

**The Genetic Basis of Male Mating Success in the Yellow  
Fever Mosquito, *Aedes aegypti***

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**Statement of Originality**

I declare that the work described in this thesis is my own. All outside contributions are described in the “Acknowledgements” section, and all literature and image sources are appropriately cited.

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## Thesis abstract

*Aedes aegypti* is responsible for the transmission of many emerging and re-emerging pathogens. Understanding *Ae. aegypti* mating behaviour and its genetic basis is important for the improvement and development of mosquito control strategies. While male mating success is intrinsically important in spreading desirable genes, sterility, or endosymbionts in reproductive control, we know relatively little about variation in the male mating success phenotype and its genetic basis. Through this thesis I investigated the variation in male mating success, using a novel assay designed to incorporate relevant sexual selection pressures, and estimated its heritability. I found evidence for a skew in male mating success, with very few males mating multiple times, despite the capacity to do so. Male mating success likely has a genetic basis, though the proportion of additive genetic variation contributing to the phenotype is low.

I went on to use an experimental evolution and whole genome sequencing approach to demonstrate the role of sexual selection in shaping the *Ae. aegypti* genome. Manipulating the strength of sexual selection resulted in rapid genome-wide changes across all three chromosomes, with populations evolved in the presence of sexual selection retaining genomic similarity to their field-derived ancestors, which are predicted to have experienced strong sexual selection. I used gene ontology and functional effect analyses to show that many of the genes that responded consistently in all experimental replicates were associated with odour perception. I also identified a small number of potentially sexually selected genes that, after expression manipulation with RNAi, were tested for effects on male mating phenotypes. Males with reduced expression of one of these genes, *pickpocket315*, showed an altered mating phenotype, with males less likely to inseminate females, and making fewer attempts to mate overall.

These results highlight that sexual selection is an important evolutionary force in *Ae. aegypti* mating systems, creating variation in phenotypes, and exerting a powerful effect on the genome. The results also have important consequences for those strategies that depend upon rearing mosquitoes en-masse for field release, and for the design of new mosquito control strategies that target mating behaviours.

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### **Author's declaration**

I declare that the research described in this thesis is entirely my own work unless otherwise stated. All chapters have been compiled in co-authorship with my primary supervisor Dr. Lauren Cator. Work presented in the first chapter was published in *Trends in Parasitology* (2021) in collaboration with Professor Laura Harrington at Cornell University. The second chapter is in preparation for submission to *Behavioural Ecology* and the third and fourth chapters were published as a single paper in *Current Biology* in 2023.

### **Publications associated with thesis**

Wyer, C.A.S., Hollis, B, Cator, L.J. (2023). Release from sexual selection leads to rapid genome wide evolution in *Aedes aegypti*. *Current Biology*.

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Cator, L. J., Wyer, C. A. S., & Harrington, L. C. (2021). Mosquito Sexual Selection and Reproductive Control Programs. *Trends in Parasitology*.

<https://doi.org/10.1016/j.pt.2020.11.009>

### **Manuscripts in preparation for publication**

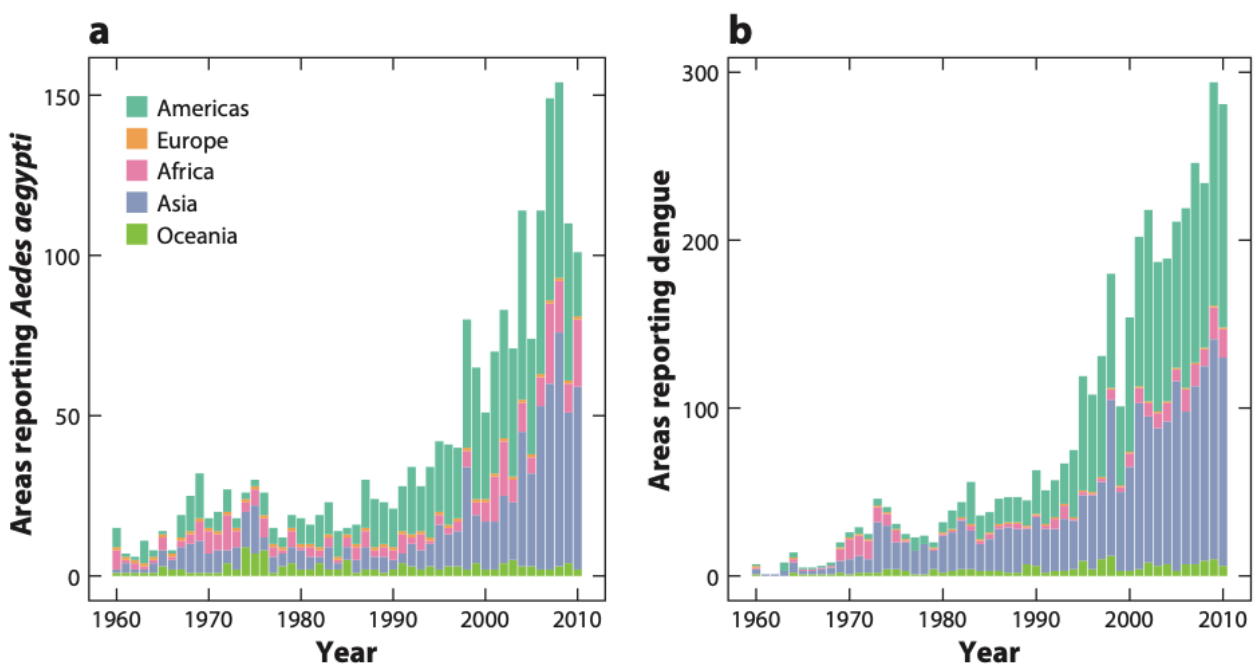
Wyer, C.A.S., Trajanovikj, V., Hollis, B., Cator, L.J. (2023). Heritability of male mating success in the Yellow Fever mosquito *Aedes aegypti*. (*In prep*)

## Chapter 1

### Sexual Selection in *Aedes aegypti*: Background and Applications for Reproductive Control

#### *Aedes aegypti*: a globally significant disease vector

The Yellow Fever mosquito, *Aedes aegypti*, is the primary vector of the viruses that cause yellow fever, dengue, Zika and chikungunya. Dengue is one of the fastest growing human infectious diseases (Figure 1.1), with an estimated 100 million dengue cases in 2017 alone, a dramatic increase from the 23 million infections reported in 1990<sup>1</sup>.



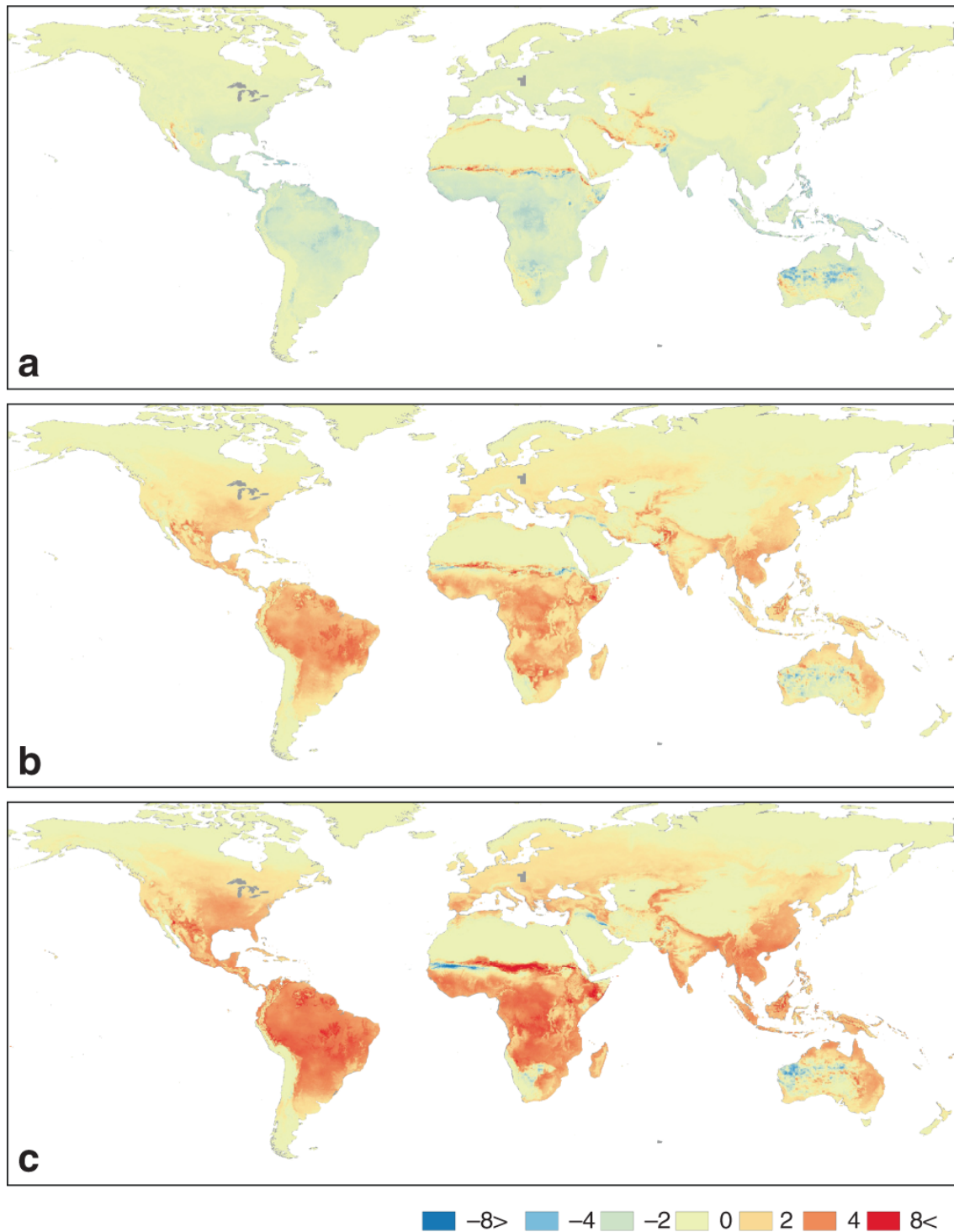
**Figure 1.1.** The rise in the global number of areas reporting (a) *Aedes aegypti* and (b) dengue. Adapted from Brady and Hay<sup>2</sup>.

The Yellow Fever mosquito, officially termed *Ae. aegypti aegypti*, is a “domestic” subspecies of *Ae. aegypti* with a preference for feeding on and living around humans, which is thought to have evolved as a result of the extended dry seasons in

the West African Sahel around 5000 years ago<sup>3,4</sup>. In such conditions the scant availability of natural bodies of water for oviposition may have selected for egg laying in artificial containers of water stored by humans<sup>5</sup>, and adaptation to blood feeding on humans thereafter<sup>6</sup>. This newly domesticated species was then transported to the Americas during the Atlantic Slave Trade<sup>4,7</sup>, and to Asia in the 19<sup>th</sup> century. *Ae. aegypti* is now present in every human-inhabited continent on earth<sup>8</sup>.

*Ae. aegypti* owes its status as a highly efficient disease vector to its remarkable life cycle. *Ae. aegypti* is highly anthropophilic, with females feeding regularly and almost exclusively on humans<sup>9–12</sup>. Multiple blood meals increase the opportunity for both acquisition and transmission of pathogens. Frequent blood feeding leads to an extended gonotrophic cycle, in which females perform what is known as ‘skip-oviposition’, distributing eggs between multiple artificial breeding sites<sup>13</sup>. Eggs can withstand desiccation, delaying hatching until conditions are optimal (in some cases by more than a year<sup>14</sup>), which can lead to explosive outbreaks of viral diseases in urban areas. Resistance to desiccation can also lead to the transportation of eggs around the world (e.g. on ships<sup>15</sup>) and the invasion of new environments. Its close association with humans has meant that urbanisation is correlated with higher *Ae. aegypti* abundance via the provision of favourable breeding sites and thus increased disease incidence<sup>16</sup>.

The continued range expansion of this prolific disease vector, driven primarily by anthropogenic climate and land use changes, is a major concern for public health. It has been estimated that the world became ~1.5 % more suitable for *Ae. aegypti* per decade between 1950 and 2000<sup>17</sup> (Figure 1.2), with increases in temperature favouring mosquito development. Alarming, it is predicted that *Ae. aegypti* will claim an estimated 19.96 million km<sup>2</sup> of territory by 2050, putting 49.13 % of the global population at risk of arbovirus transmission<sup>8</sup>. As there is no effective treatment for most of the diseases transmitted by *Ae. aegypti*, mosquito control will prove increasingly vital in the coming decades.



**Figure 1.2. Differences in *Aedes aegypti* life cycle completion (LCC) relative to LCC in 2000s (2000–2004 average).** a) Comparison with 1950s (1950–1954 average); b) comparison with 2050s (2050–2054 average) under RCP 4.5; and c) under RCP 8.5. Decreases in LCC are shown in ‘cool colours (blue and green)’ and the increases in ‘warm’ colours (orange and red). From Iwamura et al.<sup>17</sup>

## Historical and contemporary control of *Ae. aegypti* mosquitoes

Control of *Ae. aegypti* is the main method to prevent dengue and chikungunya transmission<sup>18</sup>. Environmental management, chemical, and biological control have all been used in the fight to reduce populations of *Ae. aegypti*. Environmental management involves the modification or manipulation of the environment to minimise contact between humans and mosquitoes<sup>19</sup>. This routinely involves identification and elimination or covering of sources of standing water that *Ae. aegypti* preferentially lay eggs in, or the use of house screens. Whilst such interventions are low cost and environmentally safe, breeding sites may be hard to locate, and the effects on larval and pupal density are variable<sup>20–22</sup>.

Biological control deploys arthropods, copepods or fish predators of aquatic mosquito larvae<sup>23</sup>. These methods are also generally low cost<sup>24</sup>, but again require the location of often elusive breeding sites. Further, the benefits of disease control must be carefully weighed up against the potentially harmful ecological impact of introducing these often exotic species.

Chemical insecticides are the most widespread means of reducing *Ae. aegypti*. Due to the tendency of *Ae. aegypti* to bite humans outdoors during the day, and thus the limited utility of insecticide treated bed nets, insecticides are applied directly to *Ae. aegypti* habitats. Dichlorodiphenyltrichloroethane (DDT), an organochlorine pesticide, was used pervasively for control of both agricultural insect pests and mosquito disease vectors in the mid 20<sup>th</sup> century, eradicating *Ae. aegypti* from 19 countries<sup>25</sup>. However, DDT is environmentally persistent, it had devastating effects on wildlife, and resistance in *Aedes* mosquitoes developed quickly<sup>26,27</sup>. Currently, four main classes of insecticides are used in *Ae. aegypti* control: pyrethroids, carbamates, organophosphates, and organochlorines. Of these, pyrethroids and organophosphates are the most frequently used<sup>28–30</sup>, with organophosphates commonly used as larvicides and in space-spraying to target adults (particularly during disease outbreaks), and pyrethroids used for indoor residual treatments<sup>31</sup>. Despite their historical efficacy, resistance to pyrethroid and organophosphate insecticides, mainly due to increased detoxification or target-site resistance, has been reported in *Ae. aegypti* populations around the world<sup>18,31–33</sup>. Insecticide

resistance undoubtedly presents the greatest threat to *Ae. aegypti* control and therefore disease control, creating an urgent need for new mosquito control tools.

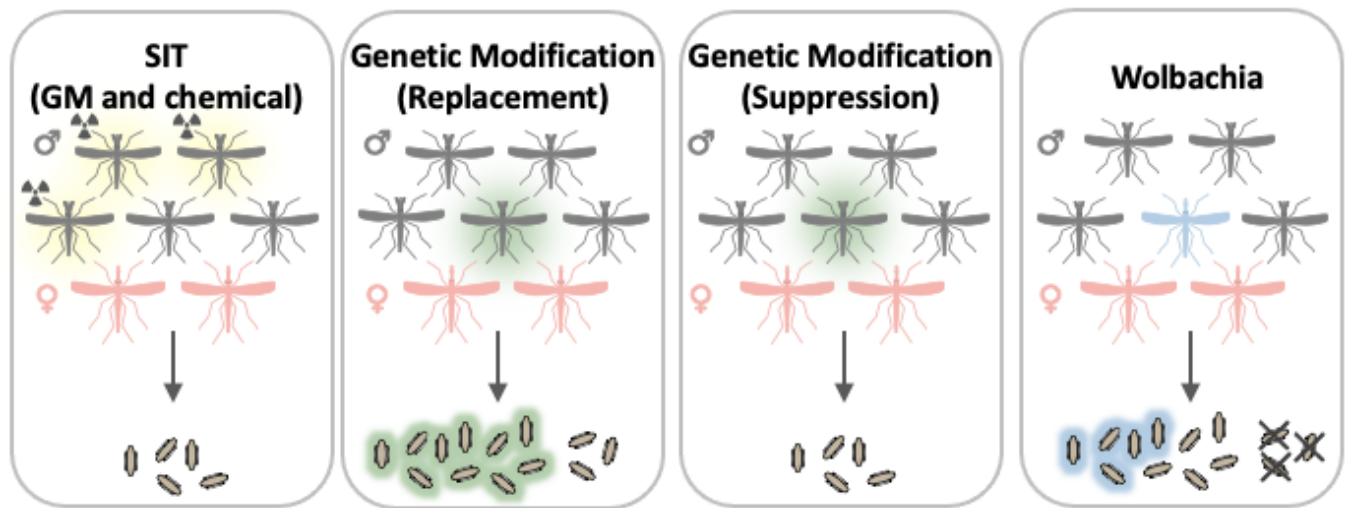
Promising new control tools have been under development in recent decades, driven by major advances in our understanding of mosquito-pathogen interactions, mosquito-associated microorganisms, sex determination and the development of sophisticated mosquito genome editing tools<sup>34</sup>. Below I describe four main categories of 'next-generation' mosquito control tools that exploit mating behaviour, also known as 'reproductive control tools', to reduce or replace wild populations (Figure 1.3).

The sterile insect technique (SIT) is a control method that aims to reduce insect pest populations by releasing sexually sterile males that mate with wild female conspecifics but produce infertile or inviable offspring<sup>35,36</sup>. SIT was famously successful in eliminating the screwworm fly, *Cochliomyia hominivorax*, from North and Central America, as well as in an outbreak in Libya in the 1980s<sup>37</sup>. Sterilisation of males is typically achieved by exposing juveniles to ionising radiation prior to release. SIT is an appealing control method since it targets specific mosquito species in confined areas and is self-limiting. Currently, the World Health Organisation (WHO) endorses SIT for mosquito control<sup>38</sup>. However, the efficacy of SIT is often limited by the significant fitness costs associated with sterilisation, shipping and release, resulting in variable success<sup>39–42</sup>. These fitness costs require the release of sterile males in huge numbers to overwhelm target populations, with initial release ratios of sterile:wild males often between 10:1 and 200:1<sup>36</sup>.

The next generation of SIT methods have sought to overcome some of these fitness costs by relying on genetic manipulation for sterilisation. The release of insects with a dominant lethal transgene (RIDL) is a recombinant DNA method that has been shown to be an effective alternative to chemical or radiation-sterilisation<sup>43</sup>. The progeny of a mating event involving a male RIDL parent mosquito will be non-viable unless the chemical repressor of the system is present, allowing transgenic individuals to be successfully reared in large numbers prior to release<sup>41,44</sup>. Commercially generated RIDL strains of *Ae. aegypti* have undergone field testing in parts of Brazil and Grand Cayman where they have been shown to reduce local populations by up to 85 %<sup>45</sup> and 80 %<sup>46</sup>, respectively.

Other *Aedes* control strategies have utilised the intracellular gram-negative bacterium *Wolbachia* to reduce wild *Ae. aegypti* populations and the pathogens that they transmit<sup>47</sup>. *Wolbachia* does not naturally infect *Ae. aegypti*, though it is the most abundant endosymbiont in arthropods<sup>48</sup>, infecting around 40 % of species<sup>49</sup>. Artificial infection of *Ae. aegypti* with *Wolbachia* can induce cytoplasmic incompatibility (CI), a phenomenon that results in embryonic death when a *Wolbachia*-infected male mates with an uninfected female<sup>50</sup>. Matings between two *Wolbachia*-infected mosquitoes, or an infected female with a non-infected male produce viable *Wolbachia*-carrying offspring, facilitating the invasion of the bacteria into a target population. *Wolbachia* is also able to reduce pathogen transmission in *Ae. aegypti*. For example, the wMelPop-CLA strain of *Wolbachia* directly influences the susceptibility of *Ae. aegypti* to pathogen infection<sup>47</sup>. Further, this same *Wolbachia* strain reduces female lifespan by half, reducing the likelihood that she will have taken an infected blood meal and undergone the extrinsic incubation period required for pathogen infectivity<sup>51</sup>. Though mass release of male *Ae. aegypti* carrying *Wolbachia* has been effective in reducing dengue transmission in some locations<sup>52,53</sup>, costs to host fitness have prevented establishment of *Wolbachia* infection in others<sup>54,55</sup>.

Gene drive technologies present an exciting opportunity to reduce the capacity of *Ae. aegypti* mosquitoes to transmit deadly pathogens (Figure 1.3). Gene drives function by pushing a genetic trait of interest to high frequencies in a population in a pattern of “super-Mendelian” inheritance<sup>56</sup>. Gene drive is possible using a range of selfish genetic elements such as meiotic drive chromosomes, transposable elements and homing endonuclease genes (HEGs)<sup>57–59</sup>, though at present, the targetable endonuclease CRISPR/Cas9 is the preferred choice for mosquitoes. In this technology a guide RNA (gRNA) directs the Cas9 endonuclease to bind to and cut a sequence-specific target site in the chromosome. This break in the DNA is repaired by the host cells by homology-directed repair which results in the copying of the drive cassette into the cut site on the wild-type chromosome. CRISPR-based gene drives in principle have the potential to spread anti-pathogen effector genes (e.g. against dengue<sup>60,61</sup>) through *Ae. aegypti* populations at transmission rates of up to 94 %<sup>62</sup>.



