	0.254	2.220	2	0.323
Attempt Period	Gamma	3.693	2	0.158	13.151	3	0.00432	0.0707	1	0.790	0.0847	1	0.771	1.259	2	0.533
Attempt Rate	Gamma	1.565	2	0.457	4.0171	3	0.260	0.795	1	0.373	2.760	1	0.0967	3.406	2	0.182
Effortful Attempt	Binomial	2.866	2	0.239	159.180	3	< 2.2e-16	2.878	1	0.0898	1.490	1	0.222	1.325	2	0.516

Table S.4.2 - outputs of the Likelihood Ratio Tests conducted on all generalised linear mixed effects models examining the explanatory variables male strain, female strain, and their interaction with timing block. These models include data from both conspecific and heterospecific crosses.

Response Variable	Distribution	Cross			Timing Block			Cross*Timing Block		
		LRT	df	*p*	LRT	df	*p*	LRT	df	*p*
Copula Formation	Binomial	9.0711	2	0.0107	4.0498	1	0.0442	0.749	1	0.387
Insemination Occurrence	Binomial	6.338	2	0.0421	4.298	1	0.0382	1.5145	1	0.219
Female Hold	Binomial	3.361	2	0.186	0.202	1	0.653	0.873	1	0.350
Copula Duration	Gamma	5.598	2	0.0609	8.199	1	0.00419	3.470	1	0.0625

Table S.4.3 - outputs of the Likelihood Ratio Tests conducted on all generalised linear mixed effects models examining the explanatory variable cross and its interaction with timing block. These models include data from only conspecific crosses.

Response Variable	Distribution	Mating Type			Timing Block			Mating Type * Timing Block		
		LRT	df	*p*	LRT	df	*p*	LRT	df	*p*
Mating Attempt Occurrence	Binomial	2.636	1	0.105	0.238	1	0.626	0.322	1	0.570
Latency to First Mating Attempt	Gamma	2.130	1	0.144	0.266	1	0.606	0.691	1	0.406
Female Kick	Binomial	21.663	1	3.250e-06	0.709	1	0.400	0.125	1	0.724
Attempt Period	Gamma	12.245	1	0.000466	0.026	1	0.872	0.0296	1	0.864
Effortful Attempt	Binomial	153.44	1	<2.2e-16	0.639	1	0.424	0.335	1	0.563

Table S.4.4 - outputs of the Likelihood Ratio Tests conducted on all generalised linear mixed effects models examining the explanatory variable mating type and its interaction with timing block. These models include data from both conspecific and heterospecific crosses.

Response Variable	Model	AICc	Weighted AIC	Model Selected
Mating Attempt Occurrence	Female and Male Strains	452.42	1.00	Female and Male Strains
	Mating Type	468.78	0.00	
Latency to First Mating Attempt	Female and Male Strains	2337.09	1.00	Female and Male Strains
	Mating Type	2352.83	0.00	
Female Kick	Female and Male Strains	214.28	0.24	Mating Type
	Mating Type	211.94	0.76	
Attempt Period	Female and Male Strains	2181.39	0.16	Mating Type
	Mating Type	2178.03	0.84	
Effortfulness	Female and Male Strains	294.96	0.69	Female and Male Strains
	Mating Type	296.58	0.31	

Table S.4.5 –For each model where male strain, or its interaction with timing block, had a significant impact on the response variable, I also formed a model examining the effect of mating type. I determined which model explained the data better using AICc and Weighted AIC, as summarised.

Chapter 5: A Systematic Review of Reproductive Interference Between *Aedes* Species

Abstract

Both within and between studies, there is variation in the rates of heterospecific insemination between *Ae. aegypti* and *Ae. albopictus*. There are multiple hypotheses to explain this variation: *Ae. aegypti* females experience a greater rate of heterospecific insemination than *Ae. albopictus* females, allopatric *Ae. aegypti* experience a greater rate of heterospecific insemination than sympatric *Ae. aegypti* females, and the geographic origin of male *Ae. albopictus* effects the heterospecific insemination rate. In this study, I aimed to determine if there is consistent evidence for these hypotheses in the literature. I conducted a systematic literature review and meta-analysis of reported heterospecific insemination rates between *Ae. aegypti* and *Ae. albopictus*. I found support for the first two hypotheses, however, I could not determine whether male geographic origin has a significant effect on heterospecific insemination rate, due to a lack of replicates of crosses where males and females were from consistent geographic origins. Thus, the impact of male geographic origin requires further investigation. Despite there being evidence for the first two hypotheses, there was still considerable variation in heterospecific insemination within groups. Part of this variation was due to differences in experimental methods between studies. I found that differences in the length of time that females were exposed to males, and total mosquito density in a cage significantly affected the rate of heterospecific insemination. This highlights

the necessity to have standardised experimental methods for determining heterospecific insemination rate, to allow valid comparisons to be made.

1. Introduction

Aedes mosquitoes transmit flaviviruses such as dengue, Zika, and chikungunya (Leta et al., 2018; Kraemer et al., 2015b), which pose a substantial threat to global public health. There are no widely available vaccines, or specific treatments for many of these viruses (World Health Organization, 2022a, 2020a, 2022c) and, therefore, controlling their spread relies on manipulating the vector population. Currently, insecticides are widely used to control *Aedes* populations (Gan et al., 2021; Garcia et al., 2018), however, globally, *Aedes* populations have developed resistance to common insecticides (Kandel et al., 2019; Maciel-de-Freitas et al., 2014; Yakob & Walker, 2016; Sene et al., 2021; Toé et al., 2022; Jangir & Prasad, 2022). Therefore, pioneering methods to control *Aedes* populations are required.

Novel control methods are based on the mass-rearing and release of modified mosquitoes into the wild, where they mate with wild mosquitoes. These techniques aim to either prevent the production of viable offspring in the next generation (population suppression), or reduce the ability of mosquitoes to spread disease (population replacement), as summarised by Alphey (2014). The extent to which the modified mosquitoes persist varies. Systems can be self-limiting, where the modified insects decrease in abundance through time, or self-sustaining, where the modified insects persist over time, and in some instances increase in frequency over generations and establish in other populations (Alphey, 2014).

During the development of these techniques, population dynamic frameworks are formed to evaluate their efficacy (for example, Atkinson *et al.*, 2007; Beaghton, Beaghton and Burt,

2016), allowing comparisons to be made between techniques (for example, Phuc *et al.*, 2007; Seirin Lee *et al.*, 2013). These models generally focus on the specific details of the control technique (for instance, details of the genetic mechanism), and only include basic life-history details (for instance, birth rate) and intraspecific interactions (for instance, intraspecific competition) of the target vector. The omission of interspecific interactions could result in inaccurate predictions of the efficacy of vector control methods, particularly where the population dynamics of multiple species are strongly coupled. Inaccurate predictions are particularly worrying for self-sustaining strategies, due to the persistence of the modified mosquitoes in wild populations.

The population dynamics of *Ae. aegypti* and *Ae. albopictus* are strongly coupled: their overlapping realized ecological niches (Gubler, Bhattacharya & Bhattacharva, 1972; Hartberg, 1971; Ponlawat & Harrington, 2005; Kauffman et al., 2017; Bagny et al., 2009; Nelson, 1986; Yuval, 2006) cause strong interspecific interactions (Lounibos and Juliano, 2018). Therefore, where their spatial distributions overlap, either competitive exclusion or stable coexistence occurs (Lounibos & Juliano, 2018; Vollans & Bonsall, 2021; Paton & Bonsall, 2019).

Interspecific interactions that strongly influence patterns of coexistence in *Ae. aegypti* and *Ae. albopictus* include competition and reproductive interference (Lounibos and Juliano, 2018; Paton and Bonsall, 2019). Reproductive interference is where incomplete species recognition results in heterospecifics engaging in mating activities which do not produce viable offspring, and cause a fitness cost to one or both of the species involved (Gröning & Hochkirch, 2008; Burdfield-Steel & Shuker, 2011). Field (Robbins et al., 2011) and laboratory

(Bargielowski et al., 2015; Honório et al., 2018; Marcela et al., 2015) studies have shown that reproductive interference can occur between *Ae. aegypti* and *Ae. albopictus* (Lounibos & Juliano, 2018). Reproductive interference has a significant impact on population dynamics: theoretical studies have shown that where two species coexist, reproductive interference is more likely to lead to exclusion than interspecific resource competition (Kuno, 1992; Kishi & Nakazawa, 2013).

In areas where *Ae. aegypti* and *Ae. albopictus* coexist, models of population control strategies should include reproductive interference. Our previous theoretical study (Vollans and Bonsall, 2021, Chapter 2) found that reproductive interference and the release of self-limiting *Ae. aegypti* act together to determine whether coexistence is maintained between *Ae. aegypti* and *Ae. albopictus* and influence the population size of each species. Thus, omitting reproductive interference may result in inaccurate predictions about the dynamic spread of the modified mosquitoes.

The impact reproductive interference can have on the population dynamics of *Ae. aegypti* and *Ae. albopictus* depends on the extent that it reduces the recruitment of each species (Vollans & Bonsall, 2021; Paton & Bonsall, 2019; Kuno, 1992). In empirical studies, the rate of heterospecific insemination, calculated as the proportion of females inseminated by heterospecifics, is frequently used as a proxy for the rate of reproductive interference. Thus, only examining heterospecific insemination omits some of the direct (for example, physical damage) and all the indirect (for example, wasted time) costs of reproductive interference. However, heterospecific insemination rates can be used to make comparisons of the strength of an important direct fitness cost between studies.

There is variation in the rate of heterospecific insemination between and within studies. One source of heterogeneity is between-species differences in female insemination rate. It has been reported that *Ae. aegypti* females are more likely to be inseminated by *Ae. albopictus* males than *Ae. albopictus* females are to be inseminated by *Ae. aegypti* males (Zhou et al., 2022). As the fitness cost of heterospecific mating is much greater to *Ae. aegypti* than *Ae. albopictus,* heterospecific mating is expected to have different effects on the population dynamics of each species.

Male geographic origin can also influence the proportion of females inseminated. Honório *et al.* (2018) crossed female *Ae. aegypti* with male *Ae. albopictus* from two cities in Brazil (Rio de Janeiro and Manaus) and *Ae. albopictus* from Vero Beach, Florida. The *Ae. albopictus* males from Rio de Janeiro and Manaus inseminated *Ae. aegypti* females at a lower rate than *Ae. albopictus* males from Vero Beach, Florida. This suggests that there are differences in male competency at inseminating heterospecific females.

Additionally, previous exposure of *Ae. aegypti* strains to *Ae. albopictus* can influence the heterospecific insemination rate. Bargielowski, Lounibos and Carrasquilla (2013) found that female *Ae. aegypti* from populations sympatric with *Ae. albopictus* were significantly less likely than nearby allopatric populations to be inseminated by heterospecific males. Furthermore, a later study by Bargielowski and Lounibous (2014) used strains of *Ae. aegypti* females which had no previous contact with *Ae. albopictus* and exposed them to *Ae. albopictus* for multiple generations. Following 1-3 generations of exposure to *Ae. albopictus* males, the heterospecific insemination rate decreased (Bargielowski & Lounibos, 2014),

suggesting that females can rapidly evolve to resist heterospecific mating, following exposure to *Ae. albopictus* males.

However, my results from *Chapters 3 and 4* seemed to contradict these findings. Here, *Ae. aegypti* females from strains with no prior exposure to *Ae. albopictus* were crossed with *Ae. albopictus* males from multiple different strains. I found no, or very low, insemination of these *Ae. aegypti* females by any strain of *Ae. albopictus*. This suggests that while there may be a level of susceptibility to heterospecific mating in females from some unexposed *Ae. aegypti* strains, this is not always the case.

To date, there has been no synthesis of studies examining reproductive interference in *Aedes* species. Here, I conduct a systematic literature review to assess variation in the rates of heterospecific insemination in *Aedes* species. I will form an overview of the studies on reproductive interference in *Aedes* species, to uncover broad biases and gaps in the literature. Furthermore, I will assess whether there is any impact of experimental methods on the reported rate of female insemination, in heterospecific crosses. I will also examine whether there is consistent evidence for four key hypotheses:

(i) There are lower rates of heterospecific insemination than conspecific insemination. As heterospecific insemination is costly, and is a result of mate misidentification, I predict that the rates of heterospecific insemination will be lower than conspecific insemination.

(i) *Ae. aegypti* females are inseminated by heterospecific males at a greater rate than *Ae. albopictus* females (Robbins et al., 2011; Zhou et al., 2022).

(ii) Sympatric females experience lower heterospecific insemination than allopatric females (Bargielowski & Lounibos, 2014; Bargielowski et al., 2015).

(iii) Geographic origins of male strains predictably influence the rate of heterospecific insemination (Honório et al., 2018).

This study will further our understanding of the characteristics of heterospecific insemination in *Aedes* and contribute to our general knowledge of *Aedes* ecology. This knowledge can be used to inform decisions when forming models examining the efficacy of *Aedes* control methods.

2. Methods

I based these methods on the PRISMA 2020 guidelines for systematic reviews (Page et al., 2021).

2.1. Literature Search

I conducted a systematic literature search examining reproductive interference between *Aedes* species. To obtain a comprehensive overview of the literature, I conducted searches in three databases: Web of Science, PubMed, and Scopus. The search was conducted on 27th May 2022. In each database, I searched the fields title, abstract and keywords, and in Web of Science I also searched KeyWords Plus (a field not available in the other databases). KeyWords Plus are words or phrases that are often in the titles of the references of the article but are not in the title of the article itself.

I began with the base search terms: *Aedes* AND (“interspecific mating” OR satyr* OR "reproductive interference" OR "mating interference" OR “heterospecific mating” OR “sexual interference”). To prevent missing relevant papers from the output, I then sequentially included the synonyms for reproductive interference listed by Gro and Hochkirch (2016) into the search term (using ‘OR’).

These synonyms are: “signal interference”, “communication interference”, “mistaken identity”, “acoustic interference”, “competition for acoustic signal space”, “interspecific

acoustic interactions", "masking interference", "cross-attraction", "interspecific sex attraction", "interspecific competition for mating territories", "inappropriate mate-selection", "interspecific mate choice", "breeding interference", "interspecific social interactions", "heterospecific pairing", "pseudocompetition", "heterospecific sexual harassment", "mating interference", "satyr effect", "satyrism", "reproductive interactions", and "sexual interference".

All terms that resulted in an increase in the number of results were kept in the search. In the different databases, this resulted in different resultant search terms:

1) **Web of Science:** *Aedes* AND ("interspecific mating" OR satyr* OR "reproductive interference" OR "mating interference" OR "heterospecific mating" OR "sexual interference").
2) **PubMed:** *Aedes* AND ("interspecific mating" OR satyr* OR "reproductive interference" OR "mating interference" OR "heterospecific mating").
3) **Scopus:** *Aedes* AND ("interspecific mating" OR satyr* OR "reproductive interference" OR "mating interference" OR "heterospecific mating").

The search outputs from each database were downloaded as a CSV, and these three CSV files were merged into one spreadsheet. I had multiple target papers that I expected to be in the output of the literature search (Bargielowski et al., 2019, Bargielowski et al., 2015; Bargielowski & Lounibos, 2014; Bargielowski, Lounibos & Carrasquilla, 2013; Marcela et al., 2015; Honório et al., 2018; Zhou et al., 2022), and all were obtained.

2.2. Screening Papers

The papers from the search outputs were put through a screening process to extract the primary literature examining reproductive interference in *Aedes* species.

I used a series of spreadsheets during screening. In the first stage of screening, the spreadsheet contained all outputs from the literature search, and for each sequential stage of screening, a new spreadsheet was produced only containing articles that were retained in the previous stage. In each screening step, each article in the spreadsheet was examined to determine whether it should be excluded from the next screening step or retained. I recorded a justification in each spreadsheet for every article excluded from the following screening step.

Furthermore, for each review article identified, I extracted the references and repeated the screening process. Alongside published work, *Chapter 3* of this thesis was included in this review. Details of the steps of the screening process, and the number of articles excluded and retained, are summarised in figure 5.1.

To prevent relevant papers being excluded by mistake, an additional reviewer, Helen Micheal Youssef Moussa (HM), independently repeated the initial search. Where there were any disagreements in retention or exclusion, this was discussed by both reviewers until the same categorisation was agreed on. This resulted in the addition of two papers. Furthermore, HM screened a random subset of 10% of the references from review articles, which resulted in no additional papers being added.

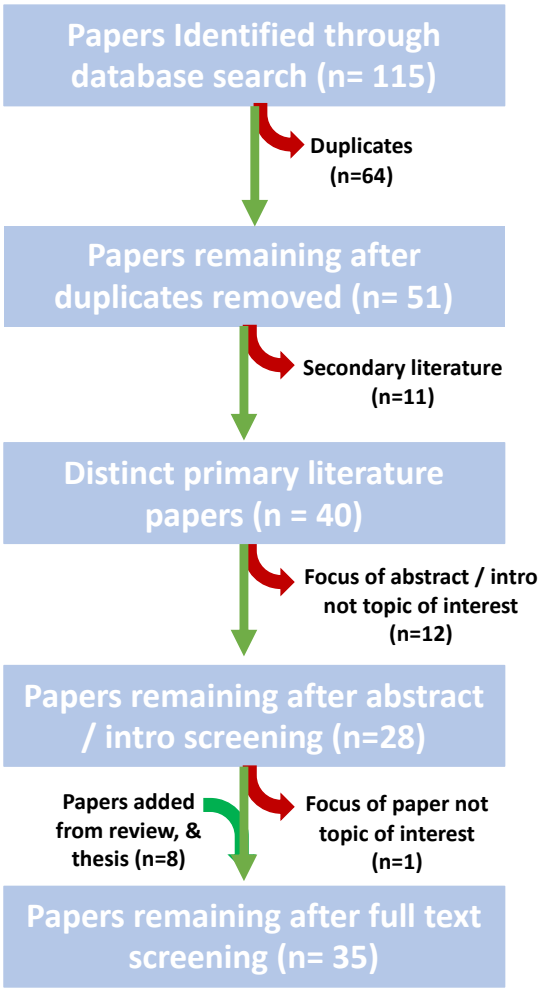

Figure 5.1 – Schematic representing the screening process, and the number of papers excluded and retained at each stage, based on the PRISMA flowchart (Page et al., 2021). *Here, the topic of interest refers to reproductive interference between Aedes species.*

2.3. Analysis

All data processing and analyses were conducted in *R* version 4.3.1 (R Core Team, 2023). Data was processed using *dplyr* (Wickham et al., 2023) and *tidyr* (Wickham, Vaughan & Girlich, 2023) and all graphs were formed using *ggplot2* (Wickham, 2016).

The package *lme4* (Bates et al., 2015) was used to fit linear and generalised linear mixed-effects models. Model simplification was conducted using the package *MASS* (Venables & Ripley, 2002) which I used to conduct likelihood ratio tests, using a chi-square test to produce p-values for the fixed effects. From each model, I removed fixed effects which had no significant effect, and then used *lmerTest* to produce summary statistics (Kuznetsova, Brockhoff & Christensen, 2017).

2.3.1. Initial Data extraction

Data were extracted manually. From all papers, I recorded: article title, journal name, authors, year of publication, main topic of study, type of data collected, study type, type of mating choice examined, and the *Aedes* species used.

2.3.2. Insemination Analysis

a) Paper selection criteria

To make valid comparisons of insemination rates between studies, study methods need to be sufficiently similar. As most studies examining heterospecific mating in *Aedes* are non-choice laboratory studies using *Ae. aegypti* and *Ae. albopictus*, I only used data from these studies in the insemination analysis.

In a small number of studies, females were exposed to males for very short periods of time (under 30 minutes) before their insemination status was assessed. As most studies exposed males and females for one day or longer, I excluded crosses where exposure was less than one day. In experimental evolution studies, I only included heterospecific insemination data from before the populations had undergone experimental evolution. The papers selected for insemination analysis are detailed in the supplementary information (Table S.5.1).

b) Data extraction

For all papers included in the insemination analysis, I extracted data on the results, and details of the cross, strains used, and experimental methods.

For the results, I extracted the percentage of females inseminated, the associated error and the error type (e.g. standard error, SE and standard deviation, SD). To examine details of the cross, I extracted data on the cross type (conspecific/ heterospecific), the type of test conducted (non-choice/ female-choice / male-choice), and the male and female species used. For each strain, I extracted data on their geographic origin, the number of generations

they had been in the laboratory and their historical contact with the heterospecific of interest (*Ae. aegypti* or *Ae. albopictus*).

To gain an overview of the experimental methods, I extracted data on the length of exposure of females to males prior to assessing their insemination status, the cage volume, the number of replicates, and the total number of mosquitoes, number of males, number of females and number of females assessed, per replicate cage.

In some instances (54/129, 41.86% of data points) data on the percentage of females inseminated and the associated error were not provided in the main text or supplementary material and instead results were presented in graphical form. Here, I used *WebPlotDigitizer* (Rohatgi, 2023) to extract data from graphs.

c) Data processing

Standardisation

To make the results of the studies comparable, where necessary I standardised the data reported and grouped variables. In most cases, insemination rate was reported as the average percentage of females inseminated, across multiple replicate cages. In the small number of cases where the raw data was reported, I calculated the arithmetic mean across the replicate cages, and the associated standard error. Error was reported as SD or SE, and I converted all errors to SE for consistency. Furthermore, I formed 6 groups, based on the ranges reported in the studies analysed, for the number of generations male and female strains were in the laboratory (F2-F5, F6-F7, F8-F12, F13-F16, F17-F19, F20+).

Calculations

I used the raw data to form two variables that I used in later analysis. The first was the ratio of male to female mosquitoes (the number of males per replicate divided by the number of females per replicate) and the second, the density of mosquitoes in each replicate cage (the total number of mosquitoes per replicate cage divided by the cage volume (cm^3)).

d) Statistical Analysis

Model overview

I examined the effect of cross type (heterospecific / conspecific) on the number of females inseminated. Furthermore, I assessed how historical contact of the female strain with heterospecifics (allopatric / sympatric) and differences in experimental methods (length of exposure, density of mosquitoes) impacted the number of females inseminated by heterospecific males, in *Ae. aegypti* females and *Ae. albopictus* females separately. I also examined differences in the number of *Ae. aegypti* and *Ae. albopictus* females inseminated in heterospecific crosses. However, I could not use statistical models to examine the impact of male or female geographic origin on the number of females inseminated [4].

I used generalised linear mixed effects models with binomial response variables and logit link functions to examine differences in the number of females inseminated. This is because, recent studies have advocated directly using binomial data in meta-analyses, rather than

[4] There were often few or no replicates of crosses where males and females were from consistent geographic origins, and frequently all replicates of a geographic origin were conducted in the same study. This resulted in the models not converging.

using effect sizes such as odds ratios (Chang & Hoaglin, 2017; Mengersen & Gurevitch, 2013), or conducting arcsine transformations of proportional data (Lin & Chu, 2020).

Most studies I examined reported the mean percentage of females inseminated for each cross. As the mean was reported, and not the percentage insemination per replicate cage, I could not fully recreate the binomial dataset. Instead, I generated a binomial data of the average number of females inseminated per replicate cage.

However, this omitted variation in number of females inseminated between replicate cages. To account for this variation, for each explanatory variable I formed an additional model: a linear mixed effects model examining differences in the standard error of the percentage of females inseminated between replicate cages.

In all models, I included study ID as a random effect, to account for between-study variation. Furthermore, in each model data were only included where the value for the explanatory variable was specified for that cross.

Determining the mean number of females inseminated

To calculate the average number of females inseminated per cross, I multiplied the proportion of females inseminated by the number of females whose insemination status was assessed for each replicate cage. The number of females not inseminated was then calculated by subtracting the number inseminated from the total number of females whose insemination status was assessed, per replicate cage.

To maximise consistency in my methods, I followed clear rules when determining the number of females whose insemination status was assessed, per replicate cage. In some papers, it was not reported how many females were assessed; in these cases, I assumed that all females in each cage were assessed. Furthermore, sometimes the number of females assessed was uneven between replicate cages (for instance, due to differences in the number of female deaths between replicates); here, I used the arithmetic mean number of females assessed per replicate cage in calculations. Where the total number of females assessed per cross was specified, but not the number dissected per cage, I assumed that an equal number of females were dissected per replicate cage, and again used the mean in calculations. As binomial analysis requires integers, where necessary I rounded the number of females whose insemination status was assessed to the nearest integer.

3. Results

3.1. Overview of Studies

The first paper published on heterospecific mating in *Aedes spp* was in 1965. Since then, there have been multiple clusters of publications on the topic, though most papers (22/34, 64.71%) have been published recently, since 2009 (figure 5.2B). Furthermore, most papers (12/16, 75.00%) with data on heterospecific and conspecific insemination rates, that I used in later insemination analysis, were published since 2011.

I found that most studies (30/35, 85.71%) examining heterospecific mating in *Aedes spp.* were conducted exclusively in the laboratory (figure 5.2A). Most (17/21, 80.95%) laboratory trials with data on the rates of heterospecific insemination used exclusively non-choice tests (figure 5.2D). Therefore, most of our knowledge of heterospecific mating in *Aedes* comes from trials with artificially high densities of mosquitoes, and where heterospecific males have no mating competition from conspecific males.

Furthermore, I found that most studies were conducted on *Ae. aegypti* and *Ae. albopictus*, two vectors with public health significance (figure 5.2C). While there are studies on other *Aedes* species (*Ae. cretinus, Ae. polynesiensis, Ae. scutellaris, Ae. triseriatus, Ae. hendersoni, Ae. brelandu, Ae. seatoi, Ae. pseudoalbopictus*) there are few replicates, preventing between-study comparisons.

An overview of all studies included in this review is in the supplementary information (*Table S.5.1*).

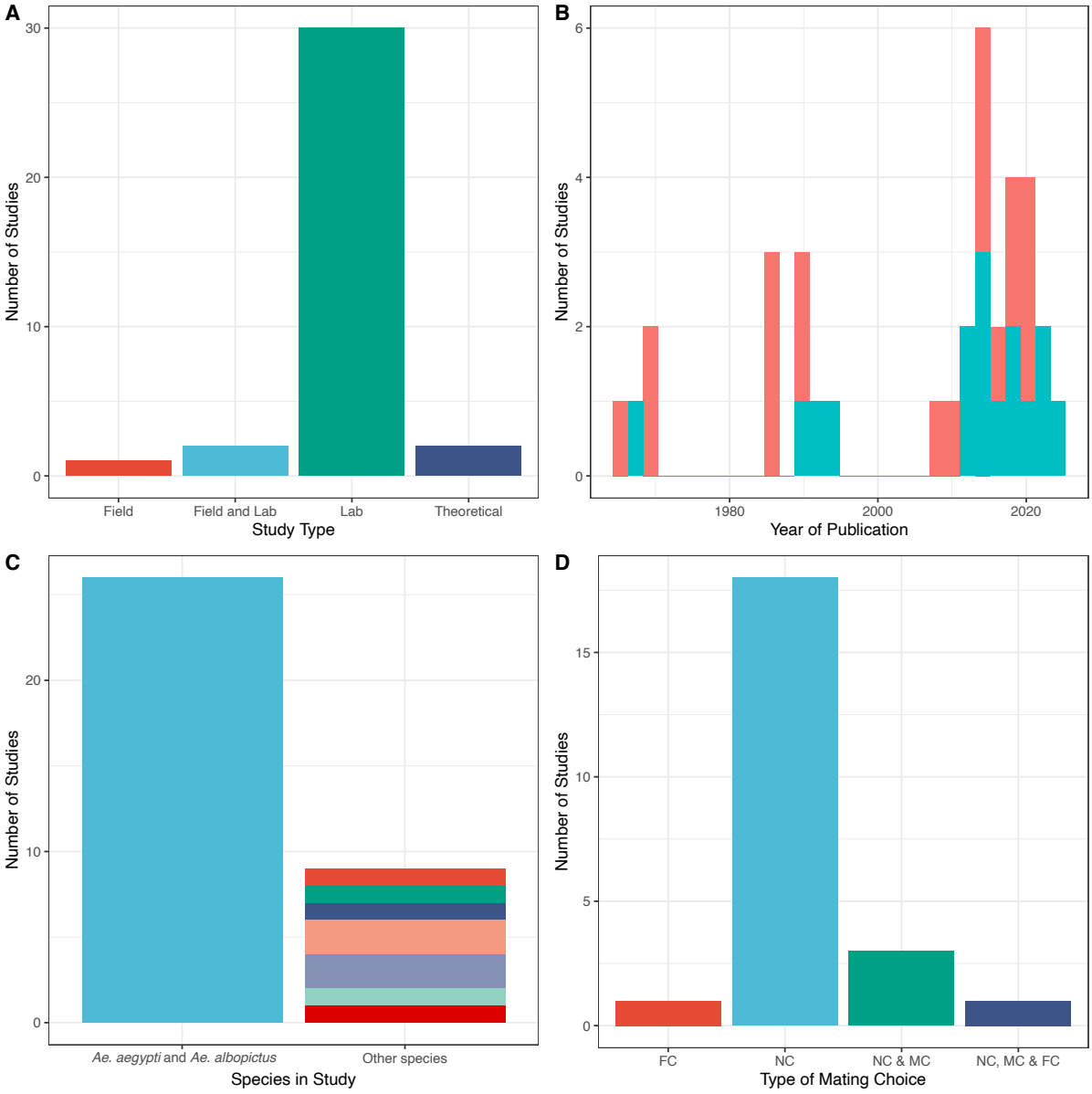

Figure 5.2 – overview of the papers selected for this review. A: Comparison of the number of each type of study: field, field and laboratory, laboratory and theoretical. B: The number of studies published each year. Blue bars represent studies with data used in later insemination analysis, and pink bars represent studies not included in that analysis. C: Comparison of the number of studies conducted on different Aedes species. The colour of the bar corresponds to the species pairing used in the study. D: Comparison of the number of laboratory studies using each type of mating choice in trials examining heterospecific mating. FC = female choice, NC = no choice, and MC = male choice.

3.2. Insemination Analysis

3.2.1. Conspecific vs. Heterospecific crosses

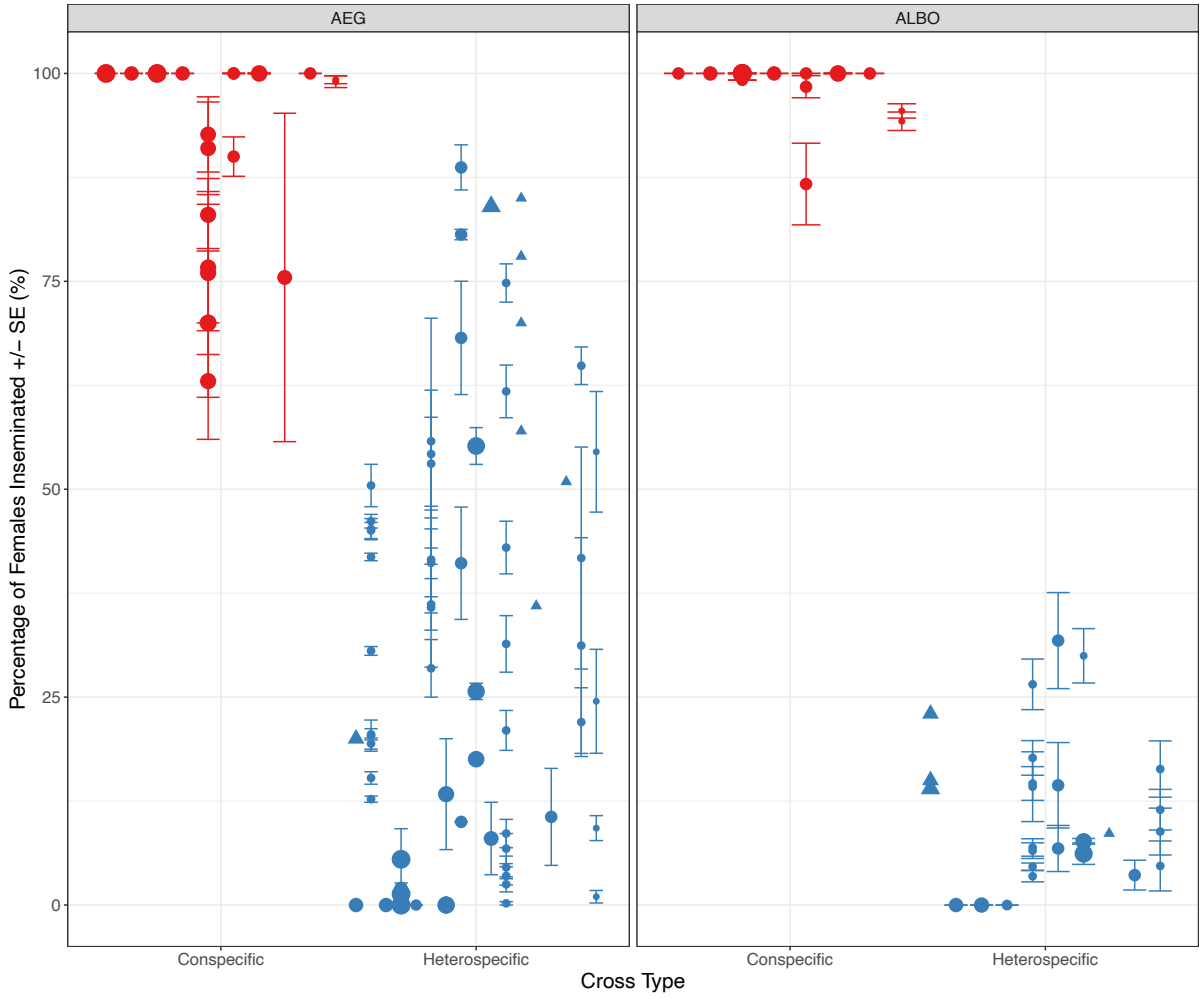

Figure 5.3 – differences in the percentage of Ae. aegypti (top) and Ae. albopictus (bottom) females inseminated, between conspecific (red) and heterospecific (blue) crosses. Each point represents the percentage of females inseminated in one cross, averaged across replicates where applicable. The size of the point corresponds to the average number of females dissected per replicate (range 20 – 200). Within each cross type, points are vertically aligned by study. Triangular points show insemination rates where the error was not reported. Error bars represent the standard error of the mean percentage of females inseminated, across replicates.

a) Number of females inseminated

I found that on average, fewer females were inseminated in heterospecific crosses than conspecific crosses, in both *Ae. aegypti* (coeff = -5.03, SE = 0.33, $P < 2.00 \times 10^{-16}$) and *Ae. albopictus* females (coeff = -5.66, SE = 0.39, $P < 2.00 \times 10^{-16}$). However, as seen in figure 5.3, there is variation in the conspecific and heterospecific insemination rates between crosses, and within replicates of the same cross. This variation cannot be clearly predicted by study identity (Figure S.5.1).

b) Variation within replicates of the same cross

I compared variation in the percentage of females inseminated between replicates of the same cross, in conspecific and heterospecific crosses. I found that in *Ae. albopictus* females, there was more variation in heterospecific crosses than conspecific crosses (coeff = 1.49, SE = 0.51, $P = 0.0062$). However, I found no difference in variation between conspecific and heterospecific crosses in *Ae. aegypti* females ($P = 0.16$).

c) Variation due to study

The impact of study on the number of females inseminated was much greater for *Ae. aegypti* females than *Ae. albopictus* females: the standard deviation due to the random effect of study was over four times greater for *Ae. aegypti* females (standard deviation of random effect: $SD_{RE} = 1.25$) than *Ae. albopictus* females ($SD_{RE} = 0.28$). Furthermore, the effect of study on variation in the percentage of females inseminated between replicates of the same cross was greater in *Ae. aegypti* females ($SD_{RE} = 2.94$) than *Ae. albopictus* females ($SD_{RE} = 0.92$). These results suggest that study design has a greater impact on the mean

proportion of females inseminated in a cross, and the variation in the proportion of females inseminated between replicates in *Ae. aegypti* females than *Ae. albopictus* females.