**Figure 1.3. Illustration of the main categories of mosquito reproductive control methods.** Modified male mosquitoes are either sterile, harbour endosymbiotic bacteria, or express genetic modifications that result in non-viable offspring or resistance to pathogens. The successful mating of laboratory-reared male mosquitoes with wild-type females is central to each of these four control strategies. Adapted from Cator, Wyer and Harrington<sup>63</sup>.

Gene drives also have the potential to be used for the suppression of wild mosquito populations (Figure 1.3), for example by spreading mutations that interfere with female reproduction<sup>64</sup>. A recent study in the malaria vector mosquito *Anopheles gambiae* demonstrated that a CRISPR-Cas9 gene drive targeting the gene *doublesex* gave rise to homozygous females that were not only sterile but could not bite, whilst heterozygous females and males were fertile, spreading the drive until total population collapse<sup>65</sup>. As yet, no studies of gene drives for either population suppression or population replacement have been carried out in the field, since there are still major scientific, ethical and logistical issues that need to be addressed<sup>66–68</sup>.

Ultimately, regardless of the mechanism, all of these aforementioned ‘next-generation’ reproductive control tools rely on the release of modified, laboratory-reared, male mosquitoes, and their success is contingent on the ability of these males to locate and successfully mate with their wild-type counterparts to spread sterility or pathogen resistance<sup>69,70</sup>. Despite the dependence of reproductive control tools on the mating ability of modified males, astonishingly little is known about male mating behaviour. This lack of understanding of male mating biology was the downfall of early attempts to use SIT to control wild mosquito populations in the 1960s and 1970s<sup>71</sup>, as the mating ability of released males was severely

compromised<sup>71</sup>. This is a cautionary tale to new methods of mosquito control, particularly since both mass-rearing and the modification of the genome or microbiome can affect the mating success of released male mosquitoes<sup>72–74</sup>. An understanding of male mating behaviour will therefore be instrumentally necessary for the successful and efficient implementation of interventions that rely on the mass-release of modified male mosquitoes. Furthermore, identifying the determinants of male mating success could lead to the development of novel control measures and sampling methods<sup>75</sup>.

Sexual selection theory can help us to understand not only the types of traits that increase a male's competitiveness to acquire mates, but how and why they evolve. In the next section I use a sexual selection framework to describe the current understanding of key components of the *Ae. aegypti* mating system and the evolutionary mechanisms that underly them (Figure 1.4). Furthermore, I highlight the relevance of specific components of the mating system for reproductive control, as well as the major gaps in the current literature. While my primary focus is *Ae. aegypti*, I also reference other mosquito species of medical significance when appropriate.

## **Sexual selection**

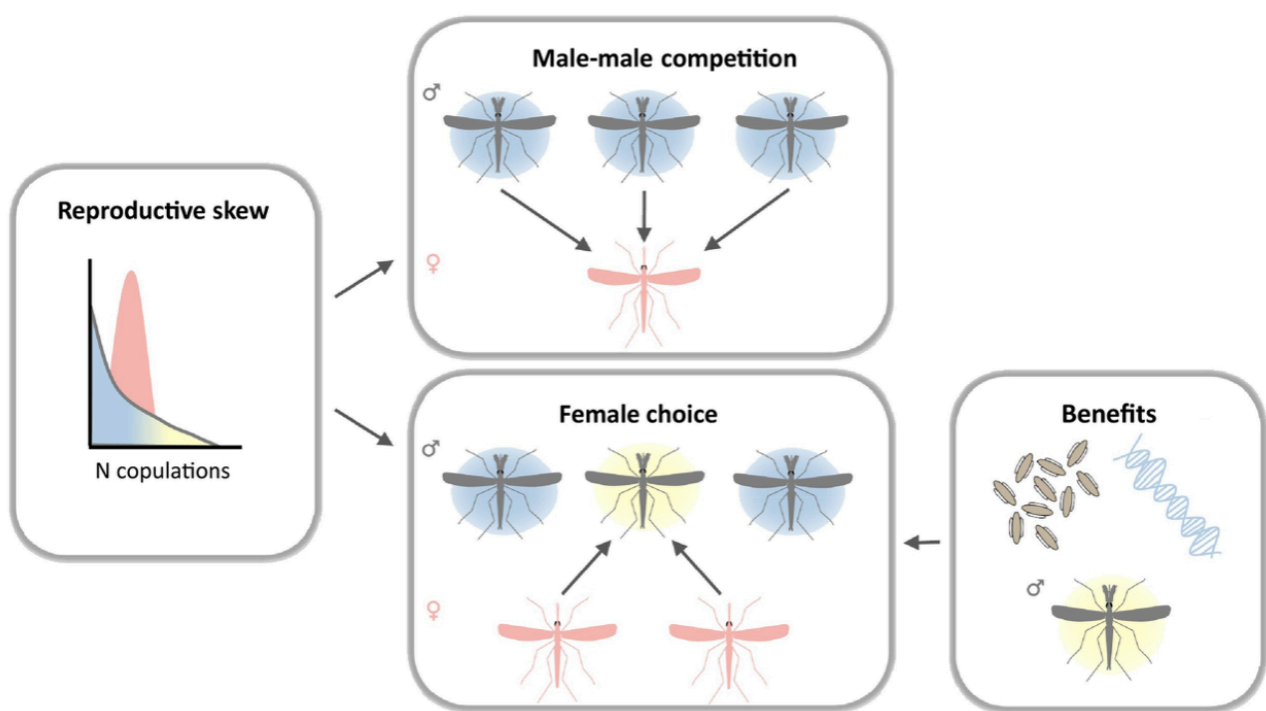
Sexual selection, first described by Darwin in 1871<sup>76</sup>, is defined as any selection due to fitness differences associated with variation among individuals in traits that affect success in competition over mates and fertilisations<sup>77</sup>. Sexual selection has led to the evolution of some of the most remarkable traits in the animal kingdom. Sexual selection in the form of male competition for access to mates can result in a great diversity of elaborate weaponry<sup>78</sup>, such as giant elk antlers or stag beetle horns<sup>79</sup>. Other sexually selected traits, such as the bright plumage of male birds of paradise, have evolved to attract choosy females<sup>80</sup>.

## **Mosquito mating systems**

In the three most medically important genera, *Anopheles*, *Aedes* and *Culex*, mating occurs in swarms<sup>81</sup>. “Swarming” describes the initiation of male pre-copulatory

behaviours and species-specific flight patterns associated with mating. Thus, a single mosquito can be said to be swarming<sup>82</sup>. When many individuals initiate swarming behaviour at the same time, they form an aggregation referred to as a swarm. Among swarming mosquito species, there is a great diversity of aggregation sizes, swarm locations, and circadian patterns<sup>83–86</sup>. *Ae. aegypti* typically form swarms with dozens of males around a human host during peak female blood-feeding activity<sup>84,87</sup>, whereas *Anopheles freeborni* have been reported to swarm in their thousands over rice fields at dusk<sup>83</sup>.

Male and females generally encounter one another in the context of these swarms. The entire mating interaction occurs in mid-air, usually lasts less than a minute, and typically ends in either successful synchronised mating flight or swift and effective rejection by the female<sup>81,88</sup>.



**Figure 1.4. Illustration of the key components of mosquito mating systems.**

Reproductive skew, a measure of the proportion of individuals of each sex that breed in a group, sets up the conditions for male-male competition and female choice to be active. Males attempt to locate and orient to females by scramble competition, and females choose males by mediating their rejection behaviours. Sexual selection models predict that females receive direct or indirect benefits by discriminating between mates. Adapted from Cator, Wyer and Harrington<sup>63</sup>.

## Reproductive Skew

Reproductive skew is one of the most commonly used descriptors of animal mating systems<sup>89</sup>. In this case, classification depends on knowing the number of copulatory partners per individual. Reproductive skew can be quantified directly by measuring the statistical variance in reproductive success within a sample of males and females<sup>89</sup>. In monogamous species, variance is expected to be low for both males and females, with all males having a roughly equal likelihood of mating. In polygynous systems, variance in male reproductive success is high, with the minority of males achieving the majority of matings, and low variance in female reproductive success (Figure 1.4).

There are several reasons to categorise mosquito mating systems as polygynous. Firstly, the operational sex ratio (OSR) is skewed towards males<sup>83,84,86,90–93</sup>. A male biased OSR means that within the mating swarm the number of sexually competing males outnumbers that of sexually receptive females. The other two lines of evidence are related to reproductive physiology. Males have the ability to inseminate multiple females over their lifetime<sup>94,95</sup> and even consecutively within a swarming period<sup>96,97</sup>. Studies in *Ae. aegypti* have reported a mean insemination capacity of 3.50 (SE  $\pm$  0.070)<sup>96</sup> to 17.60 females<sup>98</sup>, though these values were obtained from experiments of different lengths and using different methods of providing females<sup>63</sup>. Finally, females generally only mate once<sup>99</sup>, and are able to store sufficient sperm from a single insemination to last their entire lifetime. Indeed, in the three main disease vector genera, *Anopheles*, *Aedes* and *Culex*, the receipt of seminal fluid molecules from conspecifics results in refractoriness towards subsequent mating attempts. A study by Degner and Harrington<sup>100</sup> showed that the receptivity of *Ae. aegypti* females to second mating attempts decreases with time after the first mating event, with just 3 % of females remating after 20 hours. Further work has demonstrated that the female refractory state can be induced with very small amounts of seminal fluid ( $\geq$  0.031 male-equivalents) and that their effects are long-lasting (up to 34 days after injection)<sup>101</sup>. Many *Anopheles* mosquitoes have an additional physical barrier to remating, with a mating plug produced and transferred by males into the female reproductive tract during mating<sup>102,103</sup>.

Fewer data are available on female multiple mating rates in field populations. However, the available data from small field studies or semi-field cage studies indicate that a low proportion of females mate more than once. Tripet et al.<sup>104</sup> reported 1.7 % polyandry within forms of *An. gambiae* from a sample of 179 field collected females. In large field cages with stable isotope-labelled male *Ae. aegypti*, Helinski et al.<sup>105</sup> found re-mating rates of up to 14 % over multiple seasons in Mexico. In a small field study, Richardson et al.<sup>106</sup> also found limited evidence for polyandry in *Ae. aegypti*, with just 6.25 % of family groups showing evidence of multiple paternity. Boyer et al.<sup>107</sup> found evidence of polyandry in a small field study of *Ae. albopictus* where 25.9 % of females (of a total n = 27) had offspring of mixed paternal genotypes.

### **Evidence for male competition**

In a system where males outnumber females in the mating arena, and where males can mate multiply and females generally only mate once, it is likely that males compete for access to females. Males have rarely been reported interfering with one another or defending territories during swarms, so there is little evidence to support contest competition, when one competitor actively and negatively influences another competitor's access to a resource<sup>108</sup>. Instead, males likely engage in scramble competition, competing to locate and orient to females in the swarm<sup>109</sup>. Males locate females via their flight tones, responding to pure tones between 200 and 800 Hz in a behaviour known as phonotaxis<sup>110,111</sup>. Strong selection on males to locate females within the swarm is supported by the highly specialised and sexually dimorphic auditory system that enables the phonotactic response<sup>112,113</sup>. Not only are male and female antennal flagella tuned to different frequencies, but the Johnston's organ, a mechanosensitive structure in the antenna, has twice the number of auditory neurons in males than in females<sup>114</sup>. There is also evidence to suggest *Ae. aegypti* males respond to the flight tones of other males within the swarm<sup>115</sup>.

Male competitive ability is essential for reproductive control. Though it will not guarantee mating success, enhanced competitive performance would conceivably increase male mating opportunities.

## Evidence for female choice

Mate choice is the process by which females tend to mate non-randomly according to variation in traits of the opposite sex<sup>116</sup>. Until only relatively recently it was assumed that females did not show any selective behaviour towards potential mates<sup>81</sup>. However, detailed observations of female behaviour within mating swarms has revealed an array of rejection behaviours such as kicks, thrusts and abdominal tilts, strong indicators of female choice influencing mating outcomes<sup>88,117,118</sup>. In a recent laboratory study, Aldersley and Cator<sup>88</sup> used high-speed videography to show that males that received fewer kicks from females were more likely to form a successful copula, suggesting that the outcome of male-female mating interactions are contingent on female rejection responses.

The presence of female choice in *Ae. aegypti* raises the question: why are some males preferred over others? Evolutionary theories of female choice state that females can gain direct or indirect benefits from their choice of mate. The direct benefits model states that females receive immediate and tangible benefits as a result of mate choice, such as valuable resources or parental care<sup>119</sup>. In *Ae. aegypti* mating systems, and in mosquito mating systems in general, males do not appear to offer direct benefits to females<sup>117,120–123</sup>. Though there is evidence to suggest that components of *Ae. aegypti* male ejaculate confer survival benefits to the females<sup>121</sup>, it is not known if females select on variation in this trait.

Since females appear to visit swarms of males purely for mating, and males do not provide females with any direct material benefits, only genes, mosquito mating swarms may be characterised as leks<sup>77,124</sup>. Within leks, mate choice is expected to have genetic or 'indirect benefits'. Models of indirect benefits take two main forms: 'good genes' models<sup>125,126</sup>, and 'sexy sons' or Fisherian models<sup>119</sup>. In good genes models, choosy females select on variation in male traits linked to inherent genetic quality and are compensated by the increased fitness of their offspring. The sexy son hypothesis, however, states that females benefit from choosing to mate with males whose genes will confer increased attractiveness to their sons.

Studies of indirect benefits models of sexual selection in *Ae. aegypti* are limited. One study by Reitmayer et al.<sup>127</sup> showed that harmonic convergence of wing beat

frequencies in mating pairs correlates with increased immune function in offspring, supporting the 'good genes' model. However, a recent study by League et al.<sup>123</sup> did not report any positive correlation between the acoustic signals of mating pairs and the reproductive fitness of their offspring, suggesting that harmonic convergence is not a signal of 'good genes' in *Ae. aegypti*.

Further, in contrast to previous work by Cator and Harrington<sup>117</sup> that reported that male offspring of parents that harmonically converge were themselves more likely to harmonically converge and mate successfully, suggesting a Fisherian model of sexual selection, League et al.<sup>123</sup> showed that the male offspring of pairs that converged were no more likely to converge than male offspring of non-converging pairs.

Importantly, all these studies measured indirect benefits as a result of a single harmonic convergence event, and it is not clear how repeatable this measure is. Further, it is unclear whether females directly select on harmonic convergence ability in males, or whether this trait merely correlates with mating success. A better understanding of the targets of female preference and the evolutionary benefits of choice has clear applied benefits for reproductive control programmes, since those released modified males that are more attractive to females are more likely to spread their desirable genes or endosymbionts into the wild population.

## **Knowledge gaps**

Whilst recent work has highlighted the importance of male mating biology for the successful implementation of reproductive control strategies and the development of novel control methods<sup>63,93,128–130</sup>, there are still large gaps in our knowledge. Firstly, there is no consensus on what defines male mating success in mosquitoes, nor is there a single metric for it. Many studies use insemination capacity as a measure of mating success, which serves as only a proxy given that it does not incorporate the mechanisms of sexual selection (i.e. female choice and mate competition) described above. Harmonic convergence has also been used as a proxy for male mating success though, as discussed above, whether females actively select on variation in male convergence ability has not been established. Different approaches to

measuring mating success can yield very different outcomes<sup>131</sup>; establishing a method for appropriately defining and measuring variation in male mating success will not only increase our collective understanding of mating behaviour, but will have applied benefits for the development and testing of mass-reared mosquitoes.

Another major knowledge gap is the genetic basis of male mating success. In the absence of any evidence for direct benefits, female choice over mating partners suggests the presence of indirect benefits. Indirect benefits are defined by genetic correlations between male traits and female preference. Recent work demonstrating that male mating success in *Ae. aegypti* can evolve in just five generations indicates that it has a genetic basis<sup>132</sup>, yet the extent to which variation in male mating success is explained by genetic variation is not known. An understanding of the genetic basis of male mating success would benefit our understanding of how sexually selected traits evolve, and how they could be maintained in mass-rearing methods.

Finally, it is not known which male traits are the targets of sexual selection in *Ae. aegypti*. Female mate choice is often based on the assessment of multiple male traits<sup>133</sup>, and a variety of traits have been correlated with measures of male mating success in *Ae. aegypti*: body size<sup>134,135</sup>, age<sup>136</sup>, acoustic signalling<sup>117,134</sup> and pheromones<sup>137</sup> have all been suggested to be involved. However, these traits have been merely correlated with mating success (or proxies thereof), and the trait itself is rarely manipulated. Identification of the traits that are responsible for male mating success would not only help to optimise mass-rearing methods but could also generate new targets for reproductive control of *Ae. aegypti*.

## **Thesis outline and aims**

The aim of this PhD research was to characterise male mating success in the important global disease vector *Ae. aegypti*, with a specific focus on the genetic basis of this trait.

The research followed the format of three studies. Each study was designed to address a specific set of objectives as outlined below. This thesis follows the same format, with each study written up as a separate chapter.

**Aim 1: Quantify variation and heritability of the male mating success phenotype (Chapter 2)**

Objective 1: Design a new assay to determine male mating success in *Ae. aegypti* that incorporates the modes of sexual selection that are relevant for this mating system.

Objective 2: Quantify the skew in male mating success.

Objective 3: Use a full-sib breeding design to determine the total contribution of genetic components to the male mating success phenotype.

Objective 4: Use a parent-offspring regression to determine the proportion of additive genetic variance that contributes to variation in the male mating success phenotype.

**Aim 2: Identify a signature of sexual selection in the *Ae. aegypti* genome (Chapter 3)**

Objective 1: Identify patterns of genetic variation between populations of *Ae. aegypti* that had evolved with and without sexual selection with respect to the field-derived ancestral population.

Objective 2: Identify genomic regions that underwent large and consistent changes in allele frequencies between populations evolved in the presence and absence of sexual selection.

Objective 3: Identify potentially causal variants putatively under sexual selection that have functional effect on amino acid sequence.

Objective 4: Determine the biological function of areas of the genome that appear to respond to sexual selection.

**Aim 3: Characterise the behavioural phenotype of high-confidence gene candidates putatively under sexual selection (Chapter 4)**

Objective 1: Use publicly available transcriptomic data to identify candidates suitable for transient gene silencing by RNAi in adult male *Ae. aegypti*.

Objective 2: Design and perform RNAi-mediated gene silencing on target genes, quantifying the knockdown in gene expression and subsequent mosquito survival.

Objective 3: Determine the effect of transient gene silencing on the male mating phenotype.

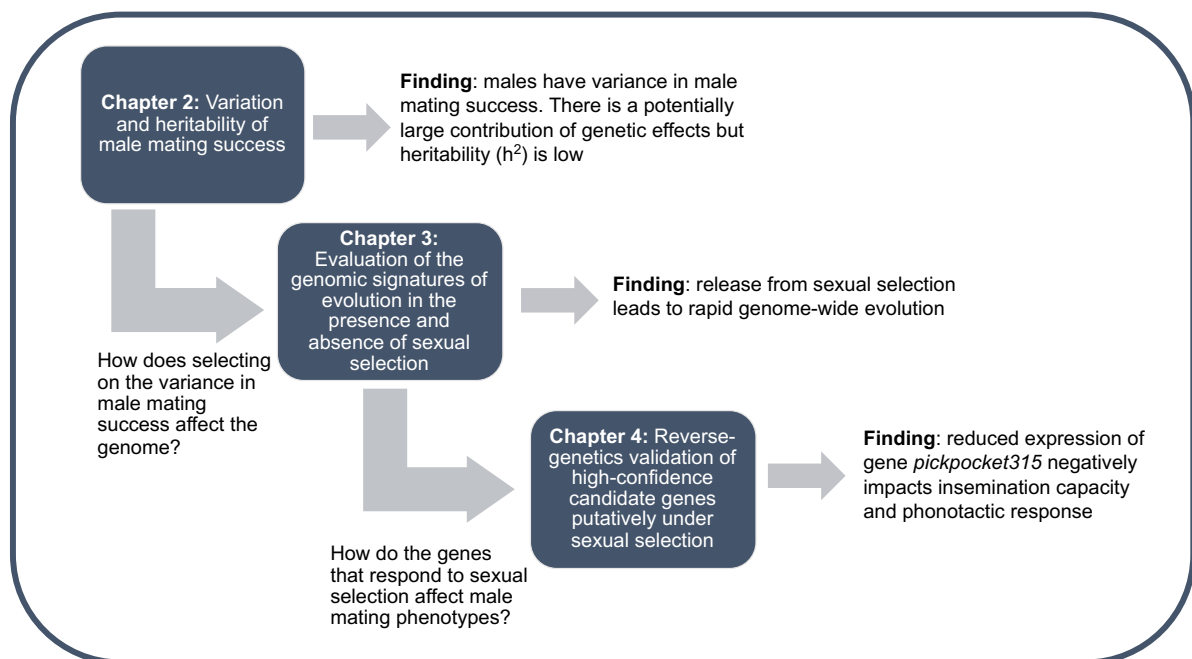


Figure 1.5. Roadmap of thesis data chapters with key finding for each chapter.