3.2.2. *Ae. aegypti* vs *Ae. albopictus* Females

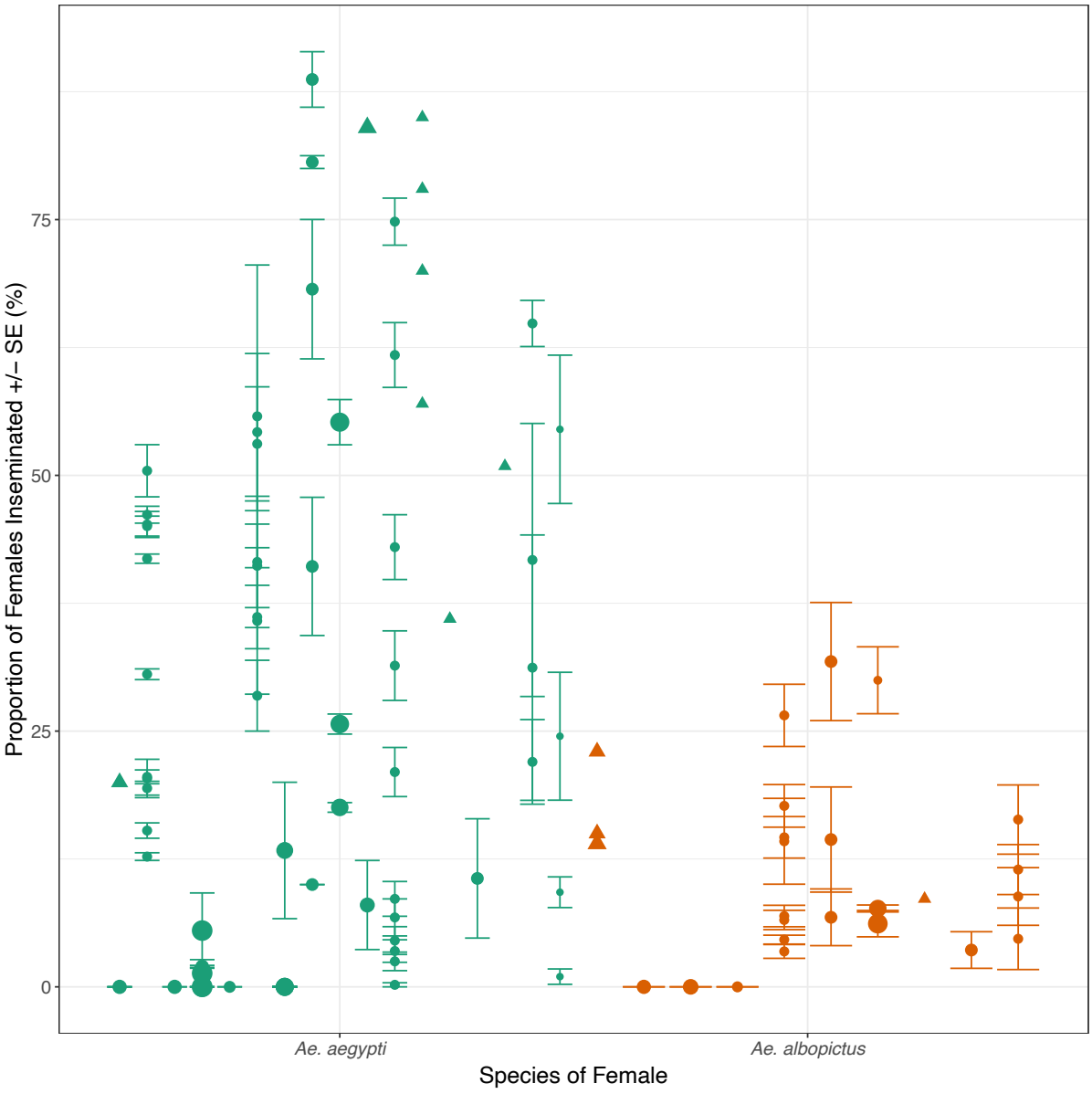

Figure 5.4 – differences in the proportion of heterospecific insemination in Ae. aegypti (red) and Ae. albopictus (blue) females. Each point represents the percentage of females inseminated in one cross, averaged across replicates where applicable. The size of the point corresponds to the average number of females dissected per replicate (range 20 – 200), in that study. Within each female species, points are vertically aligned by study. Triangular points show insemination rates where the error was not reported. Error bars represent the standard error of the mean proportion of females inseminated, across replicates.

a) Number of females inseminated

I found that more *Ae. aegypti* females were inseminated in heterospecific crosses than *Ae. albopictus* females (coeff = 1.65, SE = 0.078, $P < 2x10^{-16}$).

b) Variation within replicates of the same cross

The high variation in the proportion of females inseminated in *Ae. aegypti* females results in overlap between *Ae. aegypti* and *Ae. albopictus* females. I found that there was more variation in the percentage of females inseminated within replicates of the same cross in *Ae. aegypti* females than *Ae. albopictus* females (coeff = 2.14, SE = 0.71, $P = 0.0034$).

c) Variation due to study

Between studies there were large differences in the number of females inseminated (SD_{RE} = 1.617) and in the amount of variation between replicates of the same cross (SD_{RE} = 1.83). Thus, as seen in figure 5.4, there was considerable variation in the proportion of females inseminated.

3.2.3. Allopatric vs. Sympatric Females I

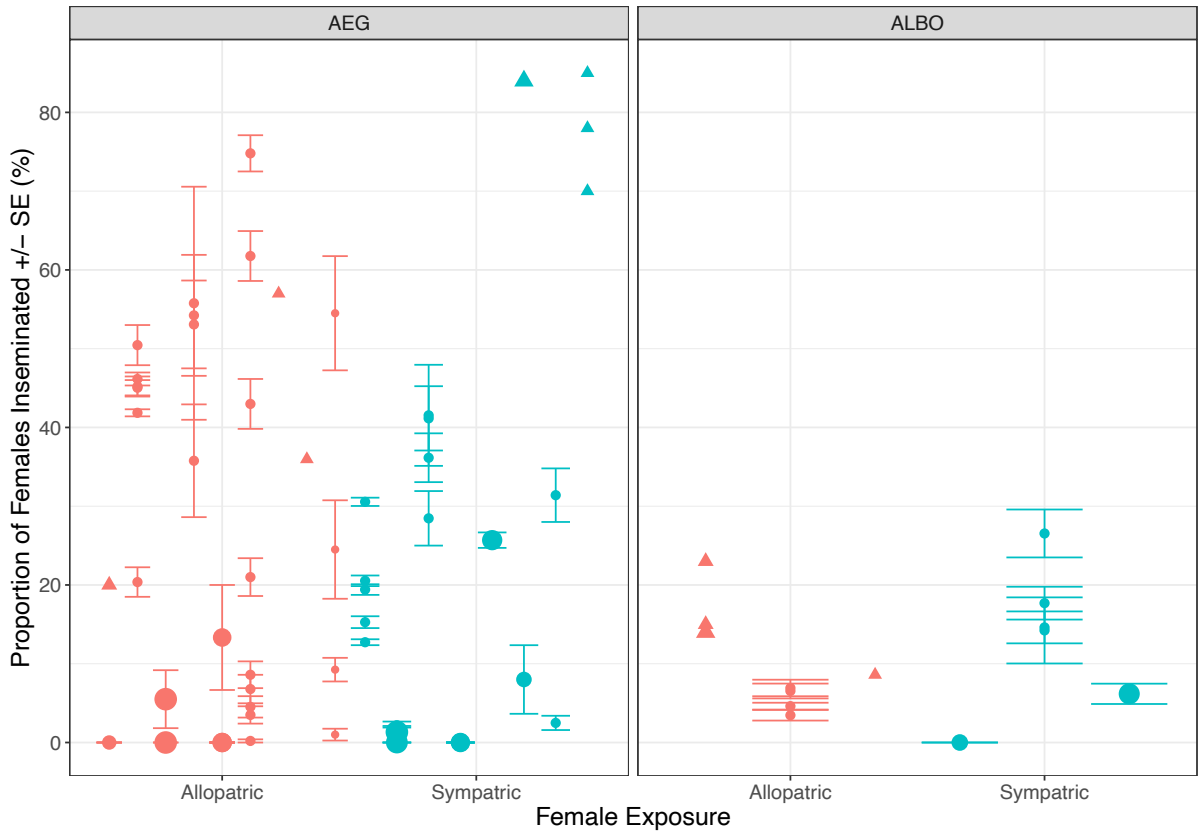

Figure 5.5 – differences in the percentage of Ae. aegypti (left) and Ae. albopictus (right) females inseminated in heterospecific crosses, where females are from strains allopatric (red) or sympatric (blue) with the heterospecifics. Each point represents the percentage of females inseminated in one cross, averaged across replicates where applicable. The size of the point corresponds to the average number of females dissected per replicate (range 20 – 200). Within each cross type, points are vertically aligned by study. Triangular points show insemination rates where the error was not reported. Error bars represent the standard error of the mean percentage of females inseminated, across replicates. Where female exposure was not detailed in the study, the insemination data was not included in this analysis.

a) Number of females inseminated

I found that in heterospecific crosses, significantly fewer *Ae. aegypti* females were inseminated from populations sympatric with *Ae. albopictus*, than populations allopatric with *Ae. albopictus* (coeff = -0.56, SE = 0.069, $P = 4.57 \times 10^{-16}$). The converse was found in *Ae.*

albopictus females; significantly more *Ae. albopictus* females were inseminated from populations sympatric with *Ae. aegypti*, than populations allopatric with *Ae. aegypti* (coeff = 1.34, SE = 0.22, $P = 5.32x10^{-10}$).

b) Variation within replicates of the same cross

In *Ae. aegypti* females, there was less variation in the percentage of females inseminated within replicates of the same cross in sympatric females than allopatric females (coeff = -1.46, SE = 0.68, $P = 0.038$). However, the opposite trend was seen in *Ae. albopictus* females; there was significantly more variation in the percentage of females inseminated within replicates of the same cross in sympatric females than allopatric females (coeff = 2.00, SE = 0.52, $P = 0.0080$).

c) Variation due to study

More of the variation in the percentage of females inseminated within replicates of the same cross was explained by the study for *Ae. aegypti* females ($SD_{RE} = 2.11$) than *Ae. albopictus* females ($SD_{RE} = 1.33$). Furthermore, study had a slightly bigger impact on the number of females inseminated, in *Ae aegypti* ($SD_{RE} = 1.55$) than *Ae. albopictus* females ($SD_{RE} = 1.075$). Therefore, once more, the study design seems to have a greater influence on both the mean number of females inseminated and the variation in the number of females inseminated between replicates of the same cross, in *Ae. aegypti* females than *Ae. albopictus* females.

3.2.4. Allopatric vs. Sympatric Females II

Bargielowski and Lounibos (2014) found that females initially resistant to heterospecific mating can become susceptible following 8 generations without exposure to heterospecific males. Thus, there may have been a change in the behaviour of sympatric females that have been bred in the laboratory without exposure to heterospecifics for 8 generations or more. To account for this, I re-examined the data, but excluded females from sympatric lines that had been in the laboratory for more than 7 generations. This resulted in the exclusion of a small number of crosses (*Ae. aegypti* = 3, *Ae. albopictus* = 5) and did not change the overall patterns observed in my results (supplementary information, Figure S.5.2), or the outcomes of my statistical analyses (supplementary information, Table S.5.2).

3.2.5. Allopatric vs. Sympatric Females III

Where mosquito colonies are maintained using a small number (≤ 100) of individuals, there can be inbreeding depression, due to a low effective population size (Ross, Endersby-Harshman & Hoffmann, 2019). Furthermore, multiple studies have found that laboratory and field strains of mosquitoes can differ in life history, morphological and physiological traits (as summarised in supplementary 1 in Ross, Endersby-Harshman and Hoffmann, 2019). Thus, I examined the effect of laboratory breeding of *Ae. aegypti* and *Ae. albopictus* on heterospecific mating in allopatric and sympatric *Ae. aegypti* and *Ae. albopictus* females.

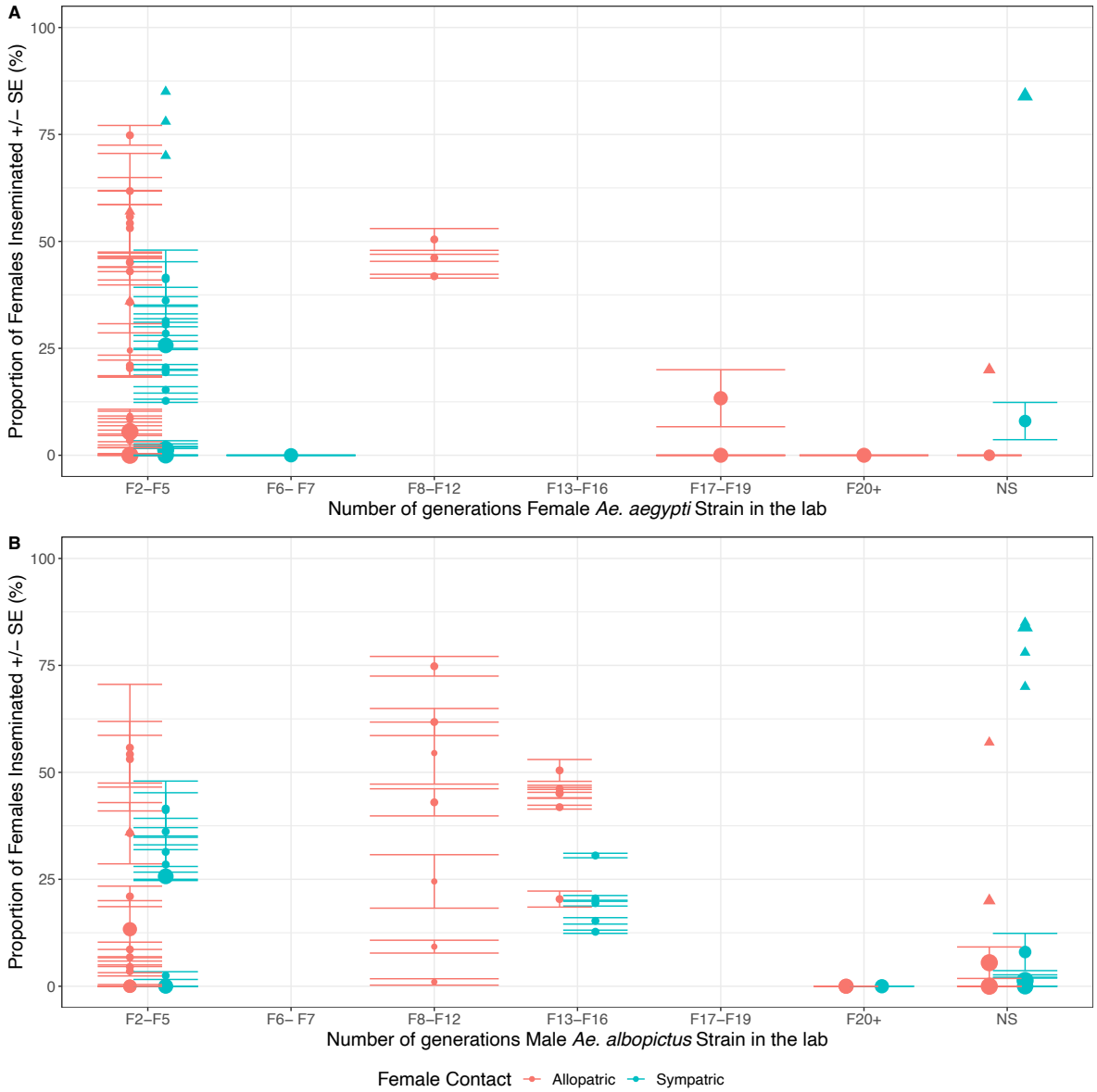

Figure 5.6 – differences in the percentage of Ae. aegypti females inseminated in heterospecific crosses with Ae. albopictus males, where females are allopatric (red) or sympatric (blue) with heterospecifics, and females (row 1, A) and males (row 2, B) have been reared in the laboratory for different numbers of generations. Each point represents the percentage of females inseminated in one cross, averaged across replicates where applicable. The size of the point corresponds to the average number of females dissected per replicate (range 20 – 200). Within each cross type, points are vertically aligned by study. Triangular points show insemination rates where the error was not reported. Error bars represent the standard error of the mean percentage of females inseminated, across replicates. Points in the NS (not specified) category represent males or females where the number of generations in the laboratory was not specified.

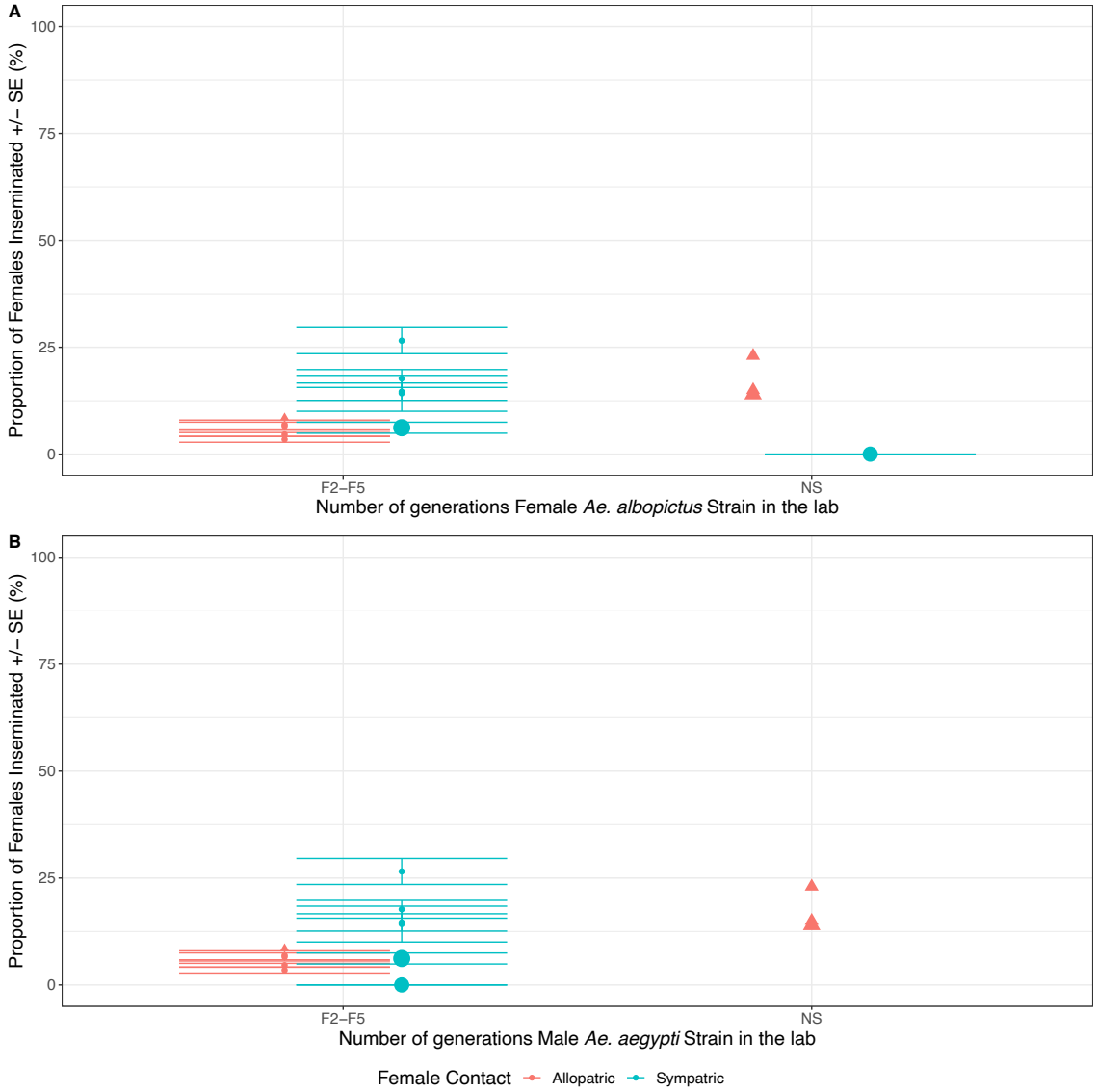

Figure 5.7 – differences in the percentage of Ae. albopictus females inseminated in heterospecific crosses with Ae. aegypti males, where females are allopatric (red) or sympatric (blue) with heterospecifics, and females (row 1, A) and males (row 2, B) have been reared in the laboratory for different numbers of generations. Each point represents the percentage of females inseminated in one cross, averaged across replicates where applicable. The size of the point corresponds to the average number of females dissected per replicate (Range 20 – 200). Within each cross type, points are vertically aligned by study. Triangular points show insemination rates where the error was not reported. Error bars represent the standard error of the mean percentage of females inseminated, across replicates. Points in the NS (not specified) category represent males or females where the number of generations in the laboratory was not specified.