## Chapter 2

### Variation and Heritability of Male Mating Success in *Aedes aegypti*

#### Abstract

Mosquito reproductive control programmes exploit mating behaviour to introduce sterility or pathogen resistance into wild populations. The effectiveness of these programmes is contingent on the laboratory-reared mosquitoes' capacity to compete against wild males to locate and mate with wild conspecific females. However, what proportion of males that mate, the number of females that they mate with, and how much of this variance can be explained by genetics, is unknown. To address this gap, I developed a novel assay that assessed individual male mating success in *Aedes aegypti*, a medically important mosquito species, while incorporating the modes of sexual selection characteristic of *Ae. aegypti* mating swarms. I compared the resemblance of male mating success between fathers and sons and between full siblings to estimate the heritability of the trait in the narrow ( $h^2$ ) and broad ( $H^2$ ) sense, respectively. The results of this study revealed that the distribution of male mating success is highly skewed, with most males remaining unmated and very few males mating with multiple females. There is some evidence for a genetic basis to the variation in the male mating success phenotype, with a low, non-significant proportion of phenotypic variation attributed to additive genetic variation. These findings enhance our understanding of male mosquito mating biology and have important implications for mass-release programmes that rely on the release of competitive males.

#### Introduction

*Aedes aegypti* is the mosquito vector of several viruses of global health importance, such as yellow fever, chikungunya, dengue, Zika, and emerging zoonotic diseases such as Rift Valley fever<sup>138</sup>. There are estimated to be around 390 million dengue infections alone every year<sup>139</sup>. *Aedes* mosquitoes and the viral pathogens that they transmit are primarily controlled with the use of chemical insecticides. However,

resistance of *Aedes* mosquitoes to the main classes of chemical insecticides is widespread<sup>140,141</sup>. The urgent need for novel control strategies has led to the development of some promising new mosquito control interventions.

Mating behaviour is a tantalising target for new mosquito control methods. Not only can mating swarms be targeted with traps or novel insecticides, but the release of laboratory-reared males can introduce pathogen resistance (to reduce the prevalence of disease in the mosquito population) or sterility (to reduce the abundance of mosquitoes) into wild populations in a strategy termed ‘reproductive control’. The success of these reproductive control tools is critically dependent on the ability of laboratory-reared males to compete with wild males and successfully mate with wild females<sup>63</sup> (see also **Chapter 1**). A thorough understanding of mosquito mating behaviour and the traits important for male mating success are essential in delivering and optimising these control tools, as well as developing new tools<sup>63</sup>. There are several key gaps in our fundamental understanding of *Ae. aegypti* mating behaviour, many of which centre around the determinants of male mating success.

*Ae. aegypti* mosquitoes form small aerial swarms in the vicinity of potential blood-meal hosts<sup>120,142</sup>. Mating occurs mid-flight and lasts just a few seconds<sup>81,84,110</sup>. While males outnumber females in the swarm and have the capacity to mate multiple times, data suggest that females are largely monandrous<sup>100,106</sup>. Thus, the operational sex ratio (OSR) is skewed towards males. Within swarms, males compete for access to females<sup>143</sup>, but it appears females ultimately choose which male to mate with, displaying decisive rejection behaviours such as kicking and abdominal tilts<sup>88,117,144</sup> (see **Chapter 1** for a comprehensive discussion of male competition and female choice in mosquito mating systems).

While high variance in male mating success is expected in polygynous mating systems<sup>77</sup>, experimental evidence for variance in mosquito male mating success is lacking. Considering the ratio of males to females in swarms we would expect that a given male has a low chance of mating and an even lower chance of mating more than once. This seems to be the case in other swarming mosquito species; a single study of wild *Anopheles gambiae* swarms estimated that 7.2 % ( $\pm 0.95$  % SE) of males in a swarm were mated each night<sup>90</sup>. While it is assumed that the minority of

males within a swarm contribute to the majority of matings<sup>63</sup>, this skew has never been empirically demonstrated in *Ae. aegypti*.

The absence of reliable estimates of male mating success are likely due to the difficulty in tracking successful mating attempts within the swarm. Instead, variance in male mating success is most commonly determined using single reproductive traits as proxies, such as insemination capacity<sup>145</sup> and sperm production and transfer<sup>136,146</sup>. However, the relationship between these proxies and male mating success is unclear<sup>63</sup>. These traits are unlikely to be representative of overall male attractiveness to females given that they are not subject to pre-copulatory sexual selection pressures, particularly given the low levels of polyandry reported for this species<sup>105,106</sup>. Further, in many taxa male mating success is based on multiple cues<sup>124,133</sup>, and making inferences about the benefits of female choice based on individual traits can introduce substantial bias<sup>147</sup>. Prokop and Drobniak argue that “true” attractiveness can only be determined by scoring female responses to potential mates<sup>147</sup>.

Male mating success itself can be determined by measuring either the number of offspring produced by each male (genetic mating success) or the number of matings each male achieves (copulatory mating success). Genetic mating success is more often used in the field where matings are not observed, but mating success can be inferred from sampling the population<sup>148</sup>. The main drawback of this method is it is influenced by maternal effects and would not capture every mating event that did not result in offspring. Copulatory mating success can be measured by the number of copulation partners or the total number of copulations. Female *Ae. aegypti* mosquitoes generally only mate once<sup>149</sup>, so the number of copulation partners a male has is a more appropriate measure here. Copulatory mating success would previously have required researchers to observe individual mating interactions, a laborious and low-throughput endeavour. However, molecular techniques have enabled the fluorescent tagging of tissue-specific genes and has led to the generation of transgenic mosquito lines with fluorescent testes and sperm<sup>150</sup>. Detection of fluorescent sperm in the female spermathecae, the sperm storage organs, is indicative of a successful mating. These tools can be used to accurately determine male mate identity in free-flying swarms<sup>131</sup> and therefore variance in copulatory mating success with greater certainty and on a larger scale.

As with any phenotypic trait, variation in male mating success arises as a product of both environmental and genetic variation<sup>151,152</sup>. The extent to which variation in a trait is determined by environmental or genetic factors is a central question in evolutionary biology. Several studies have sought to characterise the relationship between environment and male mating success (or proxies thereof) in mosquitoes. Many of these studies have focused on either larval nutrient availability<sup>131,134,153,154</sup> or larval density<sup>155</sup>. Larval rearing conditions are frequently-studied environmental factors due to their impact on life history traits in adult stages<sup>156–158</sup>, with larval diet consistently demonstrated to affect adult size in *Ae. aegypti*<sup>131,159–162</sup> and other mosquito species<sup>163–165</sup>.

Laboratory studies of *Ae. aegypti* have demonstrated that larger males produce<sup>146</sup> and transfer<sup>136</sup> more sperm than smaller males, indicating a greater reproductive potential. However, increased body size (as generated by manipulation of larval diet) was not found to translate to greater success in tethered one-on-one precopulatory mating interactions<sup>134</sup>, or to greater mating success in competition, despite increased swarm participation<sup>153</sup>. Similarly, Aldersley et al.<sup>131</sup> showed no effect of larval diet on mating outcome in laboratory or semi-field competition experiments of *Ae. aegypti*. These studies suggest that high nutrient availability, and therefore body size, does not simply translate to increased mating success. This not only begs the question of what other environmental variables might be influencing mating behaviour, but also the role of genetic variation in the male mating success phenotype.

The role that genetic factors play in the male mating success phenotype has only recently been assessed. Studies have investigated the inherent negative effects of laboratory colonisation and rearing conditions on genetic diversity and mating success<sup>166–170</sup>. Ross et al.<sup>166</sup> documented inbreeding depression in *Ae. aegypti* colonies held at low census sizes ( $\leq 100$  individuals) due to severely reduced effective population sizes ( $N_e$ ). Ross et al.<sup>166</sup> also reported that a high generation inbred colony showed a lower mating success compared to relatively low generation lines, most likely due to the erosion of genetic diversity. A recent experimental evolution study in *Ae. aegypti* highlighted the importance of sexual selection on male mating success. Qureshi et al.<sup>132</sup> reported that males evolved in the presence of sexual selection demonstrated increased mating success compared to males evolved in the absence of sexual selection, indicating a heritable genetic basis to

mating success, since the response to selection is determined by the heritability of the trait<sup>152</sup>. Cator and Harrington<sup>117</sup> reported that the male offspring of pairs that participated in an acoustic duet prior to mating were able to outcompete the male offspring of pairs that did not perform acoustic signalling. This suggested that females may be assessing potential male mates' heritable genetic quality. However, a more recent study found no evidence that female mate choice based on acoustic signals conferred genetic benefits to females or their offspring<sup>123</sup>.

Heritability is the measure of the proportion of a phenotypic trait that is due to genetic components<sup>152</sup>. Quantitative genetics methods can be used to estimate heritability and reveal the relative contributions of environmental and genetic factors to *Ae. aegypti* male mating success<sup>152</sup>. Phenotypic variance ( $V_P$ ) is expressed as the sum of genetic variance ( $V_G$ ) and environmental variance ( $V_E$ ):

$$V_P = V_G + V_E$$

(Eqn. 1)

If a large proportion of phenotypic variance is explained by environmental variation, for example, then a small proportion of the phenotypic variance is explained by genetic variation, and vice versa. Heritability in the broad sense ( $H^2$ ) can be expressed as the proportion of phenotypic variance ( $V_P$ ) that has a genetic component:

$$H^2 = \frac{V_G}{V_P} \text{ or } \frac{V_G}{V_G + V_E}$$

(Eqn. 2)

Broad sense heritability describes the extent to which phenotypes are determined by genotypes. A  $H^2$  value of 0 indicates that none of the variation in the phenotype of

the study population is determined by genetics, and a  $H^2$  value of 1 means that all the variation in the phenotype of the study population is determined by genetics.

Heritability in the narrow sense ( $h^2$ ) is the extent to which phenotypes are determined by the additive effects of genes transmitted from parents to offspring.  $h^2$  can be determined by breaking  $V_G$  down into its constituent parts: additive variance ( $V_A$ ), dominance variance ( $V_D$ ) and interaction variance ( $V_I$ ). Additive genetic variance is the additive effect of alleles. It is the component of genetic variation that is responsible for resemblance between parents and offspring and it is the basis for the response to selection<sup>152</sup>. Dominance variance is due to interactions between alternative alleles at a specific locus. Interaction variance is due to interactions between alleles at different loci.

Narrow-sense heritability ( $h^2$ ) can be expressed as the proportion of the phenotypic variance that has an additive genetic component:

$$h^2 = \frac{V_A}{V_P} \text{ or } \frac{V_A}{V_G + V_E}$$

(Eqn. 3<sup>152</sup>)

In this study I aimed to quantify the variance in a measure of male mating success ( $V_P$ ) from a recently field-derived strain of *Ae. aegypti* in competitive serial mating assays. These experiments utilised transgenic mosquitoes with fluorescent protein-tagged sperm to determine male mate identity when examining mated females, allowing free-flight mating behaviour<sup>150</sup>. I used this strain to track precopulatory competitive male mating success over multiple days. By performing experiments treating overall male mating success as the phenotype of interest and allowing free-flight females to choose their mates, the data encompass all pre-copulatory targets of sexual selection. I then assessed the heritability of the trait in both the narrow-sense ( $h^2$ ) and broad-sense ( $H^2$ ) by performing paired measures of male mating success in fathers and offspring, and of full-sib families, respectively. Since polygamous mating systems with male-biased operational sex ratios are typically characterised by a reproductive skew, I hypothesised that males would vary in

competitive mating success. I also hypothesised that the mating success of fathers would significantly predict the mating success of their sons, and that males within full-sib families would more closely resemble each other in terms of mating success than males from different families.

## Methods

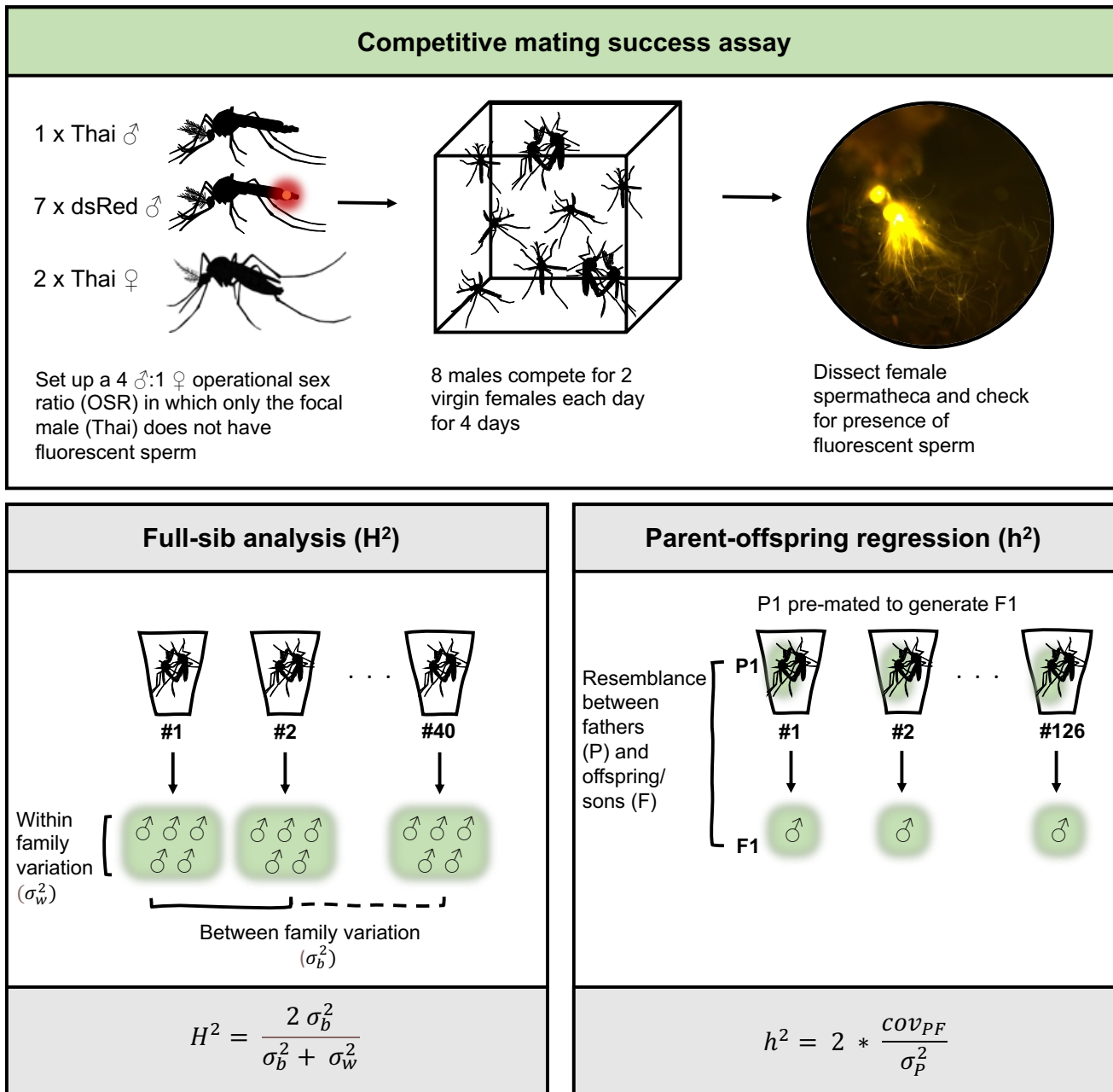
### Experimental design

Male competitive mating success was estimated using a novel assay design which incorporated the ecologically-relevant selection pressures of male mate competition and female choice. Variation and heritability in this trait were measured by estimating the resemblance between relatives using two different approaches: by comparing the competitive mating success trait within and between full-sib families ( $H^2$ ) (Figure 2.1, bottom left panel), and between father-son pairs ( $h^2$ ) (Figure 2.1, bottom right panel).

### *Aedes aegypti* lines and rearing

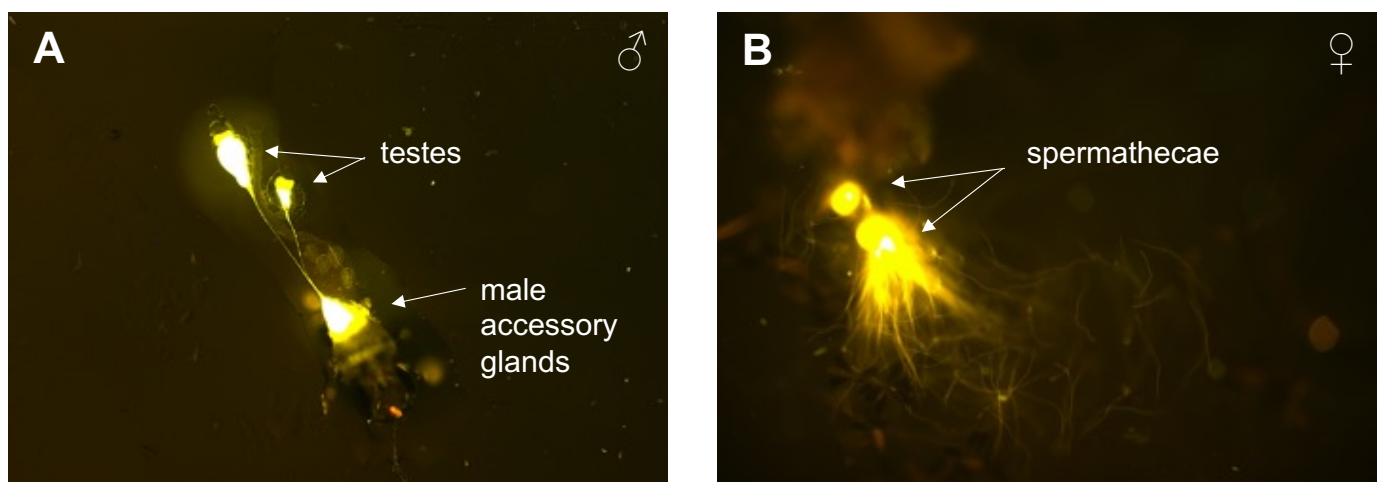
Two genetically distinct *Ae. aegypti* lines were used for this experiment. One line used was *Ae. aegypti* collected from Kamphaeng Phet Province (KPP), Thailand in 2021, which underwent just six generations in the lab (F6). The other line was a transgenic line of *Ae. aegypti* expressing two transgenes: one that labels sperm with the red fluorescent protein dsRed ( $Aa\beta 2t::DsRed$ ) expressed under a testes-specific promotor, allowing for confirmation of insemination in females mated by males of dsRed genetic background (Figure 2.2), and another that expresses green fluorescent protein (GFP) under an eye-specific promotor of larvae of both sexes ( $3xP3::GFP$ ) which serves as an integration marker<sup>150</sup>. To increase genetic similarity between the two strains, the transgenic dsRed line was backcrossed into the *Ae. aegypti* background. Briefly, around 200 homozygous males from the dsRed *Ae. aegypti* line were crossed to 200 females from the Thai *Ae. aegypti* line, the heterozygous offspring of which were screened to confirm presence of the GFP marker in the eyes larvae. Heterozygous individuals were then allowed to mate to each other, generating offspring of all three genotypes. Homozygote larvae

expressed GFP with the greatest intensity, and these individuals were selected to generate a new dsRed colony.



**Figure 2.1. Experimental design.** Schematic describing the competitive mating success assay (upper tab in green), and how it was used for estimating variation and heritability of mating success for full-sib families of males (bottom left) and parent-offspring regression of fathers and sons (bottom right). Broad-sense heritability ( $H^2$ ) is calculated in the full-sib analysis as the between-family component as a proportion of the total variance. Narrow-sense heritability ( $h^2$ ) is the covariance of offspring and parents, and  $\sigma_P^2$  is the variance of the parents. Since only one male parent is used in each family, the narrow-sense heritability is twice the slope. In both the full-sib analysis and parent-offspring regression males subjected to the competitive mating success assay are highlighted in green.

dsRed *Ae. aegypti* (hereafter dsRed) and Thai KPP (hereafter Thai) *Ae. aegypti* eggs were separately vacuum hatched for 20 minutes. The newly hatched larvae were provided with 0.1 mg of ground fish food overnight (Cichlid Gold [#04328], Hikari, Himeji City, Japan). The following day L1 larvae were separated into trays of 500 individuals in 2 L of water and provided with fish food *ad libitum*. Once pupated, both strains were sorted by sex and held in separate cages at 27 °C, 80 % relative humidity on a D12:N12 (with 30 minutes of dawn/ dusk separating each phase) circadian cycle. Adults were provided 10 % w/v sucrose *ad libitum*.



**Figure 2.2. *Ae. aegypti* dsRed phenotype.** (A) Dissected male testes and accessory glands (x 10 magnification). (B) Dissected spermathecae of an inseminated female. One spermatheca is ruptured and individual sperm are visible (x 10 magnification).

### Quantification of male competitive mating success

Male mating success was assessed using a novel assay design intended to incorporate ecologically relevant aspects of male mating behaviour (Figure 2.1, top panel), i.e. repeated measures of the same individuals and the presence of sexual selection in the form of male competition and female choice. At three days post-emergence seven virgin dsRed males were added to a 19 cm<sup>3</sup> test cage along with a focal Thai male, provided with 10 % w/v sucrose solution and allowed 24 hours to acclimate. The following day the mating competition assay began. Sucrose solution was removed, and two virgin female Thai mosquitoes were aspirated into each cage at 0930, allowing eight hours for males to compete for mating opportunities. At 1730 the two females were removed and held in a 16 oz holding cup labelled with the

same number as the test cage and provided with 10 % w/v sucrose solution. Males were provided with 10 % w/v sucrose solution and allowed to rest overnight. The following day the process of adding two virgin females was repeated for a further three days until a total of eight females had been added and removed from each test cage. After the last pair of females had been removed from the test cages, all males were collected and examined for dsRed expression in the testes and GFP expression in the eyes under a fluorescent microscope. The single non-fluorescent male was identified as the focal Thai male and stored in a 1.5 mL microcentrifuge tube at -80 °C.

The females used in the competitive mating assay were anaesthetised on wet ice and their spermathecae dissected in 1 X phosphate buffered saline (PBS) under a stereoscope and the presence or absence of sperm recorded using a brightfield microscope. Presence of sperm in the spermathecae confirmed mating status, presence of fluorescent sperm under a dsRed filter in the spermathecae confirmed insemination by a dsRed (competitor) male, presence of non-fluorescent sperm confirmed insemination by the Thai (focal) male. The number of females with non-fluorescent sperm in the spermathecae (of a possible eight) corresponded to the score of the focal male. This value was also calculated as a proportion of females that were mated over the four days, and hereafter referred to as 'competitive mating success'.

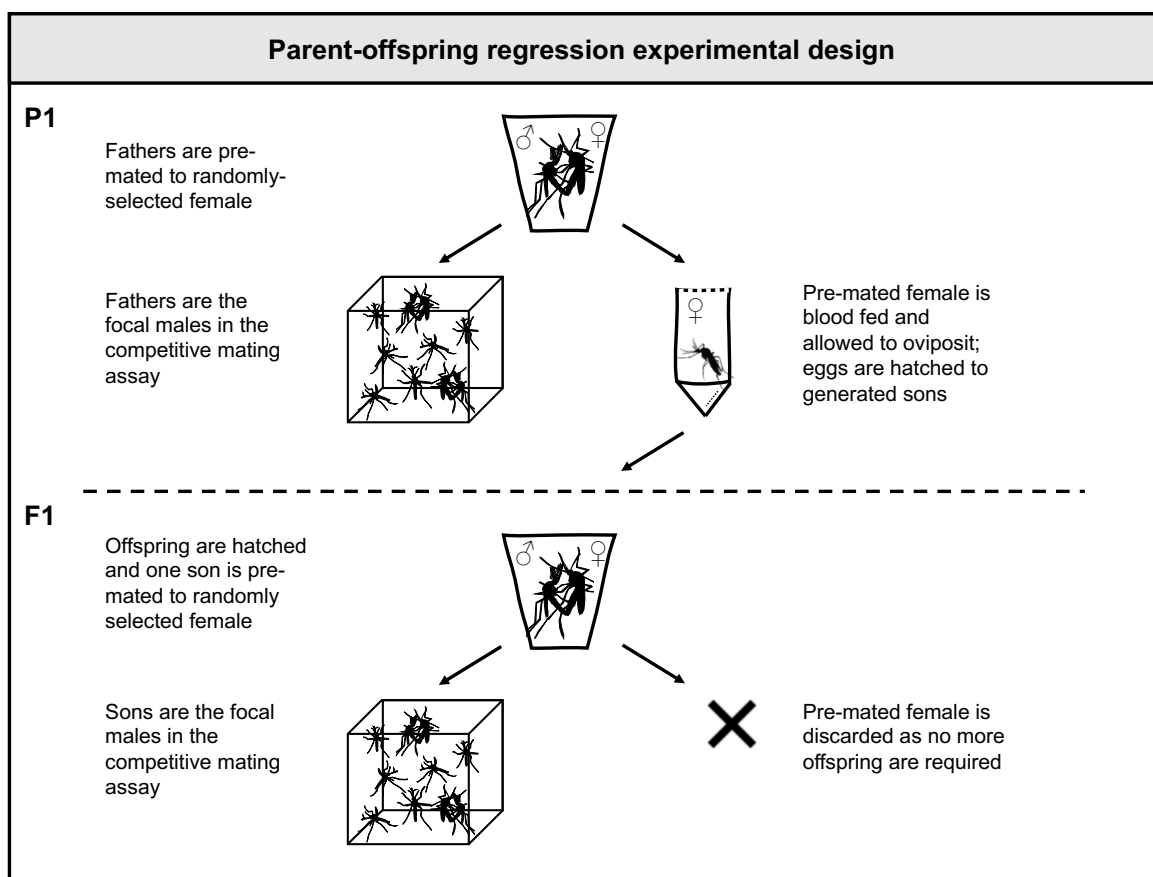
### **Full-sib breeding design**

I performed a modified full-sib breeding design adapted from a previous study in *Ae. aegypti*<sup>171</sup> to determine the broad sense heritability ( $H^2$ ) of male mating success in the Thai population of *Ae. aegypti*. A 19 cm<sup>3</sup> cage of ~75 mated, Thai females was fed on defibrinated horse blood (FirstLink, UK), and 50 visibly engorged females were aspirated into separate 50 mL centrifuge tubes with the lids modified to hold a cone of damp filter paper, on which the females deposited their eggs. The individual egg clutches were counted, dried, and stored. The offspring of those females that had laid > 30 eggs were reared as described previously to pupae, sex sorted and five males from each family kept in 16 oz holding cups and provided with 10 % w/v

sucrose *ad libitum*. In total 40 families, each consisting of five sons, were assayed using the competitive mating assay described above (and illustrated in Figure 2.1).

### Parent-offspring breeding design

Two days post-emergence single virgin focal Thai males were transferred to pre-mating cages (19 cm<sup>3</sup>) and allowed to mate two virgin Thai females. This pre-mating step was to confirm that the males were capable of mating, and to generate eggs for the F1 generation, which would also be subjected to the competitive mating assay (Figure 2.3). The following day females were removed and held in separate 16 oz cups where they were blood fed and allowed to oviposit on damp filter paper. The eggs were dried and stored as the sons from these clutches would also be assayed. Focal Thai males were then transferred to test cages for the competitive mating success assay described above.



**Figure 2.3. Schematic describing the parent-offspring regression experimental design.** Visual representation of the experimental manipulation of a single parent (P1) and offspring (F1) generation of focal Thai *Aedes aegypti* mosquitoes performed to quantify their competitive mating success. This process was repeated for 126 fathers and 126 sons in total.

To assess heritability ( $h^2$ ) of male mating success as a trait, the competitive mating success assay was repeated for the sons of the focal males. Eggs laid by one of the (randomly selected) pre-mated females were hatched and reared as described previously. Eggs were hatched in separate containers and 10 larvae/container were reared until the pupal stage when they were separated by sex. A single male offspring per father was subjected to a pre-mating to two females (though these females were subsequently discarded) and then the same mating competition assay. Females from the mating competition assay were dissected and scores assigned to each son. I measured the competitive mating success of 126 fathers and their sons in four experimental blocks.

### Statistical analysis and heritability quantifications

All statistical analyses were performed in R (version 4.0.3). Inequality (skew) in mating success was measured using Morisita's non-standardized index ( $I_\delta$ )<sup>172</sup>. The Morisita index was proposed by Wade<sup>173</sup> and Tsuji and Tsuji<sup>174</sup> to measure monopolisation of reproductive success, and has been cited as the best estimator of the potential for sexual selection<sup>175</sup>. Inequality in mating success was calculated for the P1 and F1 generation of males, and for males from all full-sib families using the following formula:

$$I_\delta = N \frac{\sum x^2 - \sum x}{(\sum x)^2 - \sum x}$$

(Eqn. 4<sup>172,174</sup>)

Where  $N$  is group size,  $\sum x$  is the sum of matings over all mating individuals, and  $\sum x^2$  is the sum of all mating counts squared.  $I_\delta = 1$  when distribution is random,  $I_\delta < 1$  based on a uniform distribution, and  $I_\delta > 1$  when distribution is skewed. The maximum possible value of  $I_\delta$  is equal to  $N$ .

Deviation from randomness can be checked by comparing the test statistic with  $\chi^2$  (d.f. =  $N - 1$ ):

$$\chi^2 = I_{\delta} \left( \sum x - 1 \right) + N - \sum x$$

(Eqn. 5 <sup>172,174</sup>)

A nested analysis of variance (ANOVA) was used to establish broad-sense heritability ( $H^2$ ) from resemblances within and between 40 full-sib families.  $H^2$  was calculated using the variance between ( $\sigma_b^2$ ) and within ( $\sigma_w^2$ ) full-sib families as:

$$H^2 = \frac{2 \sigma_b^2}{\sigma_b^2 + \sigma_w^2}$$

(Eqn. 5 <sup>152</sup>)

Narrow sense heritability ( $h^2$ ) was estimated directly from twice the slope of the parent-offspring regression of 126 father-son pairs using a linear model and the package lme4<sup>176</sup>. This analysis used the scores (as a proportion of mated females) of fathers in competition, and scores (as a proportion of mated females) of sons in competition. Additionally, the effect of mating success of fathers on that of sons as a categorical variable (mated or unmated) was assessed. The focal male was assigned 'mated' if it mated one or more females in competition. The focal male was assigned 'unmated' if no females were mated in competition. A chi-squared test was performed using the wald.test function in the aod library<sup>177</sup>.

## Results

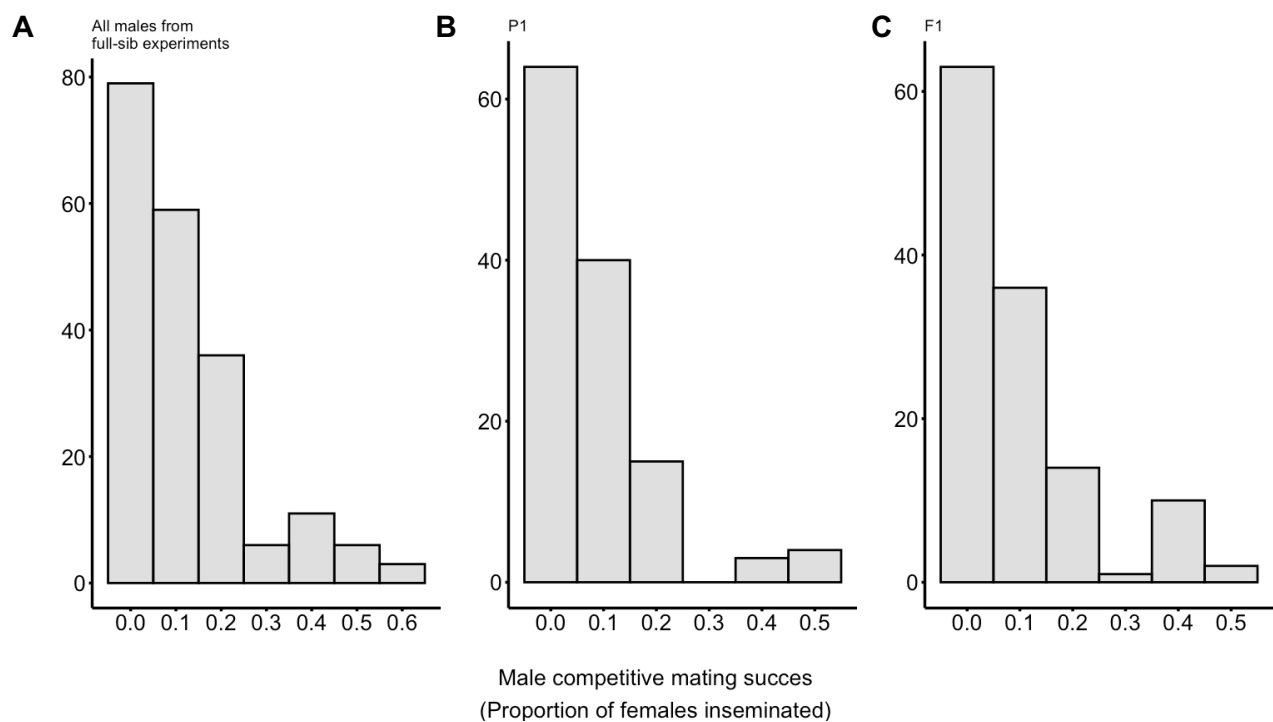
### Variation in mating success for parents and offspring

The competitive mating assay was performed on a total of 126 father-son pairs (replicate 1: n=50, replicate 2: n = 30, replicate 3: n = 29, replicate 4: n = 17).

Though males had access to up to eight virgin females, the range of the proportion of females mated was 0-0.5 for both fathers and sons. Overall, the most common outcome (the modal score) was 0; 46.83 % of fathers (n = 59) and 50 % of sons (n =

63) did not mate in competition. 34.13 % of fathers (n = 43) and 28.57 % of sons (n = 36) mated a single female, and only 19.05 % of fathers (n = 24) and 21.43 % of sons (n = 27) mated multiple females. On average focal males mated with less than one female (fathers: mean = 0.817, SD = 0.991; sons: mean = 0.825, SD = 1.028).

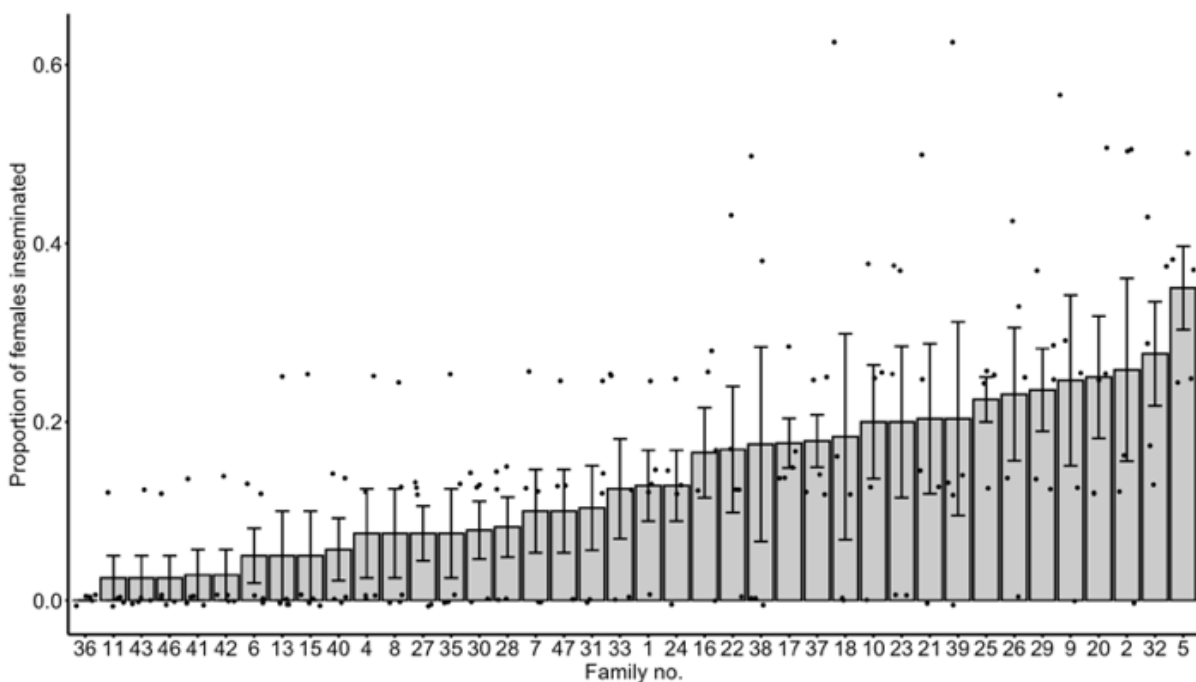
The distribution of male mating success as a proportion of available females was right-skewed, with most males not mating a single female, and very few mating multiple times (Figure 2.3A and B). The distribution of competitive mating success was similar for fathers and for sons (two-sample Kolmogorov-Smirnov test:  $D = 0.048$ ,  $p\text{-value} = 0.999$ ). Under the male-biased sex ratio of this mating assay, the Morisita index for the P1 generation (fathers) indicated a positive, but non-significant skew in mating success ( $I_\delta = 1.247$ ,  $p > 0.05$ ). The F1 generation (sons) had a positive and significant skew in mating success ( $I_\delta = 1.341$ ,  $p < 0.05$ ).



**Figure 2.4. Distribution of competitive mating success for males from parent-offspring and full-sib experiments.** (A) The distribution of competitive mating success for 200 males from 40 full-sib families. (B) The distribution of competitive mating success for 126 fathers (P1) randomly selected from a laboratory population of *Ae. aegypti* and (C) their 126 sons (F1). For all experiments competitive mating success was measured as the proportion of total females mated (of a possible eight) by the focal male.

### Variation in male mating success between full-sib families

Of the 50 mated females that took a bloodmeal, 40 survived long enough to successfully produce a clutch of eggs that resulted in five male offspring. I measured competitive mating success for five males in 40 full-sib families. The distribution of mating success for all 200 males was right-skewed (Figure 2.4C), and under the 4:1 male-biased operational sex ratio in this experiment, the Morisita index indicated a positive and significant skew in mating success ( $I_\delta = 1.219$ ,  $p < 0.05$ ). As in the P1 and F1 generations of the parent-offspring experimental design, the modal score for all males from 40 full-sib families was 0; 39.5 % of full-sib males ( $n = 79$ ) did not mate a single female, 33 % of full-sib males ( $n = 66$ ) mated a single female, and 27.5 % of full-sib males ( $n = 55$ ) mated multiple females. On average males mated with approximately one female (mean = 1.035, SD = 1.127).



**Figure 2.5. Variation in male competitive mating success.** Mean  $\pm$  SEM proportion of virgin females inseminated by focal males of  $n = 40$  *Ae. aegypti* families. Black circles represent the proportion of females inseminated by individual males ( $n = 5$ ) within each family.

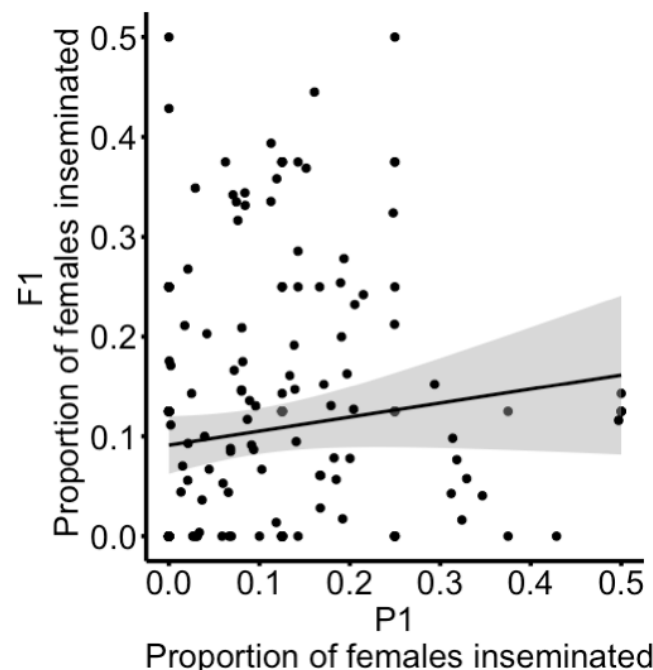
A one-way ANOVA revealed a statistically significant difference in the proportion of females mated between at least two groups ( $F(39, 160) = 2.1808$ ,  $p\text{-value} < 0.001$ ; Figure 2.5). I observed a statistically significant difference in male mating success (i.e. proportion of females inseminated) between the best and worst performing

families (two-sample  $t$ -test between family #5 and family #36:  $t = 7.4833$ ,  $df = 8$ ,  $p$ -value  $< 0.001$ ), and also between the 10 % of families from the extreme ends of the distribution of male mating success (two-sample  $t$ -test between top 10 % and bottom 10 % of families  $t = 11.21$ ,  $df = 6$ ,  $p$ -value  $< 0.001$ ).

From the full-sib analysis, I generated an estimate of broad-sense heritability ( $H^2$ ) of 0.694, suggesting that 69.4 % of the variance in competitive mating success is due to genetic differences among males.

### Relatively low contribution of additive genetic variation to mating success

In the analysis of competitive mating success for 126 father-son pairs, I observed a positive, but not significant relationship between the mating success of fathers and their sons (Figure 2.6; lm:  $R^2 = 0.017$ ;  $p$ -value = 0.141). In a father-son regression, narrow-sense heritability ( $h^2$ ) can be estimated as twice the slope of the line of regression<sup>152</sup>, giving an  $h^2$  value of 0.2, 95% CI [-0.042, 0.289]. Even when the outcome of the competitive mating success assay was converted to a binary categorical variable (i.e. where males were either 'mated' or 'unmated'), the overall



**Figure 2.6. Father-son regression analysis of competitive mating success.** Correlation between the proportion of matings achieved by fathers and the proportion of matings achieved by sons ( $R^2 = 0.017$ ;  $p$ -value = 0.141). Solid line is the linear regression of competitive mating success between fathers and sons with standard error in grey. Points have been jittered to prevent overlapping using the ggplot function `geom_jitter`.

effect of the fathers' mating success on the sons' mating success was not significant (Table 2.1:  $\chi^2 = 0.8$ ,  $df=1$ ,  $P=0.37$ ).