When examining the heterospecific cross between *Ae. aegypti* females and *Ae. albopictus* males (figure 5.6), I found that the number of generations that the strains had been in the laboratory varied, from F2-F5, to F20+. However, on average, the strains of *Ae. albopictus* males used in crosses were older than the strains of *Ae. aegypti* females. All crosses between *Ae. albopictus* females and *Ae. aegypti* males were either F2-F5, or the number of generations they had been in the laboratory was not specified (figure 5.7).

I found no clear link between the number of generations males or females were in the laboratory, and the insemination rate of allopatric and sympatric females[5]. Thus, I found no evidence for an effect of inbreeding depression on heterospecific insemination rates.

[5] I could not conduct formal statistical analysis on the impact of inbreeding on heterospecific insemination in allopatric and sympatric females, as the models failed to converge.

3.2.6. Geographic Origins of Strains

I examined the frequency of crosses in males and females from different geographic origins (figures 5.8 and 5.9). I found that the males and females were from a variety of different geographic origins, with *Ae. aegypti* males and females from 7 different countries, *Ae. albopictus* females from 5 different countries, and *Ae. albopictus* males from 8 different countries. However, I found a bias towards using strains from the USA: over 50% of *Ae. aegypti* and *Ae. albopictus* males and females originated in the USA. Furthermore, a high proportion of crosses used male and female strains that originated in Florida, in *Ae. aegypti,* 47.31% of females and 35.56% of males, and in *Ae. albopictus*, 27.50% of females and 19.74% of males. In *Ae. albopictus* males, the lower percentage can be attributed to a high percentage (27.63%) originating in East St. Louis, Illinois. Further details of geographic origins are in the supplementary information (Table S.5.2 - S.5.5).

I found no consistent effect of female or male geographic origin on the rate of insemination of *Ae. aegypti* and *Ae. albopictus* females, by heterospecific males (Figure S.5.3 - S.5.6). Thus, I frequently found great variation in the rates of heterospecific insemination in different crosses with female *Ae. aegypti* or *Ae. albopictus* from the same geographic origin. This is true when comparing crosses where the heterospecific males are from the same geographic origin, or different geographic origins.

I found the same trends when examining male geographic origin: when males from the same geographic origin were crossed with heterospecific females, there was great variation in the percentage inseminated, both when the females were from the same geographic origin or different geographic origins.

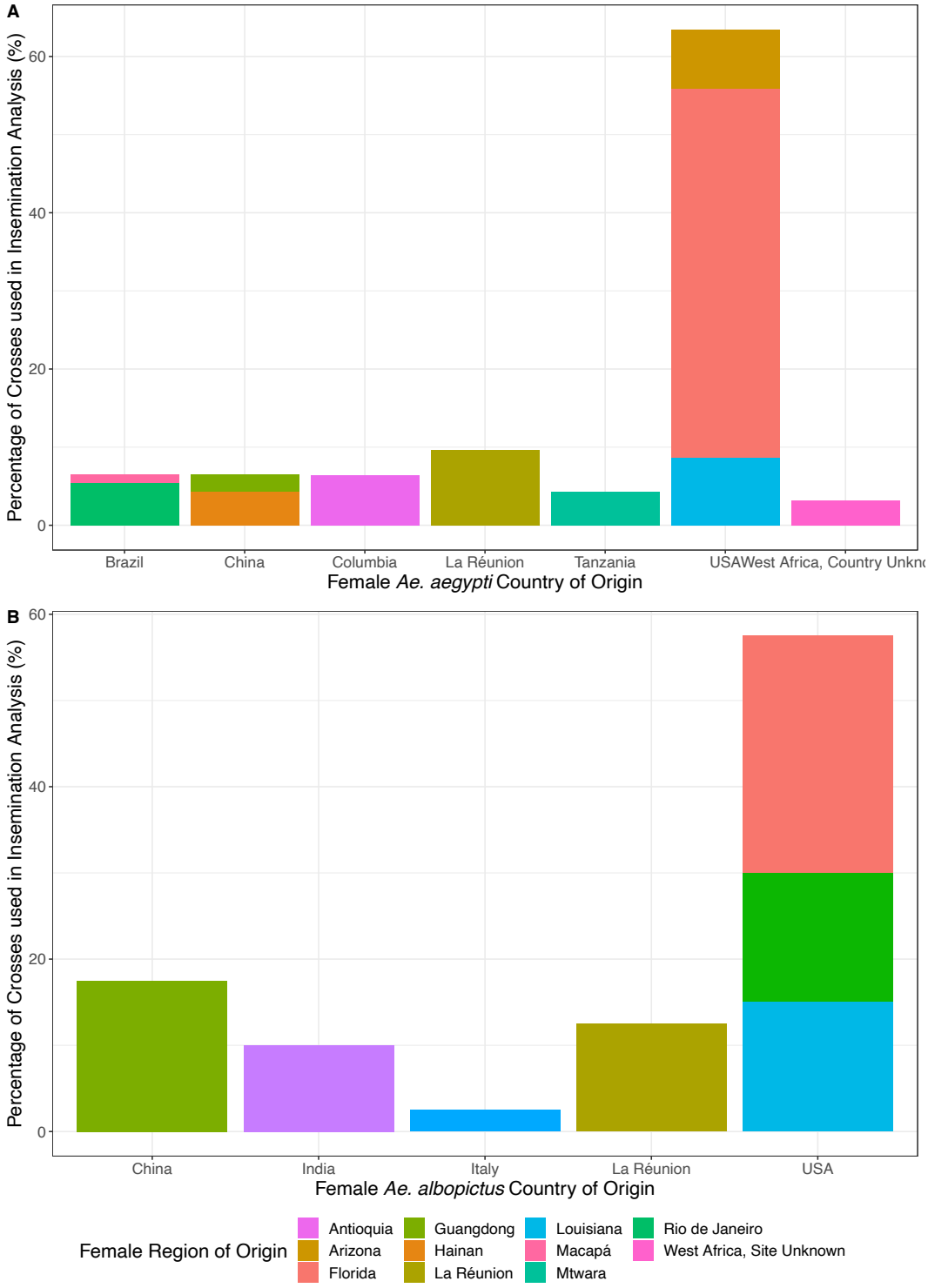

Figure 5.8 – The percentage of crosses in the insemination analysis using Ae. aegypti (A) and Ae. albopictus (B) females from different geographic origins. Geographic origins are grouped by country on the x-axis and coloured by geographic region.

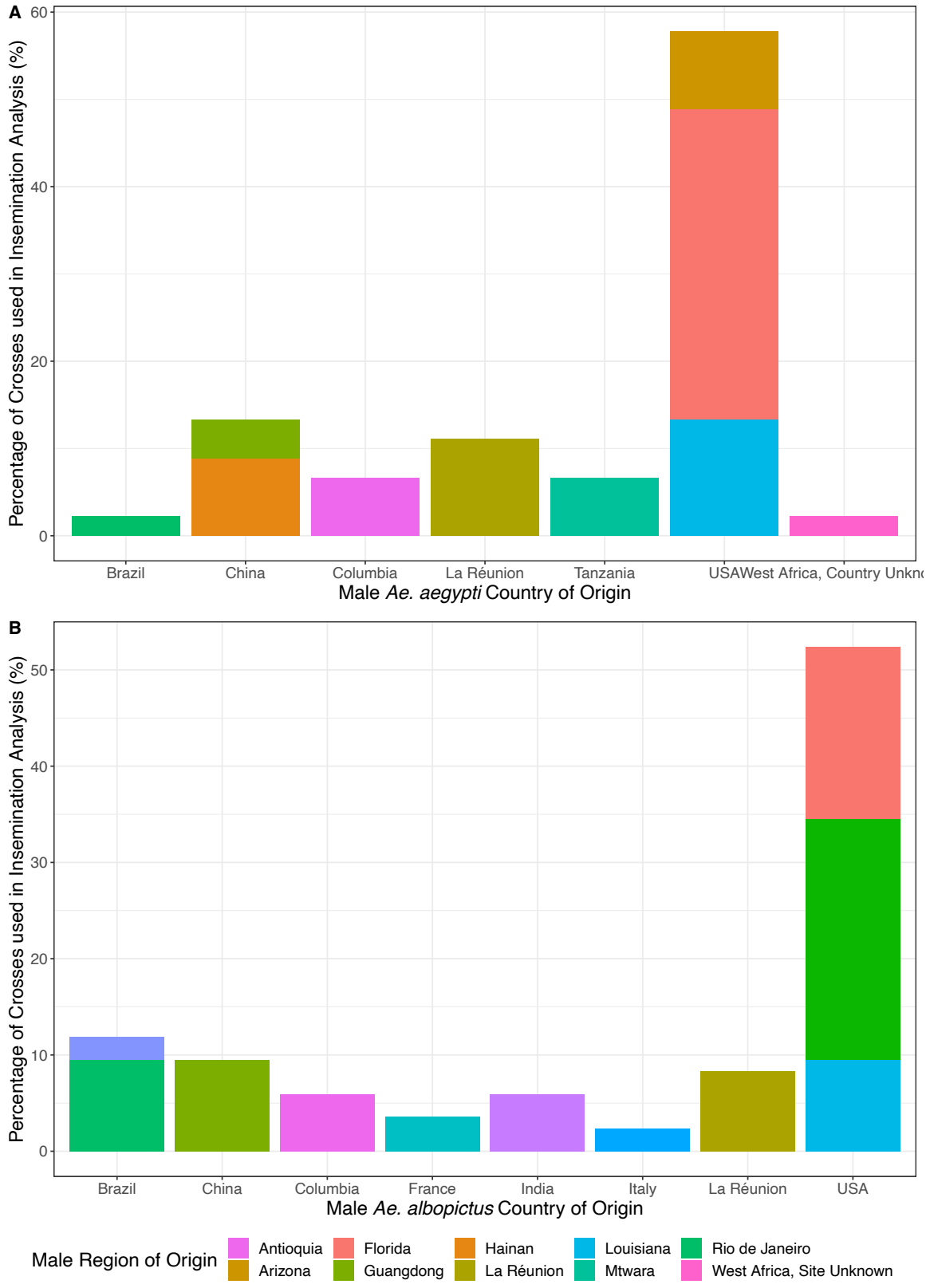

Figure 5.9 – The percentage of crosses in the insemination analysis using Ae. aegypti males (A) and Ae. albopictus males (B) from different geographic origins. Geographic origins are grouped by country on the x-axis and coloured by geographic region.

3.2.7. Differences in Study Methods

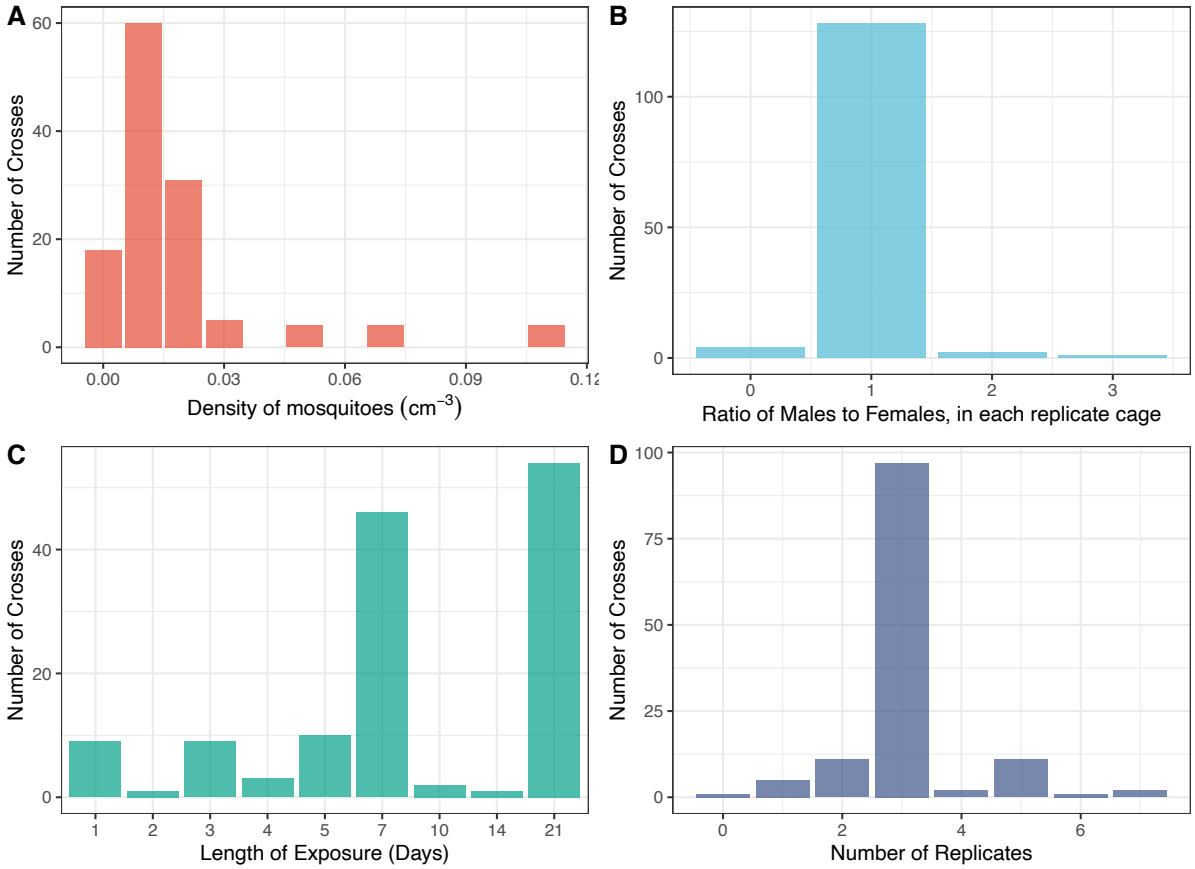

Figure 5.10 – Summary of the differences in the study methods between different crosses used in the insemination analysis. I examined differences in the (A) density of mosquitoes (cm^{-3}), (B) ratio of males to females, (C) length of exposure of male mosquitoes to female mosquitoes, prior to determining insemination status, (D) number of replicates of the cross within an experiment. Where the explanatory variable was not detailed in the study, the data was not included in this analysis.

The results so far show large differences in the rates of heterospecific insemination that cannot solely be explained by historical contact with heterospecifics, or geographic origin. Despite filtering for non-choice laboratory studies, there may be some differences in experimental methods that can explain the variation in the rates of heterospecific insemination. Thus, I examined three key experimental variables that could impact the mean

insemination rate: density of mosquitoes (Marcela et al., 2015), ratio of males to females and length of exposure (Nasci, Hare & Willis, 1989). I also examined the number of replicates of each cross within a study, due to its effect on study variance and reliability.

I found that there was great variation (between 1 and 21 days) in the length of exposure of male mosquitoes to female mosquitoes, prior to determining insemination status. Most studies either exposed males and females for 7 or 21 days, a difference of 2 weeks exposure time. Furthermore, I found variation in the density of mosquitoes, which ranged from 0.0001 mosquitoes cm^{-3} to 0.108 mosquitoes cm^{-3}: a difference of 3 orders of magnitude. However, in most studies the density is at the lower end of that scale, with the mean density 0.020 mosquitoes cm^{-3} (SE = 0.0019). While there is some variation, in most studies the ratio of males to females is 1, and the number of replicates is 3.

I further examined the impact of length of exposure, and density of mosquitoes on the number of females inseminated in heterospecific crosses (Figure S.5.7 - S.5.8). In *Ae. aegypti*, I found that increasing the length of time females were exposed to heterospecific males resulted in a significant increase in the number of females inseminated (coeff = 0.17, SE = 0.020, $P < 2x10^{-16}$), however length of exposure had no impact on the number of females inseminated in *Ae. albopictus* females ($P = 0.30$).

Furthermore, increasing the density of mosquitoes decreased the number of *Ae. aegypti* (coeff = -7.67, SE = 4.38, $P = 0.080$) and *Ae. albopictus* (coeff = -14.26, SE = 6.98, $P = 0.041$) inseminated.

4. Discussion

4.1. Overview

This review synthesised current knowledge of heterospecific insemination, an important direct fitness cost associated with reproductive interference, in *Aedes* species. I focussed on evaluating the evidence for four key explanations for heterogeneity in the rates of heterospecific insemination in *Ae. aegypti* and *Ae. albopictus* females. This review clarifies how well variation in insemination rates in *Ae. aegypti* and *Ae. albopictus* females can be explained, based on prior knowledge.