**Table 2.1. 2-way contingency table of fathers and sons mating statuses.**

|                  | P1 mating status |         |
|------------------|------------------|---------|
| F1 mating status | Mated            | Unmated |
| Mated            | 36               | 27      |
| Unmated          | 31               | 32      |

## Discussion

Understanding male mating success is critical to the effective deployment of reproductive control strategies. Here, I use a novel assay design with ecologically relevant levels and mechanisms of sexual selection to characterise the skew and heritability of male mating success for this recently colonised laboratory population of *Ae. aegypti*. A recent review categorised the mating systems of medically important mosquitoes as polygynous, with a male-biased operational sex ratio (OSR) predicted to generate a high degree of reproductive skew<sup>63</sup> (see also **Chapter 1**). It is this unequal mating success that suggests the potential for sexual selection. This is the first study to empirically demonstrate the degree of skew affecting a population of *Ae. aegypti* experiencing sexual selection in the form of female choice and mate competition. Under a 4:1 male-biased OSR, males from the P1, F1 and full-sib families all showed positive skew indexes ( $I_\delta = 1.247$ ,  $I_\delta = 1.341$  and  $I_\delta = 1.219$ , respectively). A positive skew indicates the unequal partitioning of competitive mating success among males in the population. However, the distribution of male mating success for the P1 group alone could not be detected as significantly deviating from randomness.

The unequal distribution of matings observed in this study begs the question: why might some males achieve a greater mating success than others? One possible explanation for this variation is that mating success correlates with male traits involved in female choice or mate competition<sup>178</sup>. It may be the case that males with

the highest mating success have increased expression of a trait that females prefer, and that provide her with direct or indirect benefits. For example, in some *Drosophila* and cricket species females choose mates based on the chemical signals that convey condition, and in doing so provide the couple's offspring with "good genes"<sup>179</sup>. Additionally, a male's competitive ability may also contribute to increased male mating success, as he must locate and orient to females within a frenzied swarm of competitors. In polygynous mating systems characterised by scramble competition, more mobile males are expected to have a greater mating success as they encounter females more frequently<sup>180</sup>. Whitlock and Agrawal<sup>181</sup> argue that overall health and condition are likely to determine the energy that males can dedicate to sexually selected traits, and may therefore affect mating success. Future work could determine if those males that achieve the greatest mating success in the presence of sexual selection are indeed in superior condition, or confer greater benefits to females, compared to those males with the least mating success.

Two main points should be taken into consideration when interpreting these skew index values. Firstly, that the skew in male mating success (and indices thereof) are sensitive to the OSR. Variance in male mating success is expected to increase with the intensity of mate competition, which is affected by the OSR<sup>182</sup>. There is substantial evidence to suggest that OSR is male biased in *Ae. aegypti* mating systems<sup>84,86,91,129</sup>, and in this assay I maintained an OSR of four males to each female. As such, the skew index values generated for this population are unlikely to reflect the skew in populations where the OSR differs from that employed in this assay.

Secondly, the skew indexes generated by the Morisita index ( $I_\delta$ ) cannot be compared with one another, or at least between the full-sib design and the P1 or F1 generations. That is because the maximum value of  $I_\delta$  under the highest possible skew is equal to the group size ( $N$ ), for example a group of 10 males could have a maximum  $I_\delta$  of 10, whereas a group of 100 males could have a maximum  $I_\delta$  of 100.

Finally, it should also be considered that there is no one standard way to measure inequality in mating success. A review by Kokko et al.<sup>183</sup> used 21 measures of inequality to analyse 71 datasets of unequal mating success and showed that there was little positive correlation between the different indexes of skew (24 % of

measures did not correlate). Indeed, Kokko et al.<sup>183</sup> suggest that if simply describing the data is more informative than the use of an index, then one should do so.

I would argue that data generated by the competitive mating success assay have important implications; not only do a small proportion of males achieve multiple matings, but the majority of males do not mate a single female, despite multiple mating opportunities. The result that most males do not mate in competition has important consequences. Firstly, that insemination capacity, a commonly used metric used to quantify male mating success<sup>96,98,184,185</sup>, may be somewhat misleading, given that an insemination capacity of between 3.5 ( $\pm 0.07$ ) and 11.5 ( $\pm 0.53$ ) females has been documented for this species (reviewed in Cator, Wyer and Harrington<sup>63</sup>). Secondly, if the selective conditions in this study approximate those of the field, then the majority of modified males released as part of reproductive control strategies would be unlikely to mate, which is vital for propagating their desirable (for control) characteristics into wild populations. For inundative reproductive control strategies such as sterile insect technique (SIT) to be effective, sterilised males must be released in sufficient numbers to overwhelm the target population. Sterilised males may already suffer reduced fitness and mating competitiveness<sup>186–189</sup>, so a low likelihood of mating a wild female will necessitate an even greater number of males required to achieve population suppression.

The heritability estimate of male competitive mating success in the full-sib experimental design was relatively high ( $H^2 = 0.694$ ) and indicates that most of the variation among individuals in this population is due to genetics. Other studies in insects have reported similarly high  $H^2$  estimates for traits related to mating. For example, heritability of the propensity to store apyrene sperm, the duration of the mating refractory period, and the degree of polyandry were all found to be high in the green-veined white butterfly, *Pieris napi* ( $H^2 = 0.734$ , 0.846 and 0.627, respectively)<sup>190,191</sup>. This is the first time that resemblance of mating success among full-sib relatives has been measured in *Ae. aegypti*, though several other studies have identified  $H^2$  for other traits in *Ae. aegypti*, such as thermal stress response ( $H^2 = 0.14$ )<sup>171</sup> and susceptibility to Dengue ( $H^2 = 0.4$ ) and Chikungunya ( $H^2 = 0.18$ ) viruses<sup>192</sup>. However, the heritability values generated here, and in other similarly designed studies, should be interpreted with caution;  $H^2$  estimates are complicated

by the significant potential for bias, with such biases likely the most parsimonious explanation for this high  $H^2$  result.

Possibly the most significant of these biases is the shared environment of family members; full-sib males are raised in a common environment which is likely to increase their phenotypic resemblance to one another beyond that generated by genetic effects, thereby inflating broad-sense estimates of heritability. Future studies could rear males separately to minimise the effects of a shared larval environment. Further, maternal environmental effects are a potential source of increased resemblance between full-sibs<sup>152</sup>. Maternal effects in insects can be varied and complex<sup>193</sup>. In the yellow dung fly, *Scathophaga stercoraria*, for example, male body size has been shown to covary with maternal adult feeding, though only in males reared in stressful larval environments<sup>194</sup>. Similar maternal effects have been observed in *Ae. aegypti*; maternal larval nutrition has been shown to affect both lipid investment in offspring<sup>195</sup> and offspring development time<sup>196</sup>. Though I reared all mosquito larvae with food provided *ad libitum*, it is certainly possible that other maternal effects may have influenced the phenotypic values of related males.

Dominance is an additional component of genetic variation that is shared by full-sibs, and increases the resemblance between full-sibs relative to the resemblance between parents and offspring, which is also likely to exaggerate heritability estimates<sup>152</sup>. Further, the relative contribution of genetic dominance to phenotypic variance has been shown to be higher for traits that are closely related to fitness. This is because fitness is under directional selection, and directional selection is predicted to erode additive genetic variation in such traits<sup>197</sup>. If male mating success positively varies with traits related to fitness, such as resource acquisition<sup>198</sup>, then the relative contribution of dominance variance to male mating success might be significant, and may contribute to the high estimates of total genetic variation in this study. Though full-sib experimental designs have the advantage of being able to compare the traits of relatives in the same experiment, they are often cited as the least reliable estimates of heritability, and should be considered an upper-limit of heritability<sup>152</sup>. This renders such estimates of limited use, particularly given the difficulty in parsing out the additive genetic variation from the other sources of variation I have described.

The father-son regression generated a low estimate of competitive mating success heritability ( $h^2 = 0.2$ ), which was not significantly different from zero. This indicates that of all the observed variation in phenotype, a small (yet non-significant) proportion is due to additive genetic variation. This may be explained by the additional environmental variance introduced by components of the trait that do not contribute to attractiveness, thereby obscuring underlying genetic variance<sup>147,199</sup>. The absence of significance for this estimate of  $h^2$  is most likely caused by sampling variance due to an insufficient number of father-son pairs in the assay. Sampling error of heritability estimates is inversely proportional to the relationship of individuals squared, the number of families measured, and the number of individuals within a family<sup>152</sup>. As such, thousands of observations are often required to generate precise heritability ( $h^2$ ) estimates. Whilst this assay has clear advantages in terms of ecological relevance and precision in determining the competitive mating success of individual males, it is not a high-throughput approach, and so problematic for generating enough trait values for generating reliable estimates of heritability.

Interestingly, the low estimate of  $h^2$  for male competitive mating success is consistent with the low heritability of behaviour in both *Drosophila* ( $0.18 \pm 0.03$  SE)<sup>200</sup> and across multiple taxa ( $0.235$ ; 95% CI:  $0.200$ - $0.271$ )<sup>201</sup>. It is also consistent with the low narrow-sense heritability means calculated in a meta-analysis of multi-component 'suite' sexually selected traits ( $0.12 \pm 0.05$  SE), such as attractiveness<sup>199</sup>. Though the targets of sexual selection in *Ae. aegypti* are unknown, there is evidence to suggest that several types of traits, across multiple sensory modalities might be involved, as is the case for many taxa<sup>133</sup>. Indeed, variation in cuticular hydrocarbon profiles<sup>202,203</sup>, acoustic signalling<sup>204</sup>, and body size<sup>135</sup>, have all been correlated with measures of male mating success.

This estimate of heritability ( $h^2$ ) derived from the parent-offspring regression is strikingly different to the estimate of heritability for the full-sib families ( $H^2$ ), though there are reasons to believe that this estimate of  $h^2$  is more reliable than that generated by the full-sib design. Firstly, trait covariance between fathers and sons is less likely to be inflated by a shared environment, given that measuring competitive mating success at a given age necessitates that they are assayed separately; a more variable environment reduces heritability. Secondly, offspring do not share dominance variation with their parents, as is the case for full sibs, meaning that

resemblance between fathers and sons is generated solely by additive genetic variation.

Other studies have identified similarly large differences between heritability estimates derived from full-sib families and from parent-offspring regressions. For example, in a study the sheepshead minnow, *Cyprinodon variegatus*, estimated heritability of resistance to chemical environmental contaminants was very high when obtained from a full-sib analysis (mean  $H^2 = 0.846$ ), whereas a parent-offspring regression yielded very low heritability estimates (mean  $h^2 = 0.091$ )<sup>205</sup>. The authors of this study attribute this striking disparity in heritability values to substantial dominance and common environmental effects<sup>205</sup>.

Though the results of this study contribute to our increased understanding of male mating behaviour, the extent to which they can be used to make inferences about the variation and heritability of male mating success in other populations of *Ae. aegypti*, or about *Ae. aegypti* in general, is limited. Heritability estimates apply to a given population at a given time and can vary with environmental conditions<sup>206</sup> and with changes in gene frequency, for example due to selection or inbreeding. Whilst this study population had been kept in the laboratory for under six generations, maintaining populations in laboratory colonies can quickly erode genetic diversity<sup>207</sup>, which may affect estimates of heritability, and may generate different heritability values of *Ae. aegypti* from 'older' lab colonies. Further, this population was reared under what might be considered to be favourable environmental conditions (i.e. larvae reared at relatively low densities and with nutrients provided *ad libitum*). Assessments of the impact of unfavourable and favourable environmental conditions on heritable variation vary between studies and taxa (reviewed by Hoffmann and Merilä<sup>206</sup>), and so more work is needed to address the role of environmental variation on the heritability of male mating success in this system.

Despite apparently, though perhaps un-surprisingly, low heritability ( $h^2$ ) identified by the parent-offspring regression, previous work has shown that male mating success has the potential to evolve. Qureshi et al.<sup>132</sup> showed that under intense sexual selection male mating success in *Ae. aegypti* could evolve in just five generations of experimental evolution, indicating additive genetic variation in male mating success. Whilst it is plausible that this study design was not sufficiently sensitive to detect

additive genetic variation a single generation, there is also a substantial body of evidence to suggest that heritability ( $h^2$ ), the fraction of phenotypic variance that is additive, is not an accurate measure of evolutionary potential<sup>208,209</sup>. Hansen et al.<sup>208</sup> demonstrated that the inherent positive correlation between additive genetic variance and other components of phenotypic variance (e.g. environmental or dominance variance) may cancel out the relationship between additive genetic variance and heritability, and may explain why the correlation between evolvability and heritability is essentially zero. Additionally, a low  $h^2$  estimate doesn't necessarily indicate a low level of genetic determination, it may simply be that of all the observed variation, additive genetic variation constitutes a small proportion of it.

## Conclusions

Polygynous mating systems, where males can mate multiple times and females generally mate once, are expected to exhibit a skewed mating success. The aerial mating swarms of *Ae. aegypti* have been characterised as polygynous<sup>63</sup>, though variance in mating success has previously only been estimated using proxies of this trait, often in the absence of sexual selection. This study employed a novel assay to estimate the variation and heritability of male mating success, which encompassed active female mate choice and male-male competition - two key components of pre-copulatory sexual selection. The study provides new evidence for reproductive skew, and for the potential for sexual selection. Indeed, most males fail to mate in competition, and only a small fraction of males successfully mate with multiple females. This result holds significant implications for the reproductive control of *Ae. aegypti*, as the success of such control strategies depends on the mating success of released laboratory-reared males. Additionally, this study indicated a high upper limit for heritability ( $H^2$ ) of male mating success, though a small (though non-significant) proportion of the phenotypic variance is attributed to additive genetic variation, in line with expectations of the heritability of multivariate traits. Future studies should focus on identifying the specific traits underlying male mating success, and the relative contribution of environment to competitive male mating success.

## Chapter 3

### Detection of Genomic Signatures of Evolution in the Presence and Absence of Sexual Selection

#### Abstract

Reproductive control strategies require the mass-release of sexually competitive male mosquitoes. However, in *Aedes aegypti* the genetic determinants of male mating success are unknown. In this study, I performed pooled whole-genome sequencing (pool-seq) on laboratory populations of *Ae. aegypti* recently derived from the wild that were evolved in the presence and absence of mate competition and female choice for five generations. Comparison of allele frequency data between ancestral and evolved populations revealed a greater genetic similarity of populations evolved in the presence of sexual selection to the ancestral population, from which I infer that field populations experience sexual selection at a level comparable to that of the sexual selection regime. I also identified moderate and high impact variants that consistently diverged between replicates of the selective regimes. These variants are significantly enriched for GO terms related to perception of chemical cues, which I suggest are involved in male mating success.

#### Introduction

*Aedes aegypti* mosquitoes are an ever-increasing threat to global health. *Ae. aegypti* is an efficient vector of dengue, Zika, yellow fever and chikungunya. In the absence of effective vaccines for most of these arboviral diseases, mosquito control is critical for disease control, however it is threatened by the widespread resistance of *Ae. aegypti* to the most commonly used chemical insecticides<sup>33</sup>. Of additional and increasing concern is the rapid range expansion of *Ae. aegypti* into new areas, facilitated by the extremely invasive nature of these mosquitoes and by climate<sup>17</sup> and environmental changes<sup>210</sup>.

Significant research effort has been dedicated to developing novel mosquito control tools that negate the use of chemical insecticides. One such method is reproductive

control, an umbrella term used to describe a group of strategies that involve the mass release of male mosquitoes carrying a desirable trait (such as pathogen resistance or sterility) in order to introduce this trait to the wild population via mating. Reproductive control strategies rely on mass-released laboratory reared male mosquitoes locating and successfully mating with their wild female conspecifics. Essentially, the attractiveness of a male mosquito to a wild female mosquito could make the difference between the success or failure of a reproductive control programme. In *Ae. aegypti* mating swarms, male mosquitoes engage in scramble competition to gain access to mates, while females are the more selective sex<sup>63</sup>, displaying clear rejection of presumably undesirable males through kicks or aversive flight<sup>88</sup>. Although there is much evidence to suggest sexual selection in this system is primarily driven by male competition and female choice<sup>63</sup>, pinpointing the specific traits under sexual selection remains a challenging task.

The difficulty in dissecting mosquito mating interactions has shaped the overall approach taken to identify sexually selected traits. Previous work has correlated traits such as body size, cuticular hydrocarbon profile, and acoustic signalling behaviours with male mating success based on our current understanding of mating biology and inferred importance from other systems<sup>117,202,203</sup>. However, results vary between studies and aspects of male mating biology measured<sup>146,153,154,211,212</sup>. Further, the male trait associated with increased mating success is rarely manipulated. For example, a feature of acoustic interactions between *Ae. aegypti* males and females prior to mating, known as harmonic convergence, is more likely to occur in pairs which eventually form a copula<sup>117,213</sup>. Yet, it is unclear whether harmonic convergence is a cause or consequence of copula formation. Similarly, in field populations, *Anopheles coluzzii* males caught in mating pairs have a higher cuticular carbon abundance<sup>202</sup>, but it is unclear whether the trait is sexually selected or if cuticular hydrocarbon abundance is generally associated with overall male vigour.

In a range of other organisms, direct manipulation of the strength of sexual selection over multiple generations has been used to reveal traits responding to sexual selection<sup>214</sup>. For example, experiments in yeast and *Drosophila* have reported increases in intensity of sexual signals when under strong sexual selection<sup>215,216</sup>. This experimental evolution approach can be coupled with next-generation

sequencing of pools of individuals (Evolve & Resequence<sup>217</sup>) to identify areas of the genome likely to be functionally important in producing the sexually-selected phenotype under study<sup>218</sup>. There is evidence to suggest that divergence as a result of sexual selection is due to rapid divergence in genes governing sexually selected traits<sup>218</sup>. A key advantage of this approach is that it does not require a priori knowledge about which traits are expected to respond<sup>219</sup>.

In a recent study, Qureshi et al.<sup>132</sup> experimentally evolved replicate populations of *Ae. aegypti* in the presence and absence of sexual selection. The populations evolved in the presence of sexual selection experienced male-male competition and female choice, both of which are known to occur in the natural mating system<sup>63</sup>. In these populations, five males were held with a single female during a fixed mating period. In contrast, populations evolved in the absence of sexual selection were subjected to enforced randomised monogamy during the same period. Results of this study showed that just five generations of evolution in the absence of sexual selection resulted in consistently decreased male competitive mating success<sup>132</sup>. However, the types of traits underlying this decline are unknown.

Here, I use the populations previously experimentally evolved in the presence or absence of sexual selection by Qureshi et al.<sup>132</sup>, combined with whole genome sequencing with the aim of revealing a signature of sexual selection in the *Ae. aegypti* genome. I also aimed to uncover the types of genes and therefore the phenotypic traits that are likely to be under sexual selection in this medically important mosquito species.

## **Methods**

### ***Aedes aegypti* experimental population and colony maintenance**

Mosquito samples were generated in the study by Qureshi et al.<sup>132</sup> and were maintained as follows: around 4500 eggs were collected from 17 water storage containers from two villages Muang District, Kamphaeng Phet Province (KPP), Thailand between February and April 2016. Eggs were hatched under a vacuum for 20 minutes. Newly emerged larvae were provided with 0.1 mg of ground fish food (Cichlid Gold [#04328], Hikari, Himeji City, Japan) and held in a 27 °C incubator

overnight. First instar larvae were sorted into trays of 500 larvae /1L water and provided fish food pellets *ad libitum*.

### **Experimental evolution selection regimes**

The initial egg collection was divided into six populations, three of which were subjected to a mating regime with sexual selection (+SS) and the other three to a no sexual selection (-SS) regime. In each replicate population of the +SS regime, 100 groups each with five males and single female were established. In each replicate population of the -SS regime, 100 groups each with one female and one male were established. In both sexual selection regimes, the mating period lasted for 8 hours<sup>132</sup>. Mated females were pooled for each treatment, offered a bloodmeal (FirstLink, UK) and allowed to lay eggs on damp filter paper. The number of females contributing to successive generations was controlled by monitoring the insemination rate and fecundity. This process of experimental evolution was repeated for 5 generations, after which the populations underwent a single generation of common garden rearing, to control for parental effects.

### **Genomic DNA extraction**

Seven different samples were collected from the experimental evolution set-up: a single sample from the ancestral population, a sample from each of the three replicate populations of the evolved sexual selection (+SS) regime, and a sample from each of the three replicate populations of the evolved no sexual selection (-SS) regime. Samples consisted of pools of 80 male *Ae. aegypti* mosquitoes. Genomic DNA (gDNA) was extracted from each of the seven samples using the QIAGEN DNeasy Blood & Tissue kit according to the QIAGEN protocol for purification of total DNA from insects<sup>220</sup>. To ensure approximately equal amounts of gDNA were obtained from each individual male, pools for each replicate population were subsampled in groups of 20, gDNA extracted and then equal quantities of gDNA combined from each subset.

### **DNA sequencing, aligning, and filtering**

Sequencing libraries were prepared with TruSeq DNA Nano kit (Illumina) and sequenced on the NovaSeq 6000 platform (CeGaT GmbH, Germany) with 150 base pair paired-end reads. FastQ files were quality checked using FastQC (version 0.11.9) with default settings<sup>221</sup>. Reads were mapped to the *Ae. aegypti* genome (Liverpool AGWG-AaegL5.0)<sup>222</sup> using BWA-mem (version 0.7.17)<sup>223</sup>. PCR duplicates were removed using Samtools (version 1.3.1)<sup>224</sup> and reads realigned around intervals using PicardTools (version 2.6.0) and GATK (version 3.4). Realigned sequence files were compiled using the 'mpileup' command in Samtools and then converted to 'sync' file format using the 'mpileup2sync' command in Java multi-threading from the package Popoolation2<sup>225</sup>. Allele frequency differences (\_rc file) were called using the 'snp-frequency-diff' command in Popoolation2 based on a minimum coverage of 50, maximum coverage of 500 and minimum minor allele count of 14<sup>225</sup>. A custom R script removed monoallelic and triallelic loci. The mitochondrial genome was excluded from the analysis due to excessively high coverage. I also removed sites with a minor allele frequency < 5 %. After these filtering steps, the remaining 8,704,928 SNPs were used for downstream analysis.

### **Genome-wide patterns of variation and differentiation**

Principal component analysis was run on all filtered SNPs (> 8.7 million) using the R function 'prcomp'. Next, I calculated the fixation index ( $F_{ST}$ ) to determine genetic differentiation between pairs of evolved populations, and between the ancestral and each evolved population.  $F_{ST}$  is the proportion of genetic variance in a subpopulation relative to the total variance. High  $F_{ST}$  values are indicative of a large amount of genetic differentiation between subpopulations. I first converted filtered BAM files used in earlier analyses to 'mpileup' format using Samtools<sup>224</sup>, then used a script from Popoolation2 to convert the 'mpileup' to a 'sync' format file. I used PoPoolation2<sup>226</sup> to average  $F_{ST}$  values for non-overlapping windows of 500 kb across the three chromosomes, using parameters of a minimum allele count of 14, minimum coverage of 50 and maximum coverage 500 reads.

Population size ( $N_e$ ), which greatly affects changes in allele frequency, was estimated for each evolved population using a method designed for pool-seq data which corrects for sampling at both the pooling and sequencing stages<sup>227</sup>.

Nucleotide diversity ( $\pi$ ) is the average pairwise difference between all samples, and a measure of overall genetic variation between populations. Low nucleotide diversity may indicate selection at a locus.  $\pi$  was calculated for each of the three replicates of the two evolved populations. BAM files for each sample were combined using the Samtools 'mpileup' command<sup>224</sup>, subsampled to a uniform coverage of minimum 50 and maximum 500 reads, then  $\pi$  was calculated in 10 kb non-overlapping windows using PoPoolation<sup>226</sup>. For any given window to be assigned a  $\pi$  value, at least 40 % of the window had to comply with a minimum and maximum coverage of 20 and 500, respectively<sup>226</sup>. A linear mixed effect model (lmer) using the R package lme4<sup>176</sup> was used to determine if there was a difference in  $\pi$  between evolutionary regimes genome-wide and for individual chromosomes, incorporating population and the position of the 10 kb window in the genome into the model as random effects.

To identify SNPs that had diverged due to the selective regime, I employed a generalised linear mixed model (GLMM) to identify SNPs with consistent allele frequency differences between all three replicate populations of each evolutionary regime. The GLMM compared major allele frequencies of the 8,704,928 SNPs between the two evolutionary regimes, with evolutionary regime as the fixed effect and replicate population as a random effect. The alternative and null models were then compared with a likelihood ratio test to obtain variant-level significance tests. I then calculated q-values, a determinant of significance in terms of the false discovery rate (FDR), using the method of Storey & Tibshirani<sup>228</sup> and applied a threshold of 0.1 or 10 % using the qvalue package in R<sup>229</sup>. "Significant SNPs" were those which consistently diverged between treatments with a q value < 0.1.

### **Linkage disequilibrium estimations**

Linkage disequilibrium (LD) is the non-random association of neighbouring polymorphisms due to limited recombination<sup>230</sup>. Experimental populations were evolved for five generations and so there were limited opportunities for recombination to occur. High levels of linkage due to hitchhiking of neutral variants

along with any selected variants might be expected, thus generating an inflated number of candidate SNPs, as is fairly common with pooled sequencing data<sup>231</sup>. To determine the extent to which linkage disequilibrium caused diverging candidate SNPs to evolve non-randomly the number of independently evolving clusters on each chromosome was calculated, based on a method described in Kawecki et al.<sup>232</sup> using a custom R script and the packages `dplyr`<sup>233</sup> and `data.table`<sup>234</sup>.

First, residual allele frequencies for each evolved replicate population were calculated by subtracting allele frequencies at each SNP locus from the regime mean. Then the Pearson's correlation coefficient ( $r_w$ ) was calculated between residuals of all pairs of SNPs on the chromosome. It was expected that if linkage disequilibrium was high the distribution of  $r_w$  between SNPs on the same chromosome would be more positively skewed than the distribution of  $r_w$  between SNPs on different chromosomes. Conversely if linkage was low then these two distributions would appear similar. To group SNPs into clusters, a threshold correlation coefficient and maximum distance between SNPs that could be clustered together needed to be determined. To do this the number of clusters for a range of threshold correlation coefficient values ( $r_w = 0.7, 0.8, 0.9$ ) and distances between SNPs (100 kb, 200 kb, 500 kb, 1000 kb) were calculated, and values selected for which the correlation between clusters on a single chromosomal arm was similar to the correlation between SNPs on different chromosomes. As such, SNPs were assigned to the same cluster if they were located within 200 kb of each other, and if they were found to be highly correlated ( $r_w > 0.8$ ).

### **Genetic variant effect prediction**

The programme SNPEff (version 4.3)<sup>235</sup> was used to determine the functional effects of the significantly diverged SNPs. First a custom database was created with the most recently annotated *Ae. aegypti* genome<sup>222</sup>. A Variant Call Format (VCF) file was compiled for all ~8.7 million SNPs (biallelic variants with maximum 500x coverage, minimum count 14 and global frequency > 5 %) and the subset of 50,988 significant SNPs (< 0.1 FDR) from the GLMM analysis. SNPEff requires input VCF files to contain a reference allele and an alternative allele for each variant. The reference allele was extracted from the reference genome using BEDTools<sup>236</sup> and

the alternative allele was set as both alleles present at the SNP site. SNPEff generated a list of variants with either high or moderate effect on gene function; “moderate effect” substitutions resulted in a change of amino acid, and “high effect” substitutions resulted in a stop codon. To test whether moderate and high impact variants were in genes enriched for certain functional groups, the software package Gowinda<sup>237</sup> was used to perform a gene ontology (GO) analysis. The programme performed 100,000 simulations and counted every gene with a SNP in it only once. The GO analysis was performed firstly for all genes that co-localised with moderate and high impact variants within the coding region, and secondly for all genes that co-localised with moderate and high impact variants within the coding region and regulatory regions, 2000 base pairs either side of the gene.

### **Mating and fecundity measurements**

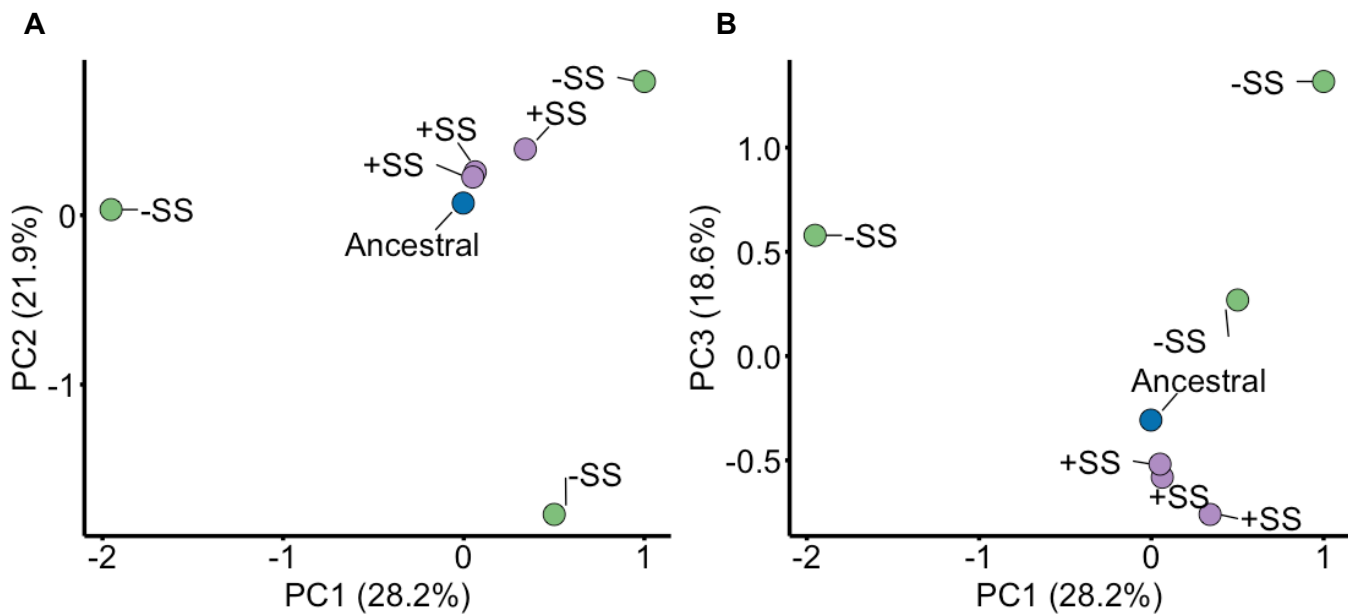
To determine if the selective regimes themselves led to changes in fecundity and insemination capacity of evolved populations, the proportion of females mating, and the number of eggs laid after mating, were measured for the -SS and the +SS treatments. 50 pairings per treatment (-SS = 1♂:1♀; +SS = 5♂:1♀) were set up in the same containers used in the original experimental evolution design<sup>132</sup>. As in the original experiment, males were transferred into containers the night before the experiment and provided with 10 % sucrose solution. Females were introduced into containers at 0700 and moved to pooled cages eight hours later. The following day females were provided with a bloodmeal and transferred into individual tubes for oviposition and provided with a 10 % sucrose solution. After six days, eggs were removed and counted. Females that failed to lay eggs were dissected to confirm insemination. Insemination data was analysed with a binomial GLM with replicate and treatment as predictors and inseminated (Y/N) as the response variable, and number of eggs produced per females was analysed with a GLM with replicate, wing length, and treatment as the predictors and eggs as response.

### **Quantification and statistical analysis**

Quantification was performed as described in the relevant method details sections above. Statistical analysis was performed with *t*-tests for comparisons of Euclidean distances in the PCA, comparison of  $F_{ST}$ , and  $N_e$ . A linear mixed effect model was used to detect differences in  $\pi$  between evolutionary regimes both genome-wide and within individual chromosomes. GLMMs were used for comparison of allele frequencies between evolutionary regimes, with treatment as a fixed effect and population as a random effect, with significance determined using Storey & Tibshirani *q* values<sup>228</sup>  $< 0.1$ .

## Results

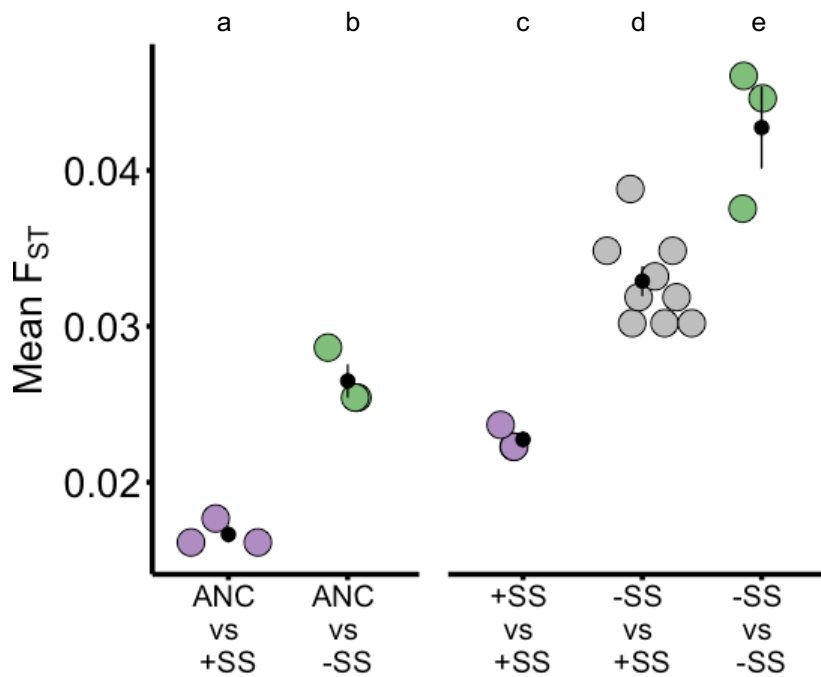
I sampled adult mosquitoes from replicate populations that had evolved either with sexual selection (+SS:  $n = 3$ ) or without sexual selection (-SS:  $n = 3$ ) and the shared ancestral population which was recently derived from the field (ANC:  $n = 1$ ). I sequenced pools of 80 males from each population to a coverage of between 50x and 500x using Illumina 150 bp paired-end reads. Sequences were aligned to the *Ae. aegypti* reference genome<sup>222</sup>. After stringent filtering, I generated a dataset of 8,704,928 SNPs from the seven populations, a large but unsurprising number given the 1.25 Gb genome size<sup>222</sup>. I found that sexual selection had a major effect on patterns of allele frequencies in evolved *Ae. aegypti* populations (Figure 3.1). Principal components analysis (PCA) of all SNPs indicated that populations evolved in the presence of sexual selection (+SS) remained more similar on the first two principal component axes to the ancestral population than populations where sexual selection was eliminated (-SS) (Figure 3.1A, independent samples *t*-test on Euclidean distance from ancestral;  $t = -5.63$ ,  $df = 4$ ,  $p\text{-value} = 0.005$ ). While these first two axes revealed movement of the populations released from sexual selection away from the ancestral population in multivariate space, the third principal component axis consistently separated populations evolving with sexual selection from those where sexual selection was eliminated (Figure 3.1B, independent-samples *t*-test on PC3;  $t = 4.20$ ,  $df = 4$ ,  $p\text{-value} = 0.01$ ). This clear separation by regime on PC3, which explains 18.6 % of the total variance, indicates some degree of parallel adaptation in response to the mating system manipulation after only five generations of evolution.



**Figure 3.1. Principal components analysis (PCA) of genome-wide allele frequencies of populations evolved in the presence (+SS, purple) or absence (-SS, green) of sexual selection.** All populations evolved from a common ancestral population (blue). (A) Populations exposed to sexual selection remained more similar to the ancestral population than populations not exposed to sexual selection on the first two principal component axes. (B) Evolutionary regimes are separated by PC3. In both PCAs all 8,704,928 SNPs were included (minimum coverage of 50 and maximum coverage of 500 in each population)

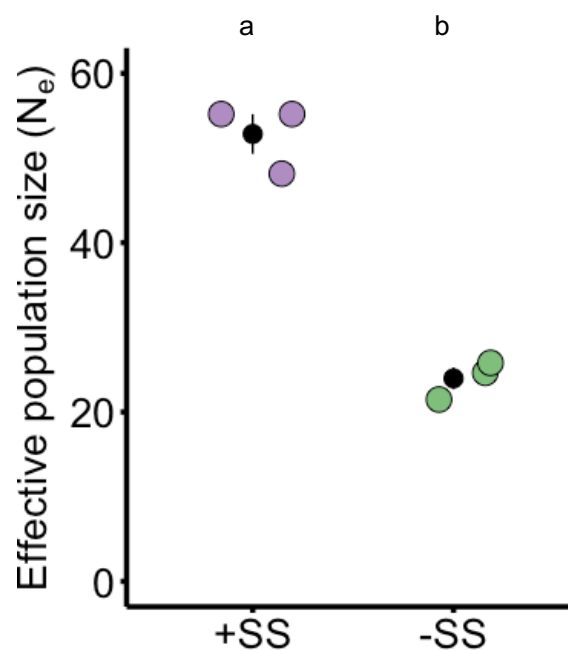
I then compared genetic differentiation between populations by calculating pairwise  $F_{ST}$  between all evolved populations, and for the ancestral population and the evolved populations, for non-overlapping windows of 500 kb across the genome (Figure 3.2). This analysis recapitulated the results of the PCA, where populations experiencing sexual selection (+SS) had relatively low differentiation from one another (mean  $F_{ST} = 0.023 \pm 0.0005$  SE) while populations evolving without sexual selection (-SS) showed higher divergence from one another (mean  $F_{ST} = 0.043 \pm 0.001$  SE, independent samples  $t$ -test on +SS vs +SS and -SS vs -SS;  $t = -7.44$ ,  $df = 4$ ,  $p$ -value = 0.002). Genetic differentiation arises due to the cumulative action of both selection and drift<sup>152</sup>. Individual populations not experiencing sexual selection appeared more genetically similar to populations from the other evolutionary regime than they did to one another in both the PCA and the analysis of genome-wide  $F_{ST}$

(independent samples *t*-test on +SS vs -SS and -SS vs -SS; *t* = -4.50, *df* = 10, *p*-value = 0.001). There was also greater differentiation between the ancestral population and populations evolved in the absence of sexual selection than there was between the ancestral population and populations evolved in the presence of sexual selection (Figure 3.2, independent samples *t*-test on ANC vs +SS and ANC vs -SS: *t* = -8.29, *df* = 4, *p*-value = 0.001).



**Figure 3.2. Genomic differentiation between populations.** Fixation index ( $F_{ST}$ ) comparisons (500 kb non-overlapping windows) between all replicate populations, grouped by within- and between-regime comparisons. Populations evolved with sexual selection showed the least divergence from one another, whilst across evolved regime  $F_{ST}$  values were significantly higher. Populations evolved without sexual selection showed the highest divergence from one another, indicating a prominent role of genetic drift over the course of experimental evolution. Between the evolved and ancestral populations, populations evolving with sexual selection showed the least divergence from the ancestral population. Coloured points indicate pairwise means for individual populations, black points indicate overall mean, error bars indicate  $\pm 1$  standard error. Lower case letters a-e indicate significant differences in overall mean.

I used temporal allele frequency data from the ancestral population and the six evolved populations to estimate effective population size ( $N_e$ )<sup>227</sup>. This analysis revealed that populations evolved in the absence of sexual selection indeed showed reduced  $N_e$  (mean =  $23.97 \pm 1.29$  SE) relative to those for which sexual selection was present (mean =  $52.84 \pm 2.35$  SE, Figure 3.3;  $t = 10.77$ ,  $df = 4$ ,  $p\text{-value} < 0.001$ ). Despite differences in estimated effective population size, when I replicated the conditions of the selective regimes, there was no statistically significant effect of treatment (+SS/-SS) on the proportion of females inseminated (Table 3.1;  $\chi^2 = 0.11$ ,  $df = 1$ ,  $p\text{ value} = 0.74$ ) or the number of eggs those females produced (Table 3.1;  $\chi^2 = 0.956$ ,  $df = 1$ ,  $p\text{ value} = 0.328$ )

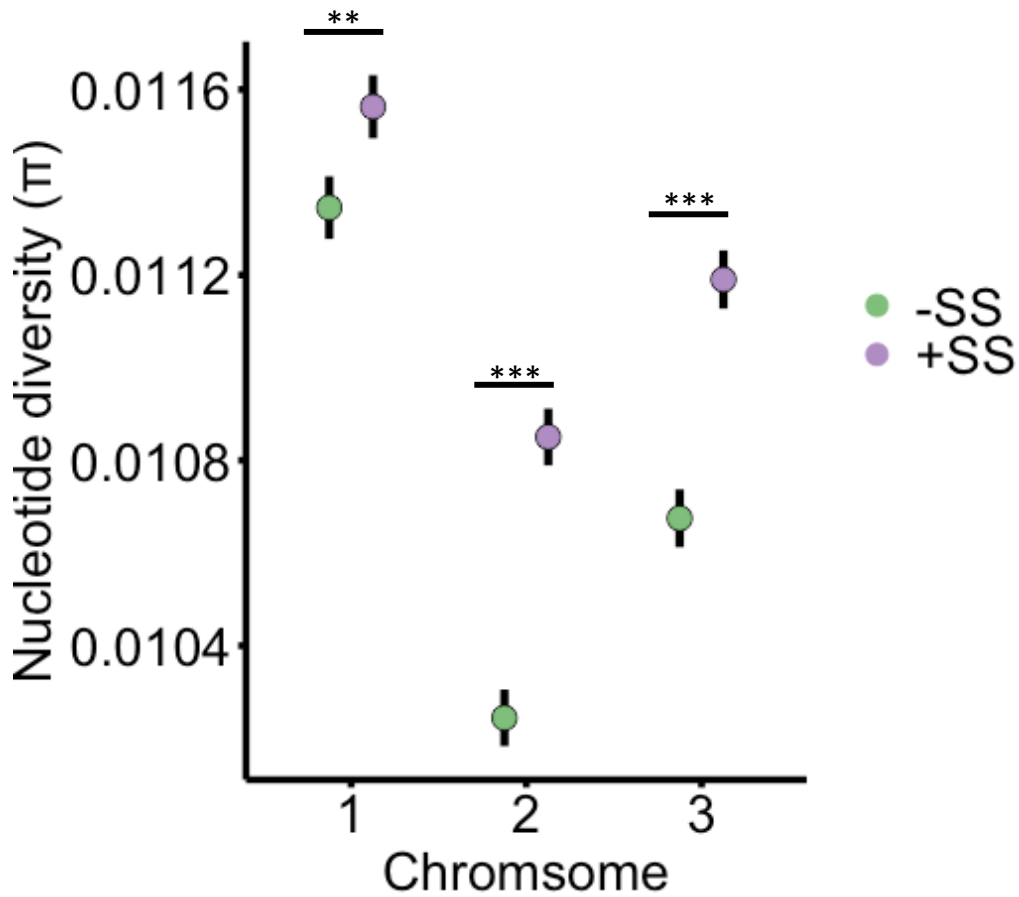


**Figure 3.3. Effective population sizes of populations evolved in the presence (+SS) or absence (-SS) of sexual selection.** +SS populations maintained a greater estimated effective population size ( $N_e$ ) than -SS populations. Coloured points indicate  $N_e$  for individual replicate populations, black points indicate overall regime mean and error bars indicate  $\pm 1$  standard error. Lower case letters indicate significant differences in overall mean.