4.2. Insemination Analysis

4.2.1. Conspecific vs. Heterospecific crosses

I found that heterospecific insemination occurs at a lower rate than conspecific insemination in both *Ae. aegypti* and *Ae. albopictus*, supporting my initial hypothesis. This suggests that there are mechanisms to promote successful mating between conspecifics and prevent mating between heterospecifics in both species. This is makes evolutionary sense; there is strong selection to mate with conspecific males, as conspecific insemination is required to produce offspring. Meanwhile, there is selection against mating with heterospecific males, due to indirect fitness costs: for instance, time wasted mating with heterospecifics, and the associated physical damage. In *Ae. aegypti* females there is an additional, direct fitness cost,

as *Ae. aegypti* females become refractory to further mating following insemination by heterospecifics (Leahy & Craig Jr., 1965; Robbins et al., 2011).

4.2.2. *Ae. aegypti* vs. *Ae. albopictus* Females

In heterospecific crosses, I found that a higher percentage of *Ae. aegypti* females were inseminated than *Ae. albopictus* females. Therefore, in *Ae. aegypti* females, the costs of heterospecific mating are greater (Leahy & Craig Jr., 1965; Robbins et al., 2011), and heterospecific mating occurs more frequently than in *Ae. albopictus* females. This supports the hypothesis that the costs of reproductive interference are asymmetric and may contribute to the displacement of *Ae. aegypti* by *Ae. albopictus* during its range expansion (Zhou et al., 2022). However, there is substantial variation in heterospecific insemination rates, especially in *Ae. aegypti* females, suggesting that there are other important ecological factors influencing the rates of heterospecific insemination experienced by both species.

4.2.3. Allopatric vs. Sympatric

One explanation for within-species differences in heterospecific insemination rates is prior exposure to heterospecifics. Previous studies have highlighted that female *Ae. aegypti* from strains sympatric with *Ae. albopictus* are inseminated at a lower rate than allopatric females (Bargielowski et al., 2015), as sympatric females have evolved resistance to heterospecific insemination following exposure to heterospecifics (Bargielowski & Lounibos, 2014). I found support for this in my review: sympatric *Ae. aegypti* females were inseminated at a lower rate by *Ae. albopictus* males than allopatric *Ae. aegypti* females. Furthermore, in sympatric *Ae. aegypti* females, there was less variation in the percentage of females inseminated than in allopatric *Ae. aegypti* females. The lower mean and variation in heterospecific

insemination rates suggests that *Ae. aegypti* females were under directional selection to evolve resistance to heterospecific mating (Walsh & Blows, 2009; Barton & Turelli, 1989).

In general, there is substantial variation in heterospecific insemination rates in both sympatric and allopatric *Ae. aegypti* females. Despite sympatric *Ae. aegypti* females having a lower average heterospecific insemination rate, in some allopatric strains there was 0% heterospecific insemination; thus, there was substantial overlap in heterospecific insemination rates between sympatric and allopatric *Ae. aegypti* females. I suggest that the low rates of insemination in some heterospecific crosses with allopatric *Ae. aegypti* females is most likely due to the *Ae. albopictus* males not attempting to mate (as I found in *Chapter 4*), or because the *Ae. aegypti* females evolved resistance to heterospecific mating through exposure to a heterospecific other than *Ae. albopictus*.

In *Ae. albopictus,* sympatric females did not have a lower rate of heterospecific insemination than allopatric females. Resistance to heterospecific insemination is costly, as it makes individuals slower to accept conspecific mates (Bargielowski & Lounibos, 2014). Because of this, when *Ae. aegypti* populations are no longer exposed to heterospecifics, resistance to heterospecific insemination is lost within 8 generations (Bargielowski et al., 2019). As *Ae. albopictus* females experience a low cost of heterospecific insemination relative to *Ae. aegypti* females (Leahy & Craig Jr., 1965; Robbins et al., 2011), and as there is a cost associated with evolving resistance to heterospecific mating, there may be no net benefit of *Ae. albopictus* females evolving resistance to heterospecific mating.

While historical contact with heterospecifics and differences between female species successfully explains some of the variation in heterospecific insemination, there is still much unexplained variation. I suggest that some of this variation originates from differences in study design. I found variation in length of time females were exposed to males, before their insemination status was examined; in most studies, the length of exposure was either 7 or 21 days, a difference of 2 weeks of exposure time. Furthermore, there was substantial variation in the density of mosquitoes in cages, with a difference of 3 orders of magnitude between studies with the lowest and highest densities. I found that increasing the length of exposure increased the number of *Ae. aegypti* females inseminated, and increasing the density of mosquitoes decreased the number of *Ae. aegypti* and *Ae. albopictus* females inseminated. This highlights that the necessity for using consistent methods, so that more reliable comparisons of heterospecific insemination rates can be made between studies.

4.3. Limitations

This meta-analysis reveals that most of our knowledge of rates of heterospecific insemination in *Aedes* is from laboratory trials, a general trend in reproductive interference research (Gröning & Hochkirch, 2008). Laboratory trials have artificially high densities of mosquitoes, resulting in higher encounter frequencies (Gröning & Hochkirch, 2008). Furthermore, most of these laboratory trials use non-choice tests, preventing heterospecific males having mating competition from conspecific males, and interspecific mate choice for both males and females.

These factors result in inflated heterospecific insemination rates in the laboratory, compared to the field, preventing the direct application of the results of laboratory trials to natural populations. Furthermore, it is unclear whether there is a correlation between the rates of heterospecific insemination in laboratory trials and in field trials in *Aedes spp*: in other animals, field and laboratory studies have sometimes yielded conflicting results (as summarised in Julia Gröning and Hochkirch, 2008). This limits the applicability of heterospecific insemination rates found in laboratory trials to natural populations, and thus their use in population dynamic models of *Ae. aegypti* and *Ae. albopictus*.

Furthermore, there are limitations in the methods I used when conducting this meta-analysis. One limitation is in my data collection: aside from *Chapter 3,* I only collected published data and omitted grey literature, so my results are susceptible to publication bias. From the data I collected, there is a further limitation: for each cross, I was only able to extract the mean insemination rate across replicate cages. Thus, I could not directly model the binary data, but instead modelled the mean number of females inseminated per cage, and the error separately. Furthermore, this review focussed on the direct costs of reproductive interference (heterospecific insemination) and did not examine indirect costs (for instance, time wasted mating with heterospecifics, and physical damage incurred).

4.4. Future Work

I suggest that standardised methods should be developed to determine heterospecific insemination rates. This study shows that there is wide variation in heterospecific insemination rates, highlighting the importance of determining heterospecific insemination rates in different crosses. Furthermore, I found great variation in experimental design between studies, and an effect of experimental design on the rate of heterospecific insemination. Standardisation of methods would allow heterospecific insemination rates in different studies to be directly compared, without any confounding effects of study design. Standardised methods for determining levels of resistance are used in other contexts; for instance, the WHO standard operating procedure for measuring insecticide resistance (World Health Organization, 2022b).

I suggest that experiments should be conducted to further our understanding of heterospecific insemination in natural populations, knowledge that can be applied when forming population dynamic models to measure the efficacy of vector-control techniques. This could be achieved directly by conducting more studies determining the insemination status and sperm species-origin in field-collected females, or indirectly by establishing methods to approximate heterospecific insemination rates in the field, using heterospecific insemination rates determined in the laboratory. The latter method would be beneficial - it would allow the large amount of data already collected from laboratory studies to be used, and it would remove the logistical constraints of fieldwork in future experiments. However, I do not know that it is possible to establish a consistent link between heterospecific insemination rates in the laboratory, and those in the field.

In this study, I was unable to clarify, using statistical techniques, whether there is an effect of male or female geographic origin on the rates of heterospecific insemination. While Honório *et al.* (2018) found differences in heterospecific insemination rates due to male geographic origin, it is not clear whether geographic origin has a broad, consistent effect on heterospecific insemination. I suggest that further studies should be conducted to examine the impact of male and female geographic origin on heterospecific insemination rates. This may account for some of the unexplained variation in heterospecific insemination.

5. Conclusion

In this review, I systematically evaluated the evidence for four key hypotheses explaining heterogeneity in insemination rates in *Ae. aegypti* and *Ae. albopictus*. I found evidence to support three of these hypotheses. Firstly, I found that females were inseminated at a greater rate in conspecific crosses than heterospecific crosses. This was expected due to the fitness gains of conspecific insemination, and the fitness costs of heterospecific insemination. Secondly, I found that *Ae. aegypti* females are inseminated by heterospecifics at a greater rate than *Ae. albopictus* females, supporting the hypothesis that reproductive interference is more costly to *Ae. aegypti* than *Ae. albopictus*. Thirdly, I found that sympatric *Ae. aegypti* females are less likely to be inseminated by heterospecifics than allopatric *Ae. aegypti* females, providing evidence for the hypothesis that *Ae. aegypti* females can evolve to resist heterospecific insemination, following exposure to *Ae. albopictus* males. However, there was considerable variation in heterospecific insemination rates which could not be explained by these hypotheses. This highlights the necessity to quantify heterospecific insemination in different populations, using standardised methods. Furthermore, I found that most studies examining heterospecific insemination in *Ae. aegypti* and *Ae. albopictus* are non-choice laboratory studies, thus further studies are required to understand heterospecific insemination under natural conditions. This would allow us to include the direct impacts of reproductive interference into population dynamic models examining the efficacy of vector control techniques and thus, increase the accuracy of their predictions.

Supplementary Information

Article Title	Authors	Journal	Publication Year	Used in Insem. Analysis?	Study Ref Number
Asymmetric Mating Interference between Two Related Mosquito Species: *Aedes (Stegomyia) albopictus* and *Aedes (Stegomyia) cretinus*	Giatropoulos A, Papachristos DP, Koliopoulos G, Michaelakis A, Emmanouel N.	PLoS One	2015	No	NA
Competitive Displacement of *Aedes (Stegomyia) polynesiensis* Marks by *Aedes (Stegomyia) albopictus* in Laboratory Populations.	Gubler DJ.	J Med Entomol	1970	No	NA
Ethological Divergence in Allopatry and Asymmetrical Isolation in the South Pacific *Aedes scuttellaris* Subgroup.	McLain DK, Rai KS, Rao PN.	Evolution	1985	No	NA
A Peptide Signaling System that Rapidly Enforces Paternity in the *Aedes aegypti* Mosquito	Duvall LB, Basrur NS, Molina H, McMeniman CJ, Vosshall LB.	Curr Biol	2017	No	NA
Accessory Gland Substance as a Stimulant for Oviposition in *Aedes aegypti* and *Ae*. *albopictus*	Leahy MG, Craig GB Jr.	Mosquito News	1965	No	NA
Barriers to Hybridization Between *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae)	Leahy MG, Craig GB Jr.	Evolution	1967	Yes	13
Coexistence of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in Peninsular Florida Two Decades After Competitive Displacements	Lounibos LP, Bargielowski I, Carrasquilla MC, Nishimura N	J Med Entomol	2016	Yes	1
Competitive Reduction by Satyrization? Evidence for Interspecific Mating in Nature and Asymmetric Reproductive Competition between Invasive Mosquito Vectors	Tripet F, Lounibos LP, Robbins D, Moran J, Nishimura N, Blosser EM.	Am J Trop Med Hyg	2011	No	NA
Could Sterile Aedes albopictus Male Releases Interfere with *Aedes aegypti* Population in Reunion Island?	Andrianjakarivony HF, Damiens D, Marquereau L, Gaudillat B, Habchi-Hanriot N, Gouagna LC.	Insects	2022	Yes	2
Demonstration of Resistance to Satyrization Behavior in *Aedes aegypti* from La Reunion island	Maïga H, Gilles JRL, Susan Lees R,	Parasite	2020	Yes	3

	Yamada H & Bouyer J.				
Differences in Male Mating Response and Female Flight Sounds in *Aedes aegypti* and *Ae. albopictus* (Diptera: Culicidae)	Duhrkopf RE, Hartberg WK.	J Med Entomol.	1992	Yes	4
Evolution of Resistance to Satyrization through Reproductive Character Displacement in Populations of Invasive Dengue Vectors	Bargielowski IE, Lounibos LP, Carrasquilla MC.	Proc Natl Acad Sci U S A.	2012	Yes	5
Induced Sterility in *Aedes (Stegomyia) polynesiensis* Marks by Cross-Insemination with *Aedes (Stegomyia) albopictus* Skuse.	Gubler DJ.	J Med Entomol.	1970	No	NA
Interspecific Mating between Louisiana strains of *Aedes albopictus* and *Aedes aegypti* in the field and laboratory	Nasci RS, Hare SG, Willis FS.	J Am Mosq Control Assoc.	1989	Yes	14
Interspecific Mating Bias may drive *Aedes albopictus* Displacement of *Aedes aegypti* during its Range Expansion	Zhou J, Liu S, Liu H, Xie Z, Liu L, Lin L, Jiang J, Yang M, Zhou G, Gu J, Zhou X, Yan G, James AA, Chen XG.	PNAS Nexus	2022	Yes	6
Interspecific Mating Effects on Locomotor Activity Rhythms and Refractoriness of *Aedes albopictus* (Diptera: Culicidae) Females	Feitoza TS, Ferreira-de-Lima VH, Câmara DCP, Honório NA, Lounibos LP, Lima-Camara TN.	Insects	2020	No	NA
Laboratory Study of Competition between United States Strains of *Aedes albopictus* and *Aedes aegypti* (Diptera: Culicidae)	Black WC 4th, Rai KS, Turco BJ, Arroyo DC.	J Med Entomol.	1989	No	NA
Linkage Maps for 20 Enzyme Loci in *Aedes triseriatus*	Matthews TC, Munstermann LE.	J Hered.	1990	No	NA
Male Accessory Gland Substances from *Aedes albopictus* affect the Locomotor Activity of *Aedes aegypti* Females	Lima-Camara TN, Codeço CT, Honório NA, Bruno RV, Peixoto AA, Lounibos LP.	Mem Inst Oswaldo Cruz.	2013	Yes	7
Male Origin Determines Satyrization Potential of *Aedes aegypti* by Invasive *Aedes albopictus*	Honório NA, Carrasquilla MC, Bargielowski IE, Nishimura N, Swan T, Lounibos LP	Biological Invasions	2018	Yes	8

No Evidence for Successful Interspecific Cross-mating of Transgenic *Aedes aegypti* (L.) and Wild type *Aedes albopictus* Skuse	Lee HL, Aramu M, Nazni WA, Selvi S, Vasan S.	Trop Biomed	2009	No	NA
Patterns of Allozyme Relationships compared with Morphology, Hybridization, and Geologic History in Allopatric Island-dwelling Mosquitoes	Pashley DP, Rai KS, Pashley DN.	Evolution	1985	No	NA
Rapid Evolution and the Genomic Consequences of Selection Against Interspecific Mating	Burford Reiskind MO, Labadie P, Bargielowski IE, Lounibos LP, Reiskind MH	Mol Ecol.	2018	Yes	9
Rapid Evolution of Reduced Receptivity to Interspecific Mating in the Dengue Vector *Aedes aegypti* in Response to Satyrization by Invasive *Aedes albopictus*	Bargielowski IE, Lounibos LP.	Evol Ecol.	2014	Yes	10
Rapid Loss of Resistance to Satyrization in Invasive Mosquitoes and the Effects of Age on Interspecific Mating Frequency	Bargielowski IE, Honório NA, Blosser EM, Lounibos LP	J Med Entomol.	2019	No	NA
Reinforcement for Ethological Isolation in the Southeast Asian *Aedes albopictus* Subgroup (Diptera: Culicidae).	McLain DK, Rai KS.	Evolution	1986	No	NA
Reproductive Interference between *Aedes albopictus* and *Aedes flavopictus* at a Place of their Origin	Sultana A, Sunahara T, Tsurukawa C, Tuno N.	Med Vet Entomol.	2021	No	NA
Reproductive Isolation between Florida strains of *Aedes aegypti* and *Aedes albopictus*	Harper JP, Paulson SL.	J Am Mosq Control Assoc.	1994	Yes	11
Satyrization without Evidence of Successful Insemination from Interspecific Mating between Invasive Mosquitoes	Carrasquilla MC, Lounibos, LP	Biol Let	2015	Yes	16
Sexual Harassment and Feeding Inhibition between Two Invasive Dengue Vectors	Soghigian J, Gibbs K, Stanton A, Kaiser R, Livdahl T.	Environ Health Insights	2015	No	NA
The Concomitant Effects of Self-limiting Insect Releases and Behavioural Interference on Patterns	Vollans M, Bonsall MB	Proc B	2021	No	NA

of Coexistence and Exclusion of Competing Mosquitoes					
The Ecological and Epidemiological Consequences of Reproductive Interference Between the Vectors *Aedes aegypti* and *Aedes albopictus*	Paton RS, Bonsall MB	J R Soc Interface	2019	No	NA
The Effects of Interspecific Courtship on the Mating Success of Aedes aegypti and Aedes albopictus (Diptera: Culicidae) Males	Bargielowski IE, Blosser EM, Lounibos LP	Ann Entomol Soc Am.	2015	Yes	12
Widespread Evidence for Interspecific Mating between *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in Nature	Bargielowski IE, Lounibos LP, Shin D, Smartt CT, Carrasquilla MC, Henry A, Navarro JC, Paupy C, Dennett JA	Infect Genet Evol.	2015	No	NA
Spermathecal Filling in Aedes aegypti and Aedes albopictus: Effects of Female and Male Body Sizes and Species	Carrasquilla MC, Lounibos LP, Honório NA, Murr S	J Med Entomol	2019	Yes	15
Chapter 3, Female Resistance to Heterospecific Insemination in *Ae. aegypti* Allopatric and Sympatric with *Ae. albopictus*	Vollans M, Bonsall MB, Cator LC, Gubbins S	NA (thesis)	2024	Yes	17

Table S.5.1 – summary of the studies selected for use in this study. The penultimate column refers to whether the study was used in my Insemination Analysis. The Study Ref numbers correspond to those in Figure S.5.1.