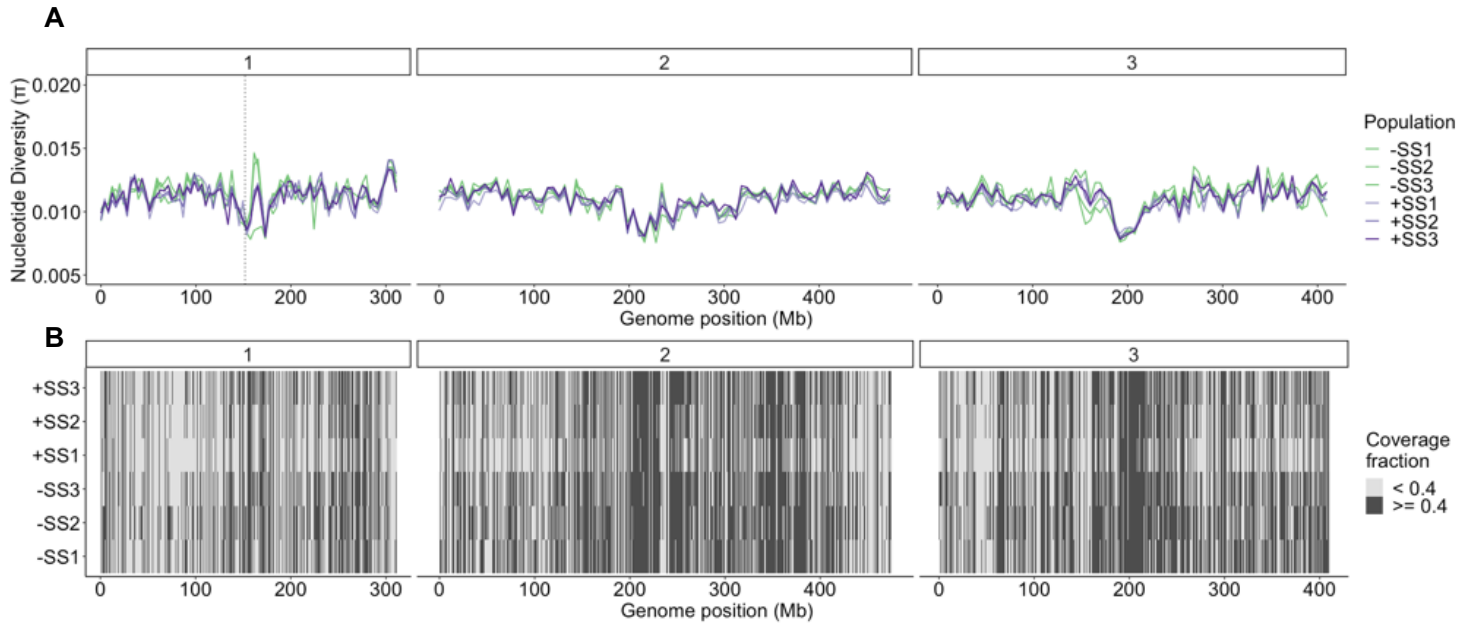
**Table 3.1. Effect of sexual selection on insemination rates and fecundity.**

| Treatment | % Inseminated $\pm$ SE [n] | Eggs/ mm wing length $\pm$ SE [n] |
|-----------|----------------------------|-----------------------------------|
| -SS       | 94.0 $\pm$ 2.7 [81]        | 16.29 $\pm$ 1.59 [29]             |
| +SS       | 93 $\pm$ 2.7 [94]          | 18.41 $\pm$ 1.04 [39]             |

Consistent with the observed differences in  $N_e$  between regimes, nucleotide diversity ( $\pi$ ) calculated in non-overlapping 10 kb windows for each replicate population also differed between evolutionary regimes. There was spatial variation across the genome in nucleotide diversity (Figure 3.4, chromosome effect:  $\chi^2 = 324.91$ , p-value  $< 0.001$ ), but a consistent effect of reduced nucleotide variation in populations that evolved in the absence of sexual selection (Figure 3.4, regime effect:  $\chi^2 = 11.47$ , p-value  $< 0.001$ ). While significant on all three chromosomes, the reduction in  $\pi$  in -SS compared to +SS is most pronounced on the 2<sup>nd</sup> and 3<sup>rd</sup> chromosomes and smaller on the 1<sup>st</sup> chromosome (Figure 3.4). Interestingly,  $\pi$  on chromosome 1 was higher than on chromosome 2 and 3 for both regimes. This elevated  $\pi$  was unlikely to be attributable to the presence of the sex-determining M locus (Figure 3.5) given that locally reduced  $\pi$  was observed in this region.

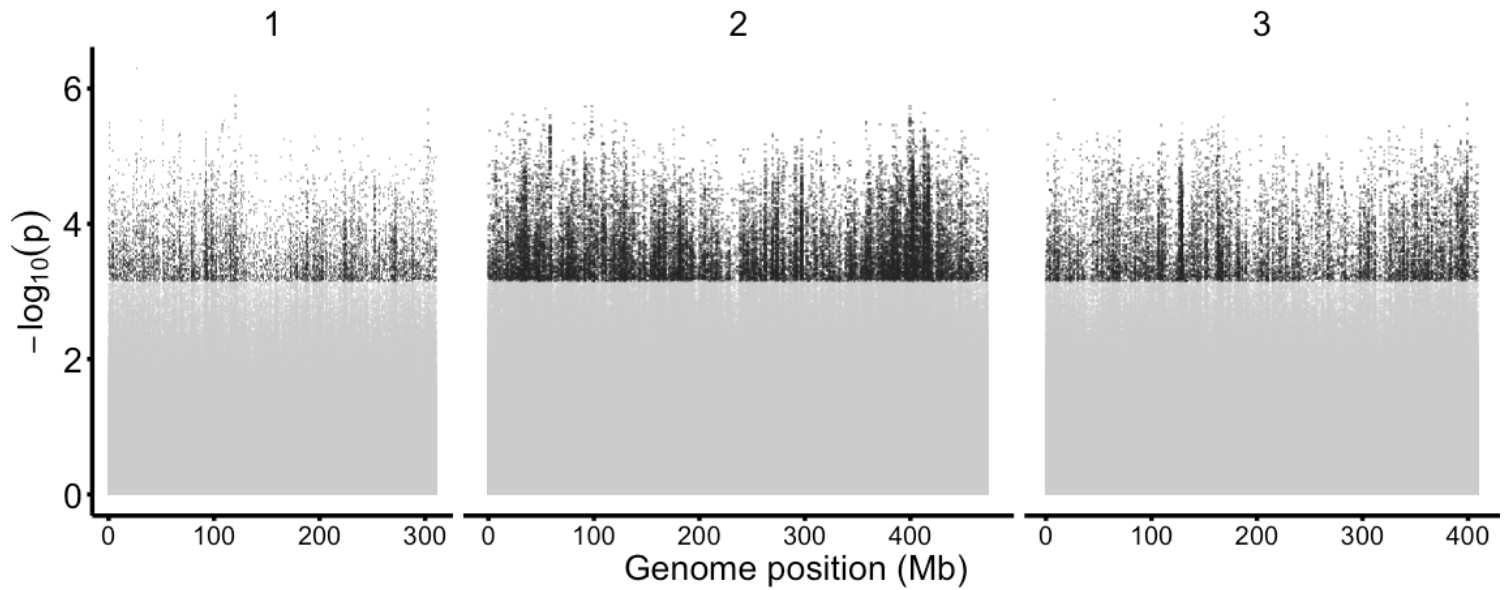


**Figure 3.4. Overall patterns of nucleotide diversity.** Estimated marginal means of chromosome-wide nucleotide diversity ( $\pi$ ) are lower in the no sexual selection (-SS) populations than the sexual selection (+SS) populations for each chromosome.  $\pi$  was calculated using sliding windows of 10 kb on sites with a where 40 % of coverage was greater than 20 and less than 500. Error bars indicate  $\pm 1$  standard error; p-values less than 0.05 are marked with one star (\*), p-values less than 0.01 are marked with two stars (\*\*), and p-values less than 0.001 are marked with three stars (\*\*\*).



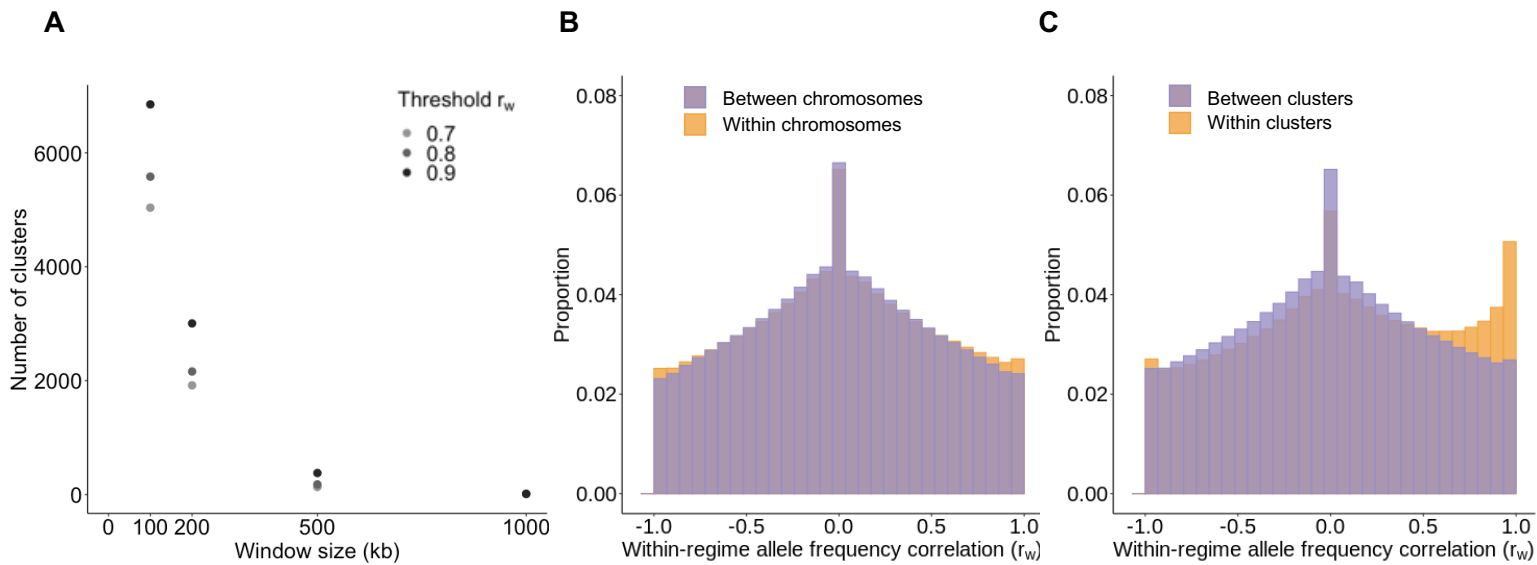
**Figure 3.5. Analysis of genome-wide nucleotide diversity.** (A) Genome-wide nucleotide diversity ( $\pi$ ) of each evolved population measured in non-overlapping windows of 10 kb. Lines in green indicate  $\pi$  values for individual replicates of the no sexual selection (-SS) evolutionary regime ( $n = 3$ ), lines in purple indicate  $\pi$  values for individual replicates of the sexual selection (+SS) regime ( $n = 3$ ). The dotted vertical line at 151.68 Mb on chromosome 1 marks the sex-determining M locus. (B) Genome-wide covered fraction of each 10 kb non-overlapping window. Windows in dark grey with a minimum coverage fraction of 0.4 were retained for  $\pi$  calculations, whereas those in light grey were excluded.

Candidate SNPs underlying the differentiation between evolutionary regimes were identified using generalised linear mixed models (GLMMs) with a false discovery rate (FDR) of 10 %. This approach identified 50,988 SNPs spanning all three chromosomes (Figure 3.6) with large and consistent differences in allele frequency between evolutionary regimes that represent either the targets of selection or linked genetic variation.



**Figure 3.6. Patterns of evolved changes in genome-wide allele frequencies in response to manipulation of sexual selection.** Manhattan plot of  $-\log_{10}$ -transformed p-values from the generalised linear mixed model (GLMM). 50,988 single nucleotide polymorphisms (SNPs) that passed the Storey-Tibshirani false-discovery threshold are coloured in dark grey. SNPs that consistently responded to the selective regime occurred across the genome.

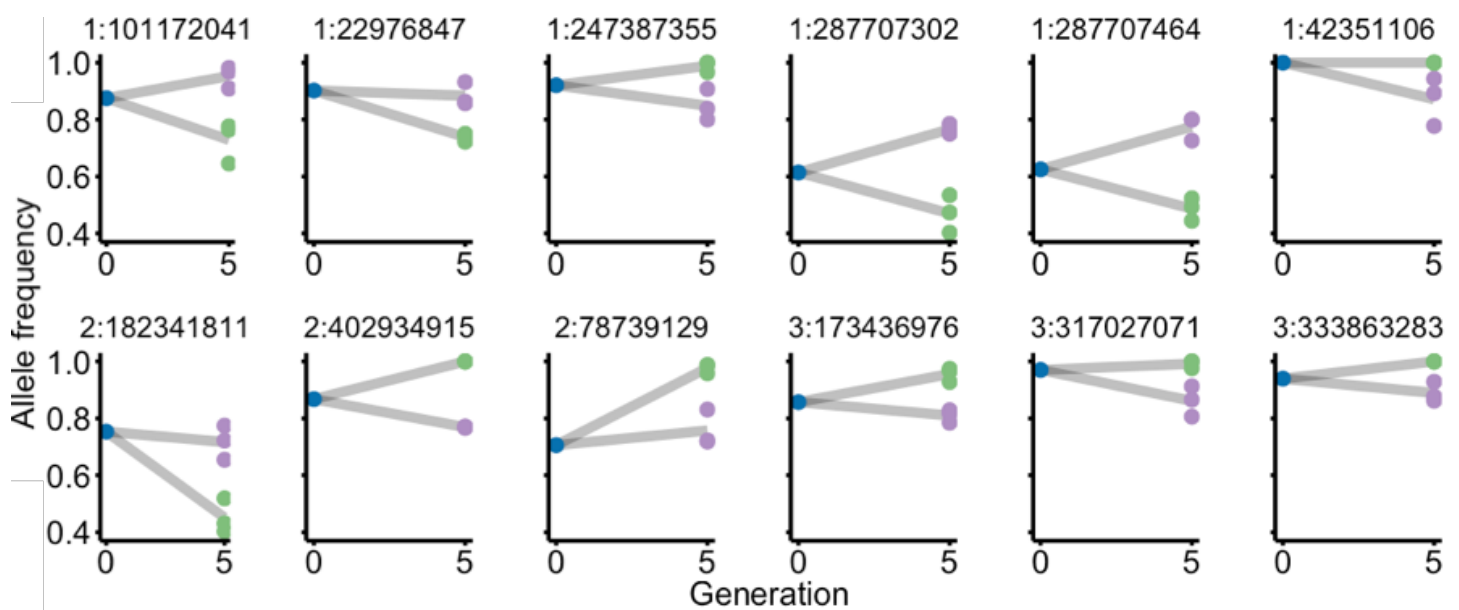
To determine to what extent reduced opportunities for recombination had inflated the number of candidate SNPs (those under the 10 % FDR threshold), the Pearson's correlation coefficient ( $r_w$ ) was calculated between residuals of all pairs of candidate SNPs on the same chromosome. The distribution of  $r_w$  for pairs of candidate SNPs on the same chromosome and the distribution of  $r_w$  for pairs of candidates SNPs on different chromosomes was similar (Figure 3.7B & 3.7C). Under stringent clustering criteria (candidate SNPs were grouped into clusters if they were located within 200 kb of one another and had highly correlated allele frequencies of  $r_w > 0.8$ ) 2,161 clusters were identified (Figure 3.7A). Further, all gene-level candidates identified with functional effect prediction (see below) were in independent clusters.



**Figure 3.7. Distributions of within-regime allele frequency correlation ( $r_w$ ) between significant SNPs.** (A) The number of individual single nucleotide polymorphism (SNP) clusters as a function of window size and correlation coefficient ( $r_w$ ). SNPs were assigned to the same cluster if they were located within the given window size left or right of the midpoint between each other, and if they were found to have a correlation coefficient greater than that of the given  $r_w$  threshold. (B) Distribution of  $r_w$  between significant SNPs on the same chromosome (orange) compared with the corresponding distribution of pairs of SNPs on separate chromosomes (purple). (C) Distribution of  $r_w$  for pairs of SNPs assigned to the same cluster (orange) and between pairs of SNPs on the same chromosome but assigned to different clusters (purple). Here, clusters were delineated as pairs of SNPs that are within 200 kb of one another and have a correlation coefficient ( $r_w$ ) of  $> 0.8$ .

A functional annotation of the 50,988 candidate SNPs identified a subset of variants with a “moderate” ( $n = 713$ ) or “high” ( $n = 12$ ) impact on amino acid sequence. Allele frequency trajectories of high impact SNPs showed a large (mean 19.4 %) and consistent average allele frequency difference between evolutionary regimes (Figure 3.8). A gene ontology (GO) enrichment analysis on high and moderate impact SNPs ( $n = 725$ ) indicated that these variants colocalised with genes enriched for GO terms related to olfactory receptor activity, sensory perception of smell, and other chemosensory terms (Table 3.2). Among the list of genes that colocalised with moderate and high impact variants were five odorant receptor genes [*or53* (aael015286), *or24* (aael017557), *or44* (aael006465), *or116* (aael025139) and *or63* (aael000628)]<sup>222</sup>. By extending the analysis to include high and moderate impact

SNPs either within genes or their predicted regulatory regions (2000 bp up or downstream of genes), the order of enriched GO terms did not change (Table 3.3).



**Figure 3.8. Evolution of high confidence SNPs.** Allele frequency trajectories of 12 candidate single nucleotide polymorphisms (SNPs) designated “high impact” by functional effect predictor program SNPEff. Individual plots indicate allele frequencies of ancestral population ( $n = 1$ , blue), +SS ( $n = 3$ , purple), and -SS ( $n = 3$ , green) populations.

**Table 3.2. Gene ontology (GO) terms of genes co-localising with moderate and high impact variants.**

| <b>GO ID</b> | <b>Description</b>                                                                                    | <b>P-value</b> | <b>FDR (&lt; 0.01)</b> |
|--------------|-------------------------------------------------------------------------------------------------------|----------------|------------------------|
| GO:0005506   | iron ion binding                                                                                      | < 0.001        | < 0.001                |
| GO:0016491   | oxidoreductase activity                                                                               | < 0.001        | < 0.001                |
| GO:0004497   | monooxygenase activity                                                                                | < 0.001        | < 0.001                |
| GO:0055114   | oxidation-reduction process                                                                           | < 0.001        | < 0.001                |
| GO:0020037   | heme binding                                                                                          | < 0.001        | < 0.001                |
| GO:0016705   | oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen | < 0.001        | < 0.001                |
| GO:0050911   | detection of chemical stimulus involved in sensory perception of smell                                | < 0.001        | < 0.001                |
| GO:0050896   | response to stimulus                                                                                  | < 0.001        | 0.00151                |
| GO:0004984   | olfactory receptor activity                                                                           | < 0.001        | 0.00151                |
| GO:0007608   | sensory perception of smell                                                                           | < 0.001        | 0.00196                |
| GO:0043022   | ribosome binding                                                                                      | < 0.001        | 0.0073                 |
| GO:0005549   | odorant binding                                                                                       | < 0.001        | 0.00747                |
| GO:0005778   | peroxisomal membrane                                                                                  | < 0.001        | 0.0293                 |

**Table 3.3. GO-terms analysis for moderate and high impact SNPs within genes and regulatory regions**

| GO ID      | Description                                                                                           | P-value | FDR (< 0.01) |
|------------|-------------------------------------------------------------------------------------------------------|---------|--------------|
| GO:0005506 | iron ion binding                                                                                      | < 0.001 | < 0.001      |
| GO:0016491 | oxidoreductase activity                                                                               | < 0.001 | < 0.001      |
| GO:0004497 | monooxygenase activity                                                                                | < 0.001 | < 0.001      |
| GO:0020037 | heme binding                                                                                          | < 0.001 | < 0.001      |
| GO:0016705 | oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen | < 0.001 | < 0.001      |
| GO:0055114 | oxidation-reduction process                                                                           | < 0.001 | < 0.001      |
| GO:0050911 | detection of chemical stimulus involved in sensory perception of smell                                | < 0.001 | 0.013        |
| GO:0043022 | ribosome binding                                                                                      | < 0.001 | 0.019        |
| GO:0004984 | olfactory receptor activity                                                                           | < 0.001 | 0.022        |
| GO:0007608 | sensory perception of smell                                                                           | < 0.001 | 0.024        |
| GO:0050896 | response to stimulus                                                                                  | < 0.001 | 0.029        |

## Discussion

In this study I used pooled sequencing of populations of the mosquito *Ae. aegypti* that had evolved for five generations in the presence or absence of sexual selection to explore genome-wide changes in allele frequencies. Previous phenotypic analyses demonstrated that the absence of sexual selection in these populations lead to a reduction in male mating success<sup>132</sup>, however the genetic basis of these differences, and how the presence or absence of sexual selection lead to evolutionary changes in the genome, was unclear. The results presented in this chapter show that evolution under opposing sexual selection regimes results in genome-wide divergence in allele frequencies, particularly in genes with chemosensory function, with populations evolving in the presence of mate choice and male-male competition retaining greater genetic similarity to the low-generation ancestral population.