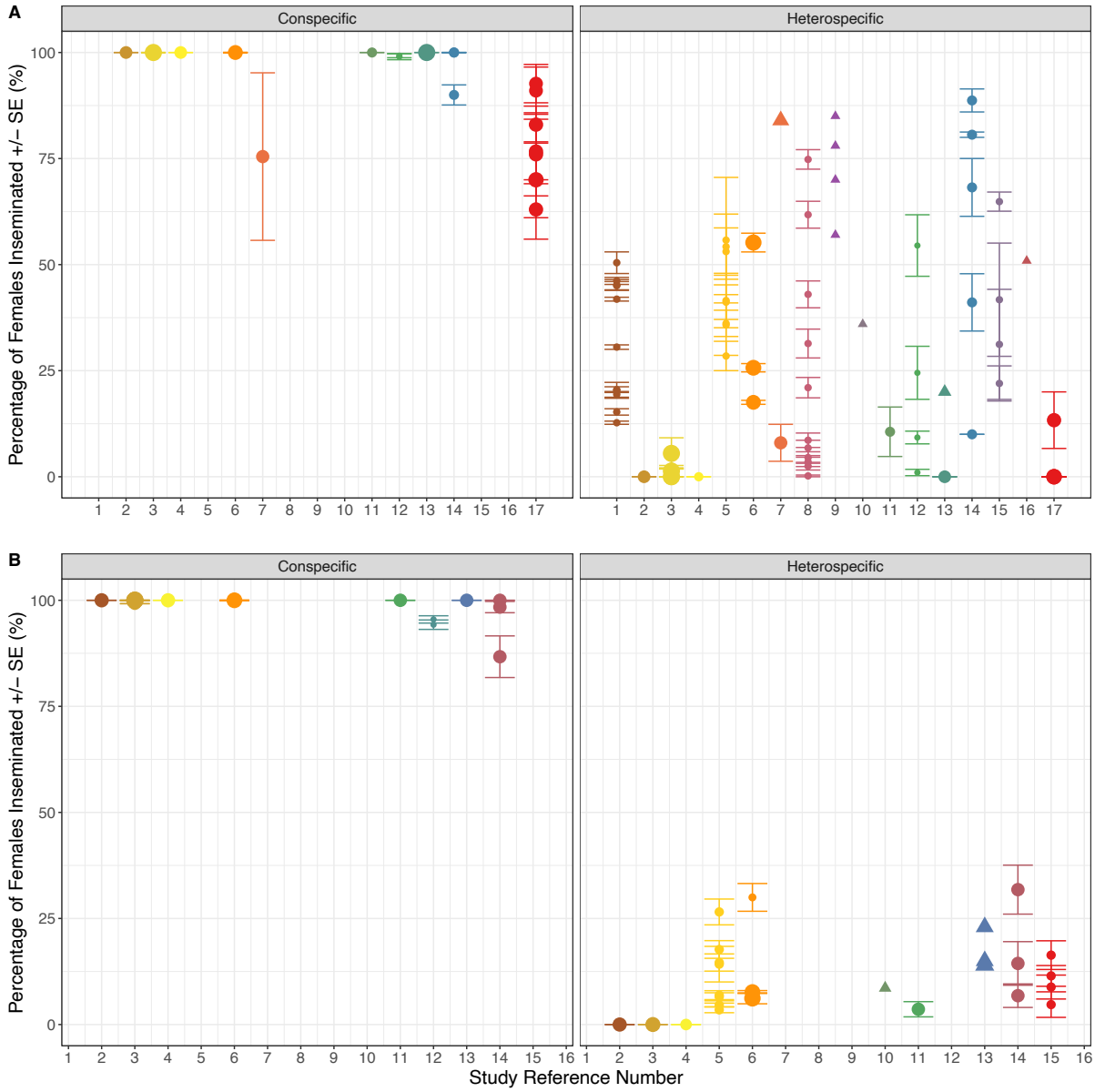

Figure S.5.1. – differences in the percentage of Ae. aegypti (top, A) and Ae. albopictus (bottom, B) females inseminated in conspecific (left) and heterospecific (right) crosses between studies. The corresponding study to the Study Reference Number is detailed in Table S.5.1. Each point represents the percentage of females inseminated in one cross, averaged across replicates where applicable. The size of the point corresponds to the average number of females dissected per replicate (range 20 – 200). Triangular points show insemination rates where the error was not reported. Error bars represent the standard error of the mean percentage of females inseminated, across replicates.

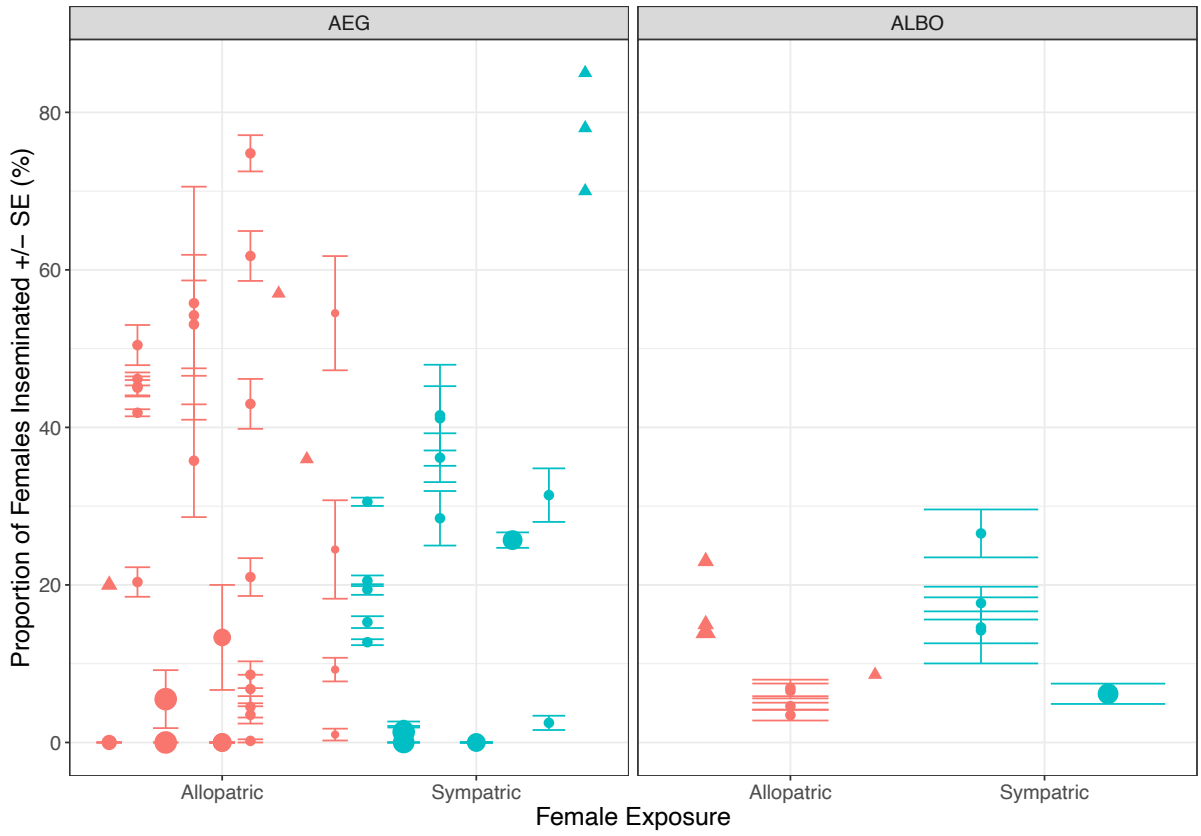

Figure S.5.2– differences in the percentage of Ae. aegypti (left) and Ae. albopictus (right) females inseminated in heterospecific crosses, where females are from strains allopatric (red) or sympatric (blue) with the heterospecifics. Sympatric females that had been bred in the laboratory for more than 7 generations were excluded from this graph. Each point represents the percentage of females inseminated in one cross, averaged across replicates where applicable. The size of the point corresponds to the average number of females dissected per replicate (range 20 – 200). Within each cross type, points are vertically aligned by study. Triangular points show insemination rates where the error was not reported. Error bars represent the standard error of the mean percentage of females inseminated, across replicates. Where female exposure was not detailed in the study, the insemination data was not included in this analysis.

Model	Distribution	Coeff	SE	p-value	Random Effect SD	Female species
Number Females Inseminated ~ Female Contact (Allopatric / Sympatric) + (1|Study)	Binomial (logit link)	-0.56	0.069	$3.75x10^{-16}$	1.59	AEG
Number Females Inseminated ~ Female Contact (Allopatric / Sympatric) + (1|Study)	Binomial (logit link)	1.32	0.22	7.82^{-10}	-0.72	ALBO
Standard Error of the proportion of females inseminated ~ Female Contact (Allopatric / Sympatric) + (1|Study)	Gaussian	-1.56	0.69	0.029	2.11	AEG
Standard Error of the proportion of females inseminated ~ Female Contact (Allopatric / Sympatric) + (1|Study)	Gaussian	1.97	0.52	0.0082	0.92	ALBO

Table S.5.2 – outputs of the statistical analysis comparing insemination of allopatric and sympatric females, when crossed with heterospecific males. In this analysis, females from sympatric lines that had been in the laboratory for more than 7 generations were excluded (Allopatric vs. Sympatric II, in the main text).

Female Strain Origin	Number of Crosses	Number of Papers
USA		
Florida		
a) Key West	24	7
b) Vero Beach	3	2
c) Miami	4	2
d) Kissimmee	2	2
e) Apopka	2	2
f) Fort Myers	2	2
g) Palm Beach County	2	1
h) Fort Pierce	2	1
i) Palmetto	1	1
j) Okeechobee	1	1
k) Orlando	1	1
Arizona		
Maricopa County	6	1
Tucson	1	1
Louisiana		
Lake Charles	8	1
China		
Guangdong		
a) Zhanjiang	2	1
Hainan		
Haikou	4	1
Brazil		
a) Macapá	1	1
b) Rio de Janeiro	5	2
La Réunion		
a) Les Trois-Bassins	2	1
b) Not Specified	7	1
Columbia		
Medellín		
Comuna 13	6	1
Tanzania		
Newala	4	1
West Africa (country unknown)		
Unknown Site [6]	3	1

Table S.5.3 – geographic origins of the strains of female Ae. aegypti used in the insemination analysis.

[6] Freetown, Sierra Leone is the most likely source of this strain (Kuno, 2010).

Female Strain Origin	Number of Crosses	Number of Papers
USA		
Florida		
a) Vero Beach	9	3
b) Polk County	2	1
Illinois		
East St. Louis	6	3
Louisiana		
Lake Charles	6	1
China		
Guangdong		
a) Foshan	4	1
b) Guangzhou	1	1
c) Zhanjiang	2	1
La Réunion		
a) Sainte-Marie	2	1
b) Not Specified	3	1
Italy		
Rimini	1	1
India		
Unknown Site	4	1

Table S.5.4 – geographic origins of the strains of female Ae. albopictus used in the insemination analysis.

Male Strain Origin	Number of Crosses	Number of Papers
USA		
Florida		
a) Vero Beach	3	2
b) Key West	7	3
c) Palm Beach County	2	1
d) Miami	2	1
e) Fort Pierce	2	1
Arizona		
Maricopa County	3	1
Tucson	1	1
Louisiana		
Lake Charles	6	1
China		
Guangdong		
a) Zhanjiang	2	1
Hainan		
Haikou	4	1
Brazil		
Rio de Janeiro	1	1
La Réunion		
a) Les Trois-Bassins	2	1
b) Not Specified	3	1
Columbia		
Medellín		
Comuna 13	3	1
Tanzania		
Mtwara		
Newala	3	1
West Africa (country unknown)		
Unknown Site [7]	1	1

Table S.5.5 – geographic origins of the strains of male Ae. aegypti used in the insemination analysis.

[7] Freetown, Sierra Leone is the most likely source of this strain (Kuno, 2010).

Male Strain Origin	Number of Crosses	Number of Papers
USA		
Florida		
a) Vero Beach	13	5
b) Polk County	2	1
Illinois		
East St. Louis	21	4
Louisiana		
Lake Charles	8	1
China		
Guangdong		
a) Zhanjiang	2	1
b) Foshan	4	1
c) Guangzhou	2	1
Brazil		
Rio de Janeiro	8	2
Manaus	2	1
La Réunion		
a) Sainte-Marie	2	1
b) Les Trois-Bassins		
c) Not Specified	5	1
Italy		
Rimini	2	1
Columbia		
Medellín		
Comuna 13	5	1
India		
Unknown Site	5	1
France		
Montpellier	3	1

Table S.5.6 – geographic origins of the strains of male Ae. albopictus used in the insemination analysis.

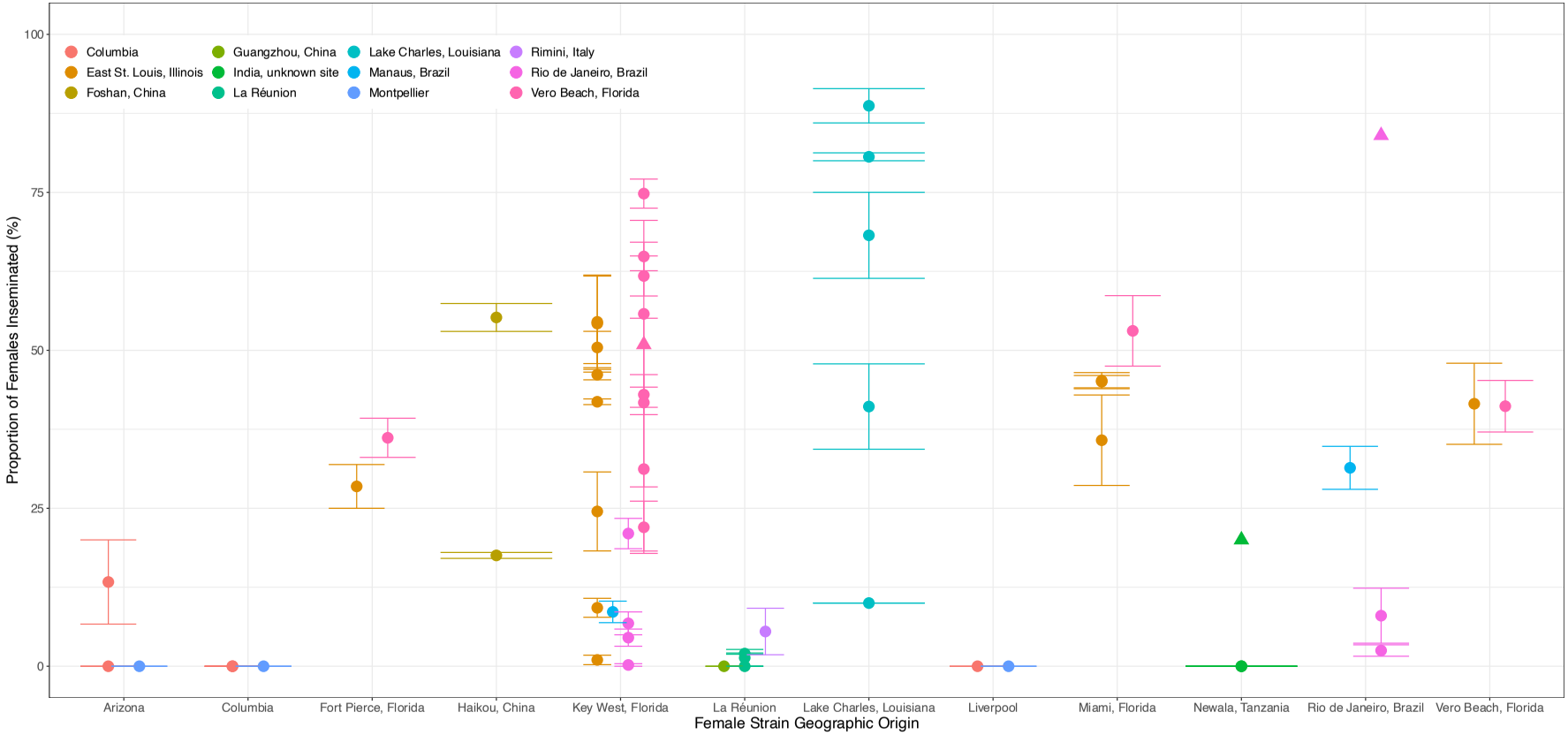

Figure S.5.3- Differences in the percentage of Ae. aegypti females inseminated in heterospecific crosses, depending on their geographic origin. I only included female geographic origins where I obtained insemination rates for multiple heterospecific crosses. Points are coloured dependent on the geographic origin of the male Ae. albopictus in the cross. Each point represents the percentage of females inseminated in one cross, averaged across replicates where applicable. Within each female geographic origin category, points are vertically aligned by male geographic origin. Triangular points show insemination rates where the error was not reported. Error bars represent the standard error of the mean proportion of females inseminated, across replicates. Where female origin was not detailed in the study, the insemination data was not included in this analysis.

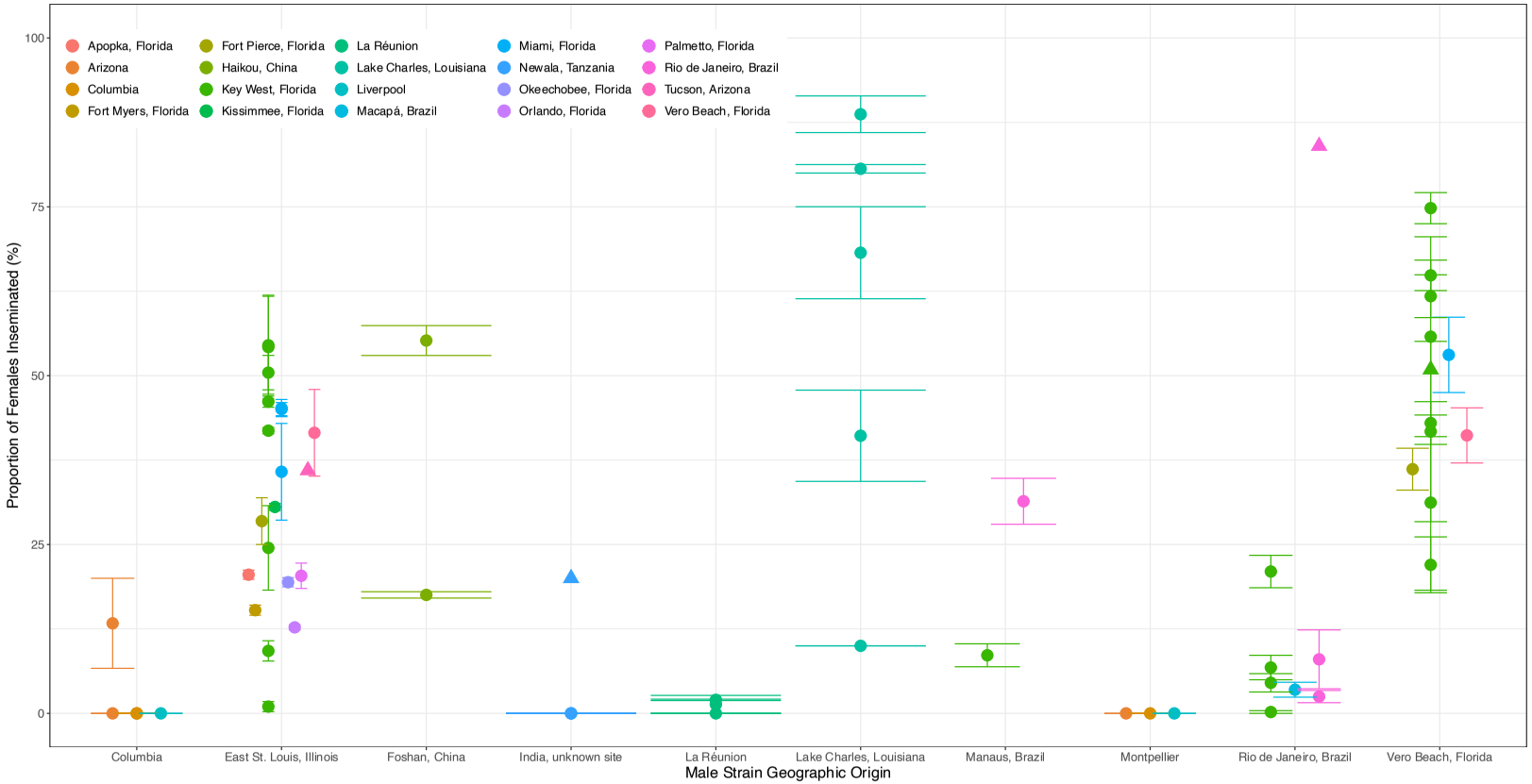

Figure S.5.4- Differences in the percentage of Ae. aegypti females inseminated in heterospecific crosses, depending on the geographic origin of the male Ae. albopictus they were crossed with. I only included male geographic origins where I obtained insemination rates for multiple heterospecific crosses. Points are coloured dependent on female geographic origin. Each point represents the percentage of females inseminated in one cross, averaged across replicates where applicable. Within each male geographic origin category, points are vertically aligned by female geographic origin. Triangular points show insemination rates where the error was not reported. Error bars represent the standard error of the mean proportion of females inseminated, across replicates. Where male origin was not detailed in the study, the insemination data was not included in this analysis.

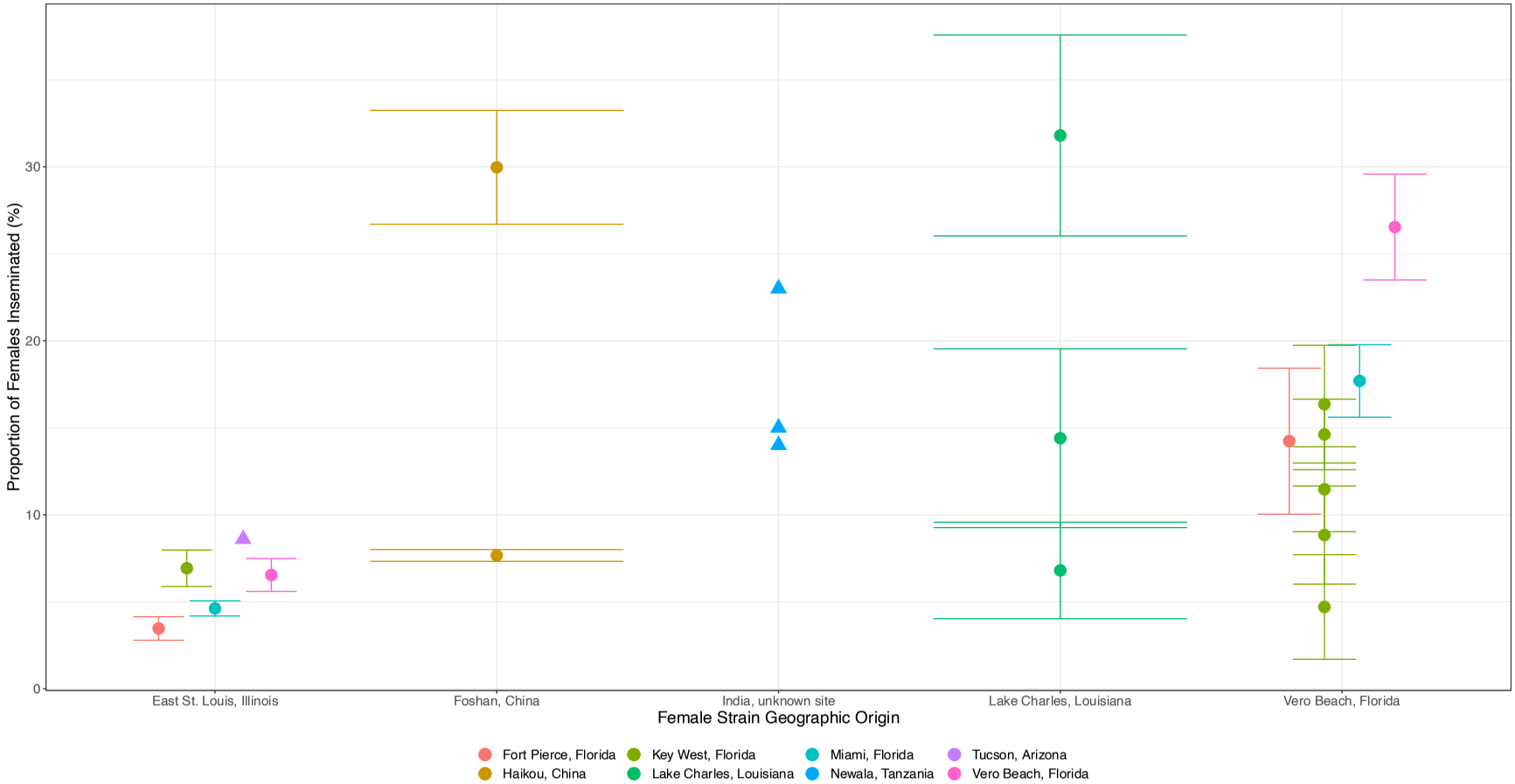

Figure S.5.5 - Differences in the percentage of Ae. albopictus females inseminated in heterospecific crosses, depending on their geographic origin. I only included female geographic origins where I obtained insemination rates for multiple heterospecific crosses. Points are coloured dependent on the geographic origin of the male Ae. aegypti in the cross. Each point represents the percentage of females inseminated in one cross, averaged across replicates where applicable. Within each female geographic origin category, points are vertically aligned by male geographic origin. Triangular points show insemination rates where the error was not reported. Error bars represent the standard error of the mean proportion of females inseminated, across replicates. Where female origin was not detailed in the study, the insemination data was not included in this analysis.

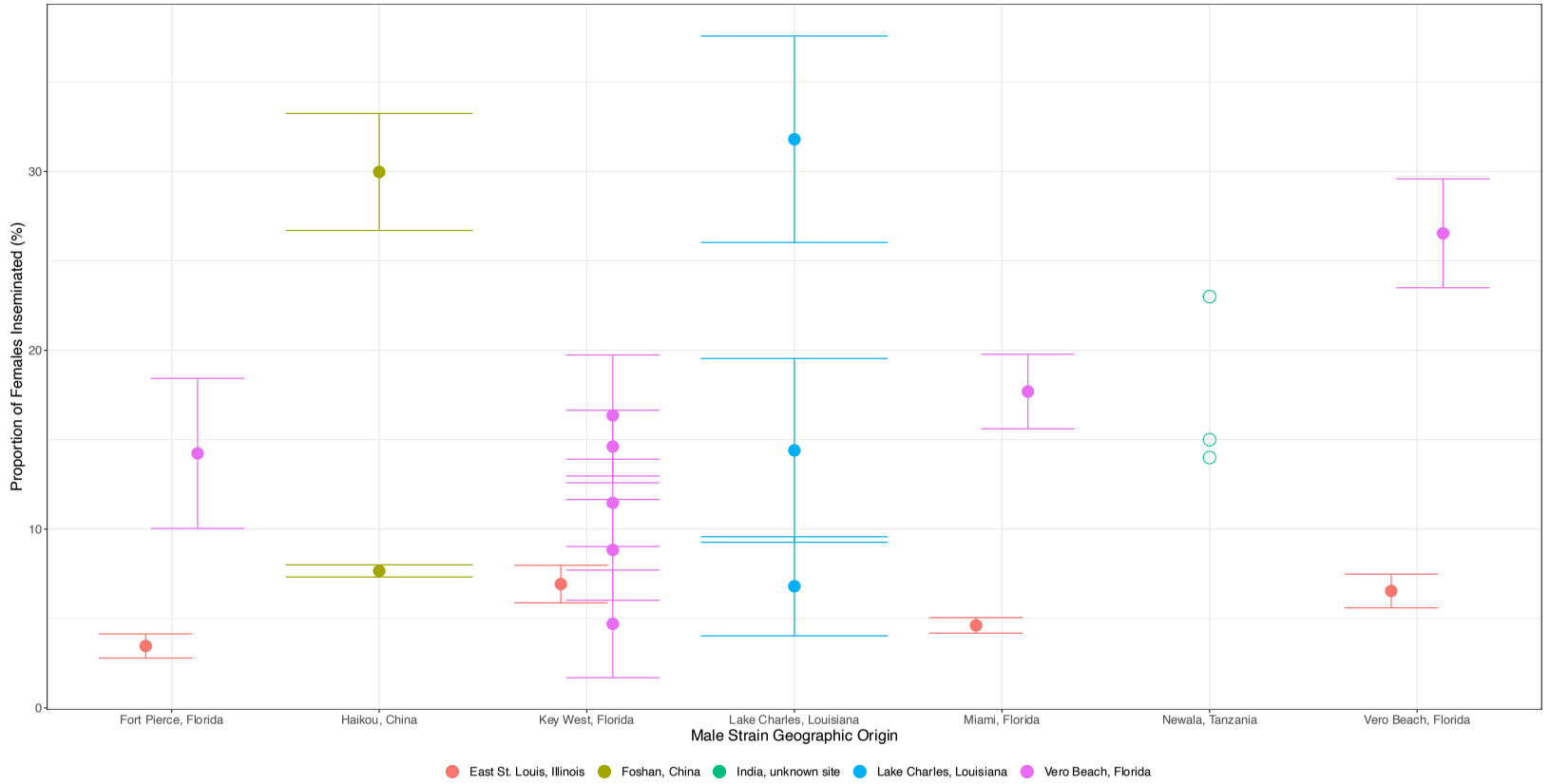

Figure S.5.6- Differences in the percentage of Ae. albopictus females inseminated in heterospecific crosses, depending on the geographic origin of the male Ae. aegypti they were crossed with. I only included male geographic origins where I obtained insemination rates for multiple heterospecific crosses. Points are coloured dependent on female geographic origin. Each point represents the percentage of females inseminated in one cross, averaged across replicates where applicable. Within each male geographic origin category, points are vertically aligned by female geographic origin. Triangular points show insemination rates where the error was not reported. Error bars represent the standard error of the mean proportion of females inseminated, across replicates. Where male origin was not detailed in the study, the insemination data was not included in this analysis.

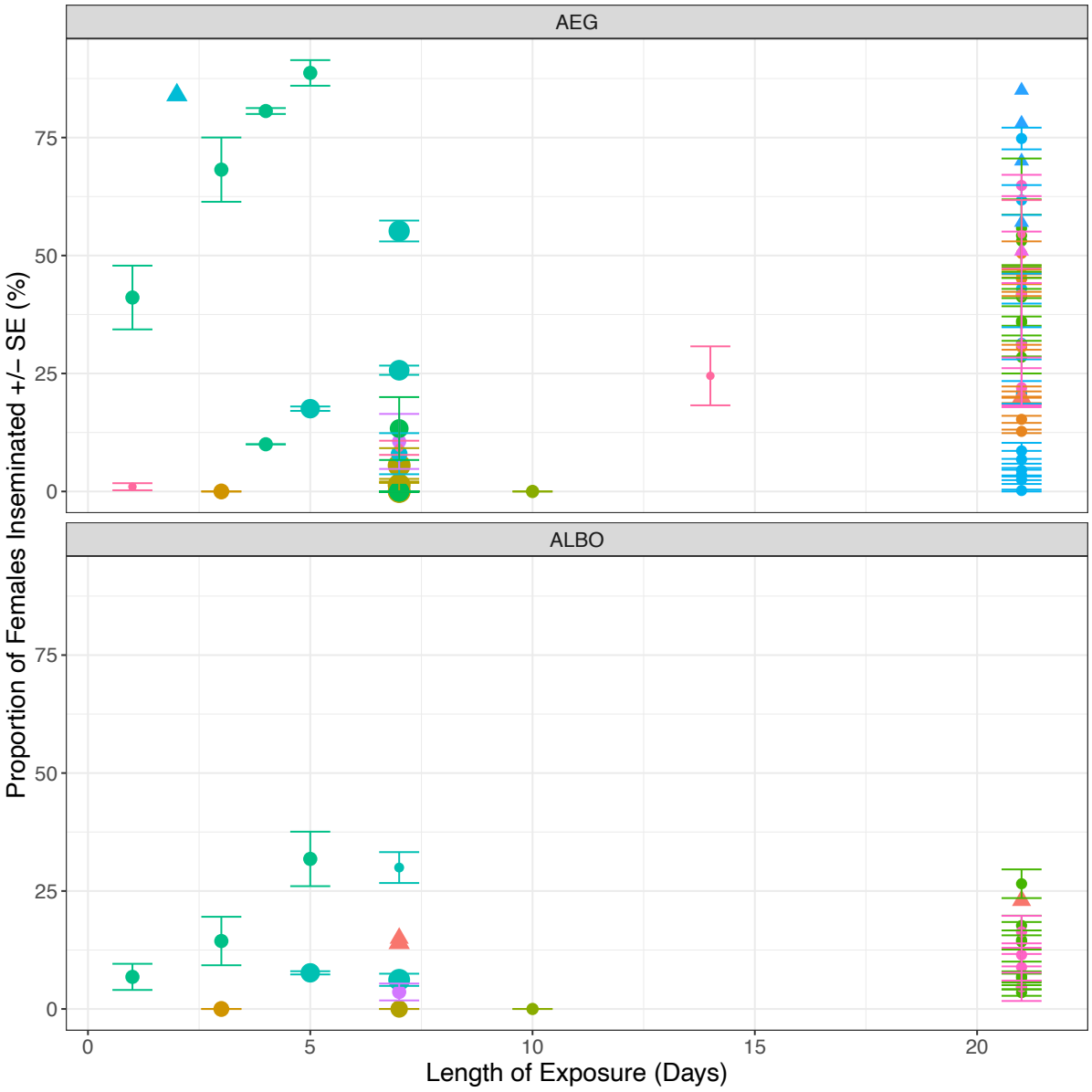

Figure S.5.7– The percentage of Ae. aegypti (row 1) and Ae. albopictus (row 2) females inseminated following different lengths of exposure to heterospecific males. Each point represents the percentage of females inseminated in one cross, averaged across replicates where applicable. The size of the point corresponds to the average number of females dissected per replicate, and the colour corresponds to the study. Triangular points show insemination rates where the error was not reported. Error bars represent the standard error of the mean percentage of females inseminated, across replicates.

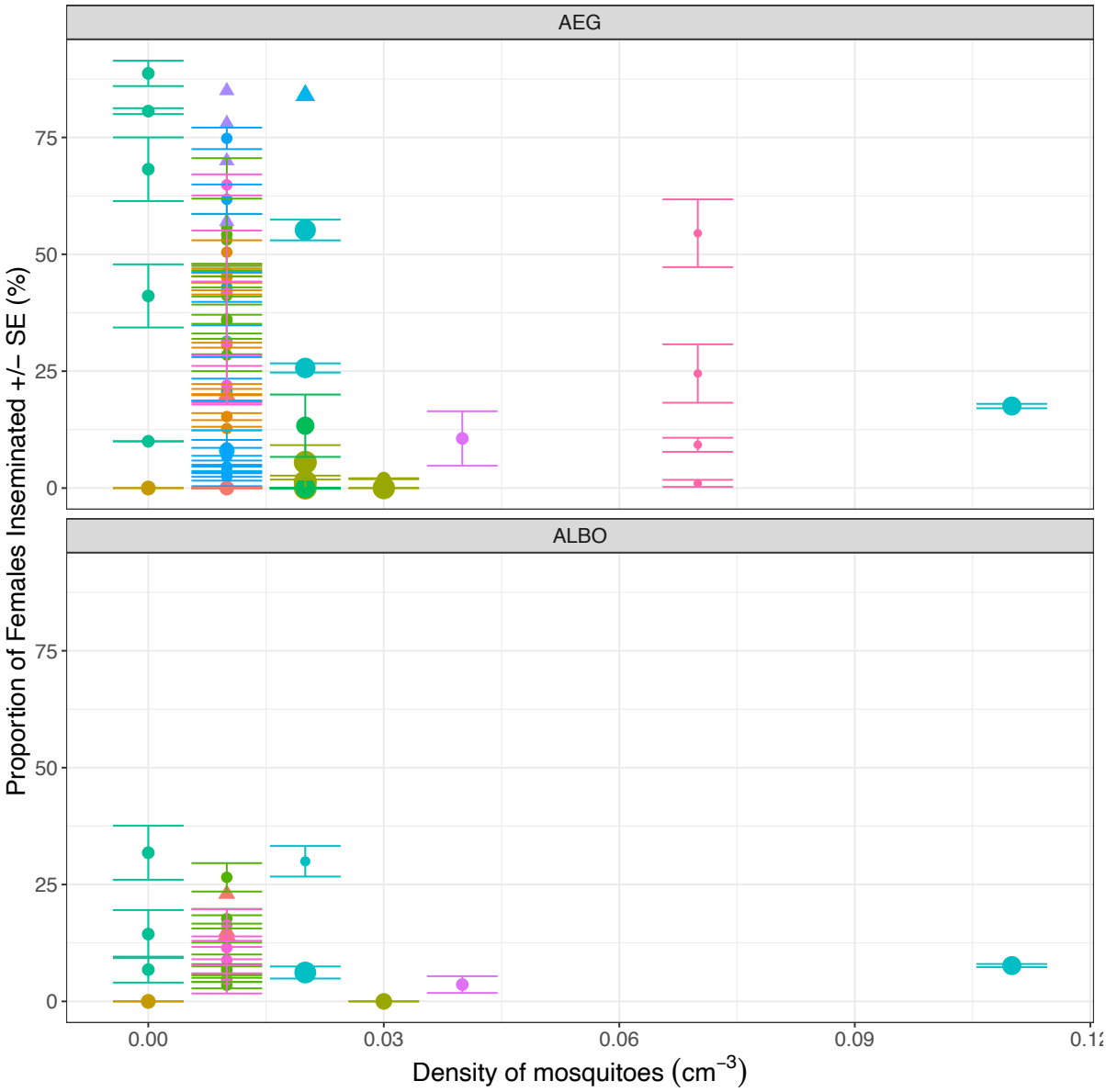

Figure S.5.8 – The percentage of Ae. aegypti (row 1) and Ae. albopictus (row 2) females inseminated, when there are different densities of mosquitoes in the cage. Each point represents the percentage of females inseminated in one cross, averaged across replicates where applicable. The size of the point corresponds to the average number of females dissected per replicate, and the colour corresponds to the study. Triangular points show insemination rates where the error was not reported. Error bars represent the standard error of the mean percentage of females inseminated, across replicates.

Chapter 6: Discussion

1. Overview

The aim of this thesis is to determine the importance of including reproductive interference into population dynamic frameworks examining the efficacy of vector control techniques, and to characterise further reproductive interference between *Ae. aegypti* and *Ae. albopictus*. Specifically, I examined:

(i) The combined effect of self-limiting mosquito release and reproductive interference on coexistence between *Ae. aegypti* and *Ae. albopictus* (*Chapter 2*)

(ii) The impact of prior exposure of *Ae. aegypti* strains to *Ae. albopictus* on the rate of heterospecific insemination of *Ae. aegypti* females (*Chapter 3 and Chapter 5*)

(iii) The behaviour of *Ae. aegypti* females and *Ae. albopictus* males, in crosses where no heterospecific insemination occurs (*Chapter 4*)

(iv) Heterogeneity in heterospecific insemination rates between *Ae. aegypti* and *Ae. albopictus* (*Chapter 5*)

In this final chapter, I discuss the principal findings and implications of this research, and end with suggesting directions for future research.

2. Principal Findings

The results of *Chapter 2* highlight the importance of including reproductive interference in models examining the efficacy of novel *Aedes* control techniques, in areas where *Ae. aegypti* and *Ae. albopictus* coexist. This is the first theoretical study to examine the combined effect of reproductive interference, and the release of modified mosquitoes on *Ae. aegypti* and *Ae. albopictus* populations. I found that the strength of reproductive interference, and the ratio of self-limiting *Ae. aegypti* released act together to determine whether stable coexistence occurs, and the population size of both *Ae. aegypti* and *Ae. albopictus*. This highlights the importance of furthering knowledge of reproductive interference between *Ae. aegypti* and *Ae. albopictus*, so that it can accurately be included into population dynamic models.

I address this in *Chapter 3*, where I determined the rates of heterospecific insemination, a direct cost of reproductive interference, in strains of *Ae. aegypti* and *Ae. albopictus* previously uncharacterised in the laboratory. The strains of *Ae. aegypti* used were either sympatric or allopatric with *Ae. albopictus*. Previous studies have shown that there are higher rates of heterospecific insemination in females from field strains of *Ae. aegypti* allopatric with *Ae. albopictus*, than sympatric with *Ae. albopictus* (Bargielowski et al., 2015). Furthermore, Bargielowski and Lounibos (2014) found that *Ae. aegypti* females previously unexposed to *Ae. albopictus* can evolve resistance to heterospecific insemination following exposure to *Ae. albopictus* males. Therefore, I hypothesised that *Ae. aegypti* females from sympatric strains would have a lower rate of heterospecific insemination than females from allopatric strains. I unexpectedly found, however, that neither *Ae. aegypti* females from

sympatric, nor allopatric strains were inseminated by *Ae. albopictus* males. These results suggest that resistance to heterospecific insemination in *Ae. aegypti* females is not always required to prevent heterospecific insemination.

In *Chapter 4*, I conducted the first study to examine the mating behaviours preventing heterospecific insemination between *Ae. aegypti* and *Ae. albopictus*, using the same strains as in *Chapter 3*. I found that male *Ae. albopictus* do not make persistent attempts to mate with *Ae. aegypti* females, from both sympatric and allopatric strains. Therefore, in heterospecific crosses between these strains, it is male behaviour rather than female rejection behaviour that prevents successful insemination. This contrasts with the dynamics of conspecific crosses, where the rate of female rejection behaviour determines the likelihood of insemination (Aldersley & Cator, 2019). As male *Ae. albopictus* do not persistently attempt to mate with *Ae. aegypti* females, this chapter highlights that in heterospecific crosses between some strains of *Ae. aegypti* and *Ae. albopictus*, there are minimal direct and indirect costs of reproductive interference.

The results of *Chapters 3 and 4* differ from other studies, which show that heterospecific mating does occur (for example, Bargielowski et al., 2015; Nasci, Hare & Willis, 1989) and *Ae. albopictus* males make persistent mating attempts to *Ae. aegypti* females (Zhou et al., 2022). In *Chapter 5*, I conducted a systematic literature review and meta-analysis to further examine heterogeneity in reproductive interference between *Aedes* species. I focussed on examining heterospecific insemination rates, the most widely used metric to examine reproductive interference. I found that *Ae. aegypti* females are inseminated at a greater rate by heterospecifics than *Ae. albopictus* females, which provides evidence that reproductive

interference is more costly to *Ae. aegypti* than *Ae. albopictus*. Furthermore, in heterospecific crosses with *Ae. aegypti* females, I found that there were lower rates of heterospecific insemination where *Ae. aegypti* strains were allopatric with *Ae. albopictus* than sympatric. This suggests that *Ae. aegypti* females evolve resistance to heterospecific mating following exposure to *Ae. albopictus* males, which has previously been shown (Bargielowski & Lounibos, 2014). Despite finding differences in heterospecific insemination rates between groups, there was also substantial unexplained variation within groups. Furthermore, I found differences in the methods used across studies, some of which had a significant impact on the insemination rate.

3. Implications

3.1. Public Health

Ae. aegypti and *Ae. albopictus* are principal vectors of dengue, a disease that poses a substantial public health threat (World Health Organization, 2023). Reducing the public health burden of dengue relies on controlling the vector population, as dengue has no vaccine or specific treatment (World Health Organization, 2023). The adverse environmental impacts of insecticides (for example, Weston *et al.*, 2005; Boyce *et al.*, 2007; Ristyadi, Andrew and Waugh, 2013; Hua and Relyea, 2019), and widespread insecticide resistance in *Aedes* mosquitoes (for example, Kandel et al., 2019; Maciel-de-Freitas et al., 2014; Yakob & Walker, 2016; Sene et al., 2021; Toé et al., 2022; Jangir & Prasad, 2022) means that novel techniques are required. During development, the efficacy of these novel techniques needs to be evaluated, using population dynamic frameworks (for example, Atkinson *et al.*, 2007; Beaghton, Beaghton and Burt, 2016).

This thesis highlights the necessity to include ecological interactions, specifically reproductive interference, in these population dynamic frameworks. In *Chapter 2*, I found that in areas where *Ae. aegypti* and *Ae. albopictus* coexist, the strength of reproductive interference acts concomitantly with the release ratio of self-limiting *Ae. aegypti,* to determine the population size of *Ae. aegypti* and *Ae. albopictus*, and whether coexistence can occur. These results highlight that the correct strength of reproductive interference needs to be included in models, to accurately determine the efficacy of vector control

programmes. Furthermore, in *Chapter 5* I found that the rate of heterospecific insemination between *Ae. aegypti* and *Ae. albopictus* varies greatly between crosses. Thus, a constant parameter value cannot be used for reproductive interference, and it is necessary to determine the strength of reproductive interference between specific, target populations of *Ae. aegypti* and *Ae. albopictus*.

3.2. Ecology of *Aedes* Mosquitoes

This thesis contributes to current knowledge of interspecific interactions between *Ae. aegypti* and *Ae. albopictus*, which is important to understanding invasion dynamics (Lounibos & Juliano, 2018). Both *Ae. aegypti* and *Ae. albopictus* are highly invasive, which has led to rapid geographic range expansions (Kraemer et al., 2015a, 2015b). In areas of overlap, outcomes are variable: *Ae. aegypti* can outcompete *Ae. albopictus* (Lounibos & Juliano, 2018), *Ae. albopictus* can outcompete *Ae. aegypti* (O'meara et al., 1995), or the two species can stably coexist (Braks et al., 2004; Simard et al., 2005). In *Chapter 5*, I found that there is substantial variation in the strength of reproductive interference between *Ae. aegypti* and *Ae. albopictus*. Furthermore, previous studies (Kuno, 1992; Kishi & Nakazawa, 2013; Paton & Bonsall, 2019), and *Chapter 2* of this thesis, show that reproductive interference strongly impacts *Ae. aegypti* and *Ae. albopictus* population dynamics. I suggest, therefore, that variation in the strength of reproductive interference between *Ae. aegypti* and *Ae. albopictus* populations may influence the outcome of an invasion.

While some strains may initially be better invaders, others may rapidly evolve to improve their success. In *Chapter 5*, I found that females from *Ae. aegypti* strains sympatric with *Ae.*

albopictus are inseminated at a lower rate by *Ae. albopictus* males than allopatric *Ae. aegypti* females, across available published studies. Thus, sympatric strains of *Ae. aegypti* may be more likely to successfully invade an area where *Ae. albopictus* is present, as they experience a lower rate of heterospecific insemination, and thus a lower associated cost (Leahy & Craig Jr., 1965; Robbins et al., 2011). However, Bargielowski and Lounibos (2014) previously found that resistance to heterospecific insemination can evolve in *Ae. aegypti* females within 1-3 generations of exposure to *Ae. albopictus*. Therefore, due to the rapid rates of evolution of mating behaviour in *Ae. aegypti* (Bargielowski et al., 2019; Bargielowski & Lounibos, 2014), *Ae. aegypti* females from strains originally susceptible to heterospecific mating may be able to rapidly evolve resistance and persist. However small, invading populations may require areas of refugia, with low or no heterospecific insemination, to prevent their extinction prior to the evolution of resistance.

4. Future Directions

4.1. Further Theoretical Modelling

I suggest further developing the theoretical model in *Chapter 2* to examine how reproductive interference influences the ability of *Ae. aegypti* and *Ae. albopictus* to spread through space. Due to the short dispersal distances of *Ae. aegypti* (Edman et al., 2005; Hemme et al., 2010) and *Ae. albopictus* adults (Bellini et al., 2010; Marni et al., 2010; Lacroix et al., 2009), and only some areas containing suitable oviposition sites (Tedjou et al., 2019; Honório et al., 2009; Bennett, McMillan & Loaiza., 2019), *Aedes* have a patchy distribution. I suggest that models should be developed to examine how the strength of reproductive interference influences the ability of *Ae. aegypti* and *Ae. albopictus* to establish in new patches from a small initial population size. This would further our knowledge of how reproductive interference influences the invasion dynamics of *Ae. aegypti* and *Ae. albopictus*.

Furthermore, I suggest that demographic stochasticity should be included in these models. This allows for the inclusion of random variation in demographic processes: individuals in the model would have a probability of dying, producing offspring, and dispersing, rather than each being included at a constant rate. It is important to include demographic stochasticity into models examining small population sizes, which is true of invading species, as demographic stochasticity has a greater effect on small populations (Lande, Engen & Saether, 2003).

4.2. Develop a Standardised Method for Determining Heterospecific Insemination Rate

The results of the systematic review (*Chapter 5*) highlight that there are key differences in the methods used to determine the rate of heterospecific insemination, and that some of these have a significant impact on the rate of heterospecific insemination determined. Therefore, I suggest a standardised method for determining heterospecific insemination rates in the laboratory should be developed. Standardised methods are used in other contexts, for example the WHO have a standard operating procedure to test for insecticide susceptibility in adult mosquitoes (World Health Organization, 2022b). Developing a standardised method would allow valid comparisons of heterospecific insemination rates to be made between different experiments.

4.3. Determine the Rate of Heterospecific Insemination in Nature

Most studies examining heterospecific insemination between *Ae. aegypti* and *Ae. albopictus* are non-choice laboratory trials (*Chapter 5*), where mosquitoes are at artificially high densities, and heterospecific males have no mating competition from conspecific males. For these reasons, the heterospecific insemination rates determined in these trials are likely to be inflated and cannot be used in models of natural populations of *Ae. aegypti* and *Ae. albopictus*. This highlights the necessity to expand our knowledge of the strength of heterospecific insemination in nature, between different strains of *Ae. aegypti* and *Ae.*

albopictus. This would allow reproductive interference rates to be accurately included into population dynamic frameworks examining the efficacy of *Aedes* control techniques.

One way to achieve this is to conduct more studies examining heterospecific insemination in the field. The two most effective methods to collect *Aedes* mosquitoes in the field are Human Landing Catches (HLCs) and BG-Sentinel traps (Wu et al., 2023). As HLCs use humans as bait, this method causes ethical concerns which are heightened in areas where mosquito-borne diseases are prevalent, and vector control techniques are likely to be implemented. While BG-Sentinel traps are a safer option, they have been lost or stolen in multiple previous studies (e.g. Bargielowski et al., 2015; Unlu & Farajollahi, 2012), hindering mosquito collection and preventing standardised methods from being maintained. Following the collection of *Ae. aegypti* and *Ae. albopictus* females, sperm is extracted from sperm bundles for PCR analysis. Extraction is technical (as specified in Tripet et al., 2011), and following extraction sperm must be maintained at -20°C until PCR analysis is conducted. Maintaining sperm at this temperature may be difficult in some circumstances, especially where there is no local PCR machine, and samples need to be sent afar for analysis. Therefore, there are challenges to determining heterospecific insemination rates in field-collected mosquitoes, in a range of locations.

Another approach, is to establish a consistent method to approximate heterospecific insemination rates in the field, using laboratory data on heterospecific insemination. This has key benefits: due to the desiccation resistance of *Aedes* eggs, they can be easily transported to an appropriate laboratory (Diniz et al., 2017; Mayilsamy, 2019; Urbanski et al., 2010; Zheng et al., 2015). The eggs can then be reared to adults and a simple,

standardised method can be used to determine heterospecific insemination rates. While spermathecae dissection (as detailed in figure 1, Parsana, Nanfack-Minkeu & Sirot, 2022) is required to determine the insemination status of the female, this is simpler than sperm extraction, and PCR analysis is not necessary. Furthermore, the ability to approximate heterospecific insemination rates in the field using laboratory data would allow a large amount of data already collected in laboratory studies (as summarised in *Chapter 5*) to be converted to field rates.

However, this method requires a consistent link between the rates of heterospecific insemination determined in the laboratory, and in the field. It is currently unclear whether this is the case: in other animals, field and laboratory studies have sometimes yielded conflicting results (as summarised in Gröning and Hochkirch, 2008). I suggest that further studies examining the rates of heterospecific insemination in the laboratory and field are conducted, in multiple strains of *Ae. aegypti* and *Ae. albopictus*, to determine whether a consistent link exists.

4.4. Examine Reproductive Interference between Wild Mosquitoes, and Modified Mosquitoes

Alongside determining the rates of reproductive interference between wild mosquito strains, I suggest future work should examine the rate of heterospecific insemination between modified mosquitoes, and local, wild strains. There is great variation in heterospecific insemination rates between *Ae. aegypti* and *Ae. albopictus* (*Chapter 5*), and genetically modified mosquitoes generally have a decreased fitness compared to wild mosquitoes (as reviewed in Dilani et al., 2022). For both reasons, it is too simplistic to assume that the strength of reproductive interference between modified mosquitoes and the local, wild mosquitoes will be the same as the strength of reproductive interference between the two local, wild mosquito strains. I suggest that following the development of a standardised protocol, it should be used to assess the rate of heterospecific insemination between wild, local strains and the modified mosquitoes. This would allow reproductive interference between wild mosquitoes and modified mosquitoes to be accurately included into models examining the efficacy of novel vector control techniques.

5. Conclusion

In this thesis, I highlight the importance of including reproductive interference into population dynamic frameworks examining the efficacy of *Aedes* control techniques, in areas where *Ae. aegypti* and *Ae. albopictus* coexist. Furthermore, I find that there is great variation in the strength of heterospecific insemination experienced by *Ae. aegypti* and *Ae. albopictus*. In some strains of *Ae. albopictus*, males do not persistently attempt to mate with *Ae. aegypti* females; thus, there is no heterospecific insemination, and negligible physical damage and time wasted. Meanwhile, previous studies have shown high rates of heterospecific insemination between other strains of *Ae. aegypti* and *Ae. albopictus*. I find some trends in the rate of heterospecific insemination: *Ae. aegypti* females are inseminated by heterospecifics at a greater rate than *Ae. albopictus* females, and strains of *Ae. aegypti* females allopatric with *Ae. albopictus* are inseminated at a greater rate than sympatric *Ae. aegypti* females. However, while there are significant differences in the rate of heterospecific insemination between these groups, I find that there is still substantial variation within groups. Therefore, the strength of reproductive interference between strains of *Ae. aegypti* and *Ae. albopictus* cannot be accurately predicted. Future work should focus on developing a simple protocol to determine heterospecific insemination rates between different strains, as differences in experimental methods affect the heterospecific insemination rate determined. This protocol could be used to characterise heterospecific insemination between target strains of *Ae. aegypti* and *Ae. albopictus*. Therefore, heterospecific insemination could be accurately included in population dynamic frameworks examining the efficacy of novel vector control techniques on the strains of *Ae. aegypti* and *Ae. albopictus* in

a specific region. This would allow these highly invasive mosquitoes, which carry diseases with substantial public health threats, to be controlled more effectively.

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