The results of this study show greater genetic similarity between the replicate populations exposed to elevated sexual selection in the +SS regime and the ancestral population, as demonstrated in the PCA analysis (Figure 3.1A, B).  $F_{ST}$  estimations show that there is more genetic differentiation within the -SS regime than there is between the two opposing evolutionary regimes (Figure 3.2). Only relatively recently has it become accepted that male mosquitoes experience intense sexual selection due to the presence of male mate competition and female choice<sup>63</sup>. These results suggest that sexual selection is important for retaining genetic similarity to field populations, indicating that ancestral populations likely experienced a level of sexual selection more like that of the +SS regime than of the -SS regime. Together, the results of the PCA and the  $F_{ST}$  analysis suggest elevated genetic drift after the elimination of male-male competition and female choice and provides an explanation for the high levels of genetic differentiation among these populations.

If the relaxation of sexual selection freed populations to evolve in unique or arbitrary directions, then a reduction in variance effective population size ( $N_e$ ) would be expected because these estimates are based on the magnitude of allele frequency changes over time. This observed reduction in  $N_e$  (Figure 3.3) is unlikely to be attributed to variation in offspring production or fewer breeding individuals of one sex<sup>238</sup> arising from the experimental design, because in both previous work<sup>132</sup> and in supporting experiments for this study (Table 3.1) there was no evidence that female mating probability or fecundity differed between selective regimes. Similarly, because *Ae. aegypti* females are largely monandrous<sup>63</sup> and the time spent with males was limited each generation, it is unlikely that evolutionary regimes differed in the number of contributing males. The estimates of effective population size, combined with patterns of genetic differentiation between the evolved populations, strongly support the idea that evolutionary change occurred via elevated genetic drift after the relaxation of sexual selection. A less likely alternative explanation is that the removal of sexual selection caused populations to rapidly evolve towards new allele frequency optima, which would also result in a lower estimated  $N_e$ . However, there is little evidence of consistent changes in replicate -SS populations that would support this alternative (Figure 3.1A).

The difference in nucleotide diversity ( $\pi$ ) between the -SS and +SS regimes is detectable on all chromosomes in the *Ae. aegypti* genome (Figure 3.4). Significantly

reduced diversity in the -SS replicate lines is symptomatic of the effects of genetic drift, whereby single alleles become fixed in the population. The results of the  $\pi$  analysis also show elevated diversity for both regimes on chromosome 1 compared to chromosomes 2 and 3. A noteworthy feature of chromosome 1 is the presence of the sex-determining M locus. However, recombination is thought to be suppressed at the sex-determining region<sup>222</sup> and the results of the genome-wide  $\pi$  analysis show a corresponding reduction in local nucleotide diversity (Figure 3.5A), so this is unlikely to be the driver of elevated  $\pi$  on chromosome 1. Other studies reporting a relatively elevated  $\pi$  on chromosome 1 in *Ae. aegypti*<sup>239</sup> suggest that additional features of the chromosome such as relatively low gene and transposable element density, or a high number of satellites<sup>240</sup> might be responsible for driving this pattern. Altogether, the analyses of genome-wide allele frequency data show that removal of sexual selection both leads to loss of genetic diversity and drives rapid evolutionary change away from the ancestor.

Broad genomic divergence between evolutionary treatments was identified with the GLMM. Significantly diverged SNPs were identified in large numbers across all three chromosomes of the genome (Figure 3.6). This pattern may reflect the polygenic nature of sexually selected traits, such that manipulating the intensity of sexual selection affects many loci at once<sup>241</sup>. This genome-wide response to the opposing selection regimes may also be, in part, due to patterns of linkage disequilibrium<sup>242</sup>. Fewer generations of evolution mean reduced opportunities for recombination in the genome. However, since the distribution of  $r_w$  for pairs of candidate SNPs on different chromosomes was similar, the level of linkage was determined to be low (Figure 3.7B & 3.7C). Candidate SNPs assigned to different clusters appear to be no more correlated to one another than they are to candidate SNPs on different chromosomes, appearing effectively independent (Figure 3.7B & 3.7C). In addition, all genes that colocalised with high impact candidate SNPs (i.e. those that caused a null allele) identified in this analysis are located in different independent clusters. This excludes the likelihood that linkage disequilibrium caused these genes to evolve together and is consistent with the likely polygenic architecture of quantitative traits. This pattern is consistent with typical 'islands' of divergence seen in comparable experimental evolution studies in *Drosophila pseudoobscura*<sup>218</sup>, though with

predictably lower resolution that might be expected given fewer recombination events in five generations.

The functional annotation of 50,988 candidate SNPs revealed that genetic variants with high and moderate effects on amino acid sequence colocalise with genes enriched for functions related to olfaction, chemosensation and sensory perception (Table 3.2 and Table 3.3), suggesting that odour perception may be a target of sexual selection. Sensory systems underpin key components of mosquito mating behaviour<sup>243,244</sup>. Considerable attention has been paid to the role of acoustic interactions between males and females<sup>213,245</sup>, though relatively little is known about the sexual chemical ecology of (particularly male) mosquitoes<sup>246,247</sup>. Laboratory studies of male *Ae. aegypti* support the production of an aggregation pheromone to recruit both females and other males to mating swarms<sup>248,249</sup>. In close-range interactions, the cuticular hydrocarbon (CHC) heptacosane has been shown in to be involved in male attractiveness to females in laboratory populations of *An. stephensi*<sup>203</sup>, and benzene extracts from conspecific males are important in attracting females in multiple species of *Culex* mosquitoes<sup>250</sup>. In a field study of *An. gambiae*, Adams et al.<sup>202</sup> show that mated males have a higher CHC abundance (by 1.37-fold) than unmated males, suggesting a possible role of CHCs in male mating success. Alongside this previous experimental work, the results presented in this chapter implicating sensory pathways as rapidly evolving targets of sexual selection make a compelling case for further investigation into the role of chemosensation in mating behaviour.

Since this experiment evolved populations in the presence or absence of sexual selection in its entirety, it is not clear whether female choice, mate competition, or a combination of the two, are driving the genomic divergence in chemosensory genes. In a recent large-scale analysis of patterns of sexual selection across taxa, female choice was cited as the most common mechanism of sexual selection associated with olfactory traits in arthropods<sup>251</sup>. However, more work is needed to identify the relative contribution of mate competition and female choice to the evolution of sexually selected traits.

As in other studies manipulating sexual selection<sup>218,252–254</sup>, there was a limited period during the life cycle where social dynamics differed between the opposing sexual

selection regimes. During the limited mating period the local density of males, and of total individuals, differed between the two sexual selection treatments (+SS:  $n = 6$ ; -SS:  $n = 2$ ). There is evidence from studies of *Drosophila* to suggest that altered social environments might favour different behaviours (e.g. longer mating duration in response to the perception of more intense mate competition<sup>255,256</sup>) and may have led to the changes in allele frequencies in this study. However, in contrast with much of the work on the sociosexual environment, the manipulations in these experimentally evolved populations were strictly targeted to just eight hours<sup>132</sup>. In *D. melanogaster* the ability of males to respond to the presence of rivals by adjusting mating duration time required 29 hours of exposure<sup>256</sup>. Though it may not be appropriate to compare *D. melanogaster* and *Ae. aegypti* here given that *Ae. aegypti* females are largely monoandrous<sup>63</sup>, and sperm competition is likely to be low, whereas *D. melanogaster* are polyandrous, the potential effects of an altered social experience should be considered in the design of future experiments. Future work could also investigate the effects of social dynamics on the *Ae. aegypti* reproductive biology and the subsequent effect on the genome.

Whilst this study identified several high confidence candidate genes putatively under sexual selection, functional validation is required before causality can be assigned to these variants<sup>257</sup>. In the next and final chapter of this thesis I aim to identify behavioural phenotypes associated with manipulation of these candidate genes.

## Conclusion

It is increasingly recognised that male mosquitoes experience intense sexual selection due to the presence of male mate competition and female choice<sup>63</sup>. The majority of research on traits involved in male mosquito mating success has focused on the contribution of environmental factors (reviewed in Cator, Wyer and Harrington<sup>63</sup>). Here I provide evidence, as suggested by previous phenotypic observations<sup>132</sup>, that mating success also has genetic determinants which can rapidly evolve. Many previous investigations of sexual selection in mosquitoes have been observational and have focused on correlations between traits and mating success<sup>63</sup>. In addition to the limits on inference that are imposed by work relying on correlations, observational studies are also challenged by the fact that they rely on

researchers to have some idea a priori which traits might be targeted by sexual selection and should therefore be measured. The use of evolve and resequence to investigate sexual selection in mosquitoes is a powerful complement to this kind of work because the genetic basis of mating success can instead be identified by the cumulative and parallel action of selection. In addition, this approach allows estimation of the net effect of sexual selection on patterns of genetic variation. This technique shows great potential for providing novel insights into control-relevant aspects of mosquito behaviour and evolution.

Here, I show that populations evolving with sexual selection that retained competitive sexual performance<sup>132</sup> were more genetically similar to the wild populations from which they originated and harboured more genetic variation. This suggests that the selective environment, including intensity of sexual selection, experienced by lab-reared populations destined for use in reproductive control could have significant impacts on male mating success and control efficacy.

## Chapter 4

### Behavioural Characterisation of Candidate Genes Putatively Involved in Male Mating Behaviour

#### Abstract

*Aedes aegypti* is the primary mosquito vector of Zika, yellow fever, dengue, and chikungunya. As the need for effective mosquito control continues to grow, techniques that manipulate or exploit mosquito mating behaviours are becoming increasingly vital for managing populations and reducing disease transmission. A deeper understanding of the genetic factors that underpin sexually selected traits could prove invaluable for developing more targeted and effective mosquito control strategies. In the previous chapter, evolve and resequence experiments were used to identify 11 genes that showed rapid divergence in response to sexual selection. Here, I conducted a reverse-genetics screen which identified four genes expressed in adult male tissues that could be silenced with RNAi. Functional analysis revealed that one of these genes, *pickpocket315*, plays a crucial role in male mating behaviour. *Pickpocket315*-deficient males exhibited an abnormal mating phenotype, displaying reduced attempts to mate with females, and a decreased phonotactic response. This study supports the finding that *pickpocket315* is under sexual selection in *Ae. aegypti* and highlights the power of evolve and resequence studies coupled with functional genetics to greater understand the genetic basis of sexual selection in *Ae. aegypti*.

#### Introduction

The determinants of male mating success are of significant interest to mosquito biologists, though they are poorly characterised<sup>63,70,129,130</sup>. Male *Ae. aegypti* mosquitoes are thought to be subject to intense sexual selection to outcompete rival males and mate with choosy females<sup>63</sup>. However, surprisingly, it remains unclear which types of traits give *Ae. aegypti* males a competitive advantage in the mating arena. Previous studies have correlated body size<sup>129,131,135</sup>, cuticular hydrocarbon (CHC) profiles<sup>258</sup> and harmonic convergence of wing beat frequencies<sup>117,127</sup> with

male mating success in *Ae. aegypti*. However, such traits are largely inferred from other systems, and rarely is the trait in question manipulated, preventing causality from being assigned to the trait.

Evolve and resequencing (E&R) has been used to great effect to identify the genomic response to selection in experimentally evolved replicate populations<sup>218,259</sup>. E&R has been used to uncover the genetic basis of courtship song<sup>260,261</sup>, cuticle pigmentation<sup>259</sup> and resistance to *Drosophila C virus*<sup>262</sup> in *Drosophila melanogaster*. A recent E&R study by Wiberg et al.<sup>218</sup> evolved *Drosophila pseudoobscura* in the presence and absence of sexual selection and found that areas of genomic divergence contained genes with mutant phenotypes related to courtship and mating. This approach has allowed those working in these other systems to identify the genetic basis of traits of interest through the cumulative and parallel action of selection.

In **Chapter 3** I showed how experimental evolution in the presence and absence of sexual selection led to rapid genome-wide evolution in *Ae. aegypti* and identified several gene candidates putatively under sexual selection, given their large and consistent divergence in allele frequencies between the sexual selection (+SS) and no sexual selection (-SS) regimes (Figure 3.6). However, despite a well-annotated reference genome<sup>222</sup>, the phenotypes of these candidate genes, and many of the other 14,000 protein-coding genes, are unknown. It is crucial to confirm the functional relevance of an identified variant before concluding that it is truly causal<sup>257</sup>. This is because genetic variants may be associated with a trait for other reasons. For example, a variant may respond to the selection treatment because it is in linkage disequilibrium with a true causal variant.

Functional validation of a candidate gene's role in trait expression is often performed using reverse genetics. Reverse genetics involves modifying the activity of a target gene to determine its phenotypic effect. This is typically achieved by direct modification of the genome using techniques such as CRISPR, or by modifying gene expression with RNA interference (RNAi)<sup>257</sup>. The CRISPR-based approach involves both a guide RNA (gRNA) and a CRISPR-associated (Cas) enzyme. The gRNA molecule directs the Cas enzyme to a specific site in the genome, where it then cleaves the target DNA, resulting in a double-stranded break. The cellular repair that

follows this break frequently introduces frameshift mutations in the DNA, which result in disruption of the gene. Whilst CRISPR-based approaches are increasingly common in *Ae. aegypti*<sup>263</sup>, they require significant technical expertise and are relatively time-consuming, so may not be suitable for screening a panel of multiple gene candidates. Further, CRISPR-mediated gene knockout of essential genes is lethal, and so is not suitable when the gene of interest has a crucial role in survival. Mating success is likely to be closely associated to fitness and overall condition (see **Chapter 2** for discussion)<sup>181</sup>, so this may increase the likelihood of a genetic knockout having survival effects.

RNAi is a biological process by which gene expression is suppressed by double stranded RNA (dsRNA). Work in *D. melanogaster* identified the role of Dicer in the enzymatic cleavage of dsRNA into small interfering RNAs (siRNA)<sup>264</sup>, the strands of which unwind and the antisense strand of siRNA binds to the RNA-induced silencing complex (RISC) which allows the targeting of complementary messenger RNA (mRNA) for degradation. RNAi has become an important and commonly used reverse-genetics tool for the transient silencing of gene expression to determine gene function in model and non-model insects.

dsRNA can be synthesised *in vitro* with a strand complementary to the mRNA of the gene of interest and delivered with relative ease, traditionally with intrathoracic injection (for example, see Magalhaes et al.<sup>265</sup> and Franz et al.<sup>266</sup>), knocking down expression of the target gene at the post-transcriptional level, and the resulting phenotype examined<sup>267</sup>. RNAi was first performed in mosquitoes by injection of dsRNA into the haemolymph to deplete expression of the *defensin* gene in *Anopheles gambiae*<sup>268</sup>. It has been used to great effect since, silencing a range of genes involved in key processes and behaviours such as host-seeking<sup>269</sup>, nutritional signalling<sup>270</sup>, immunity to dengue infection<sup>271</sup> and sperm development<sup>272</sup>.

RNAi is not without its technical challenges; silencing efficiency in mosquitoes can vary greatly between genes<sup>273</sup>, tissues and species. For example, genes expressed in salivary glands require higher doses of dsRNA to achieve RNAi in *An. gambiae*, possibly due to the relatively low permeability to dsRNA and reduced expression of Dicer in salivary gland tissue<sup>274</sup>. However, several studies have described methods to optimise silencing efficiency in *Ae. aegypti*<sup>273,275</sup>, with intrathoracic injection of

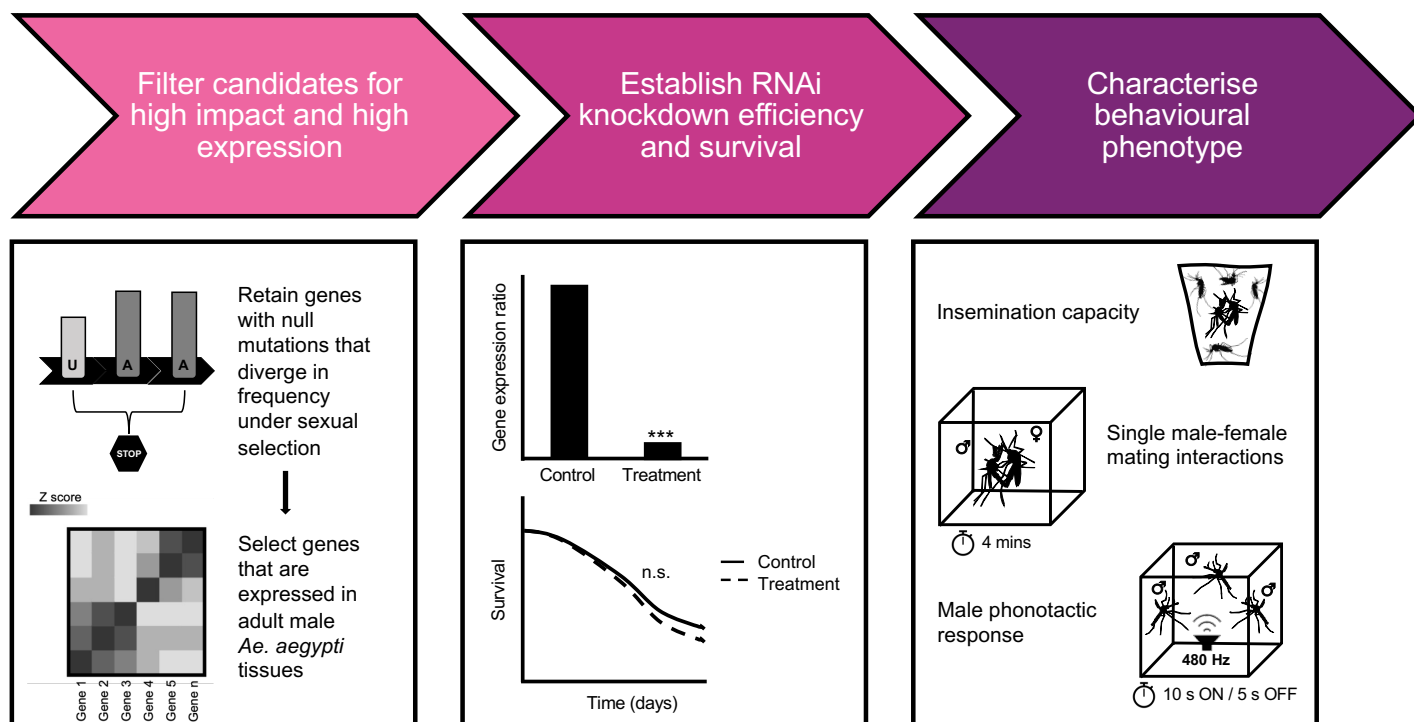
dsRNA still arguably the most simple, high throughput, and readily customisable method for functional analysis of genes of interest in mosquitoes<sup>275</sup>.

In this chapter I performed RNAi-mediated gene knockdown on a subset of high confidence candidate genes identified in the analysis of pooled DNA sequence data from populations of *Ae. aegypti* evolved with and without sexual selection in **Chapter 3**. Candidate genes that were effectively silenced with RNAi were subject to a mating capacity assay, and any genes that responded were characterised with a panel of behavioural assays. I hypothesised that silencing of candidate genes that responded to evolution under opposing sexual selection regimes would result in a mating-related phenotype.

## Methods

### Experimental plan

These methods describe the sequence of experiments performed to assess the effect of expression knockdown of a pool of candidate genes on the male mating phenotype (Figure 4.1). The methodology outlined below begins with the filtering of candidate genes based on their expression levels in adult male tissues. The subsequent knockdown of suitable genes and broad assessment of survival and insemination capacity was performed using the *Ae. aegypti* Liverpool reference strain. The behavioural characterisation experiments that assess single male-female mating interactions and the male phonotactic response were performed using a low-generation (< F7) Thai strain of *Ae. aegypti*.



**Figure 4.1. Schematic of Chapter 4 experimental design.**

### Selecting pool of putative targets of sexual selection for RNAi

Of the total ~8.7 million SNPs identified in the bioinformatics analysis of pooled sequencing data from samples of male *Ae. aegypti* mosquitoes from populations evolved with and without sexual selection (see **Chapter 3** for full detailed methods of variant calling and filtering), 50,988 variants were found to have significantly diverged between sexual selection (+SS) and no sexual selection (-SS) treatments. Variant effect predictor  $\text{SNPEff}^{235}$  was used to determine the effect of the 50,988 SNPs on amino acid sequence. Those SNPs that resulted in a change in amino acid were termed “moderate impact mutations”, and those that resulted in a stop codon were termed “high impact mutations”. Within the subset of significantly diverged variants were 12 high impact mutations in 11 genes (Supplementary Table 1). In order to determine suitability for RNAi-mediated gene silencing these 11 genes were further filtered to exclude those with relatively low expression in adult male tissues according to publicly available transcriptomic data from Matthews et al<sup>276</sup>.

## dsRNA synthesis

To achieve gene knockdown total RNA was extracted from adult *Ae. aegypti* Liverpool mosquitoes using Trizol (ThermoFisher), and residual genomic DNA (gDNA) removed using the TURBO DNA-free Kit (Life Technologies). Complementary DNA (cDNA) was generated using LunaScript RT SuperMix (NEB). Target regions (200-600 bp) were amplified from cDNA by polymerase chain reaction (PCR) using CloneAmp HiFi PCR Premix (Takara) and custom primers flanked with the T7 promotor sequence TAATACGACTCACTATAGGG (Table 4.1). The targeted genes were *adam* (AAEL004881), *rolling pebbles* (AAEL005908), *gustatory receptor GR11* (AAEL011174), *pickpocket315* (AAEL000863), and 3 unknown genes (AAEL019639, AAEL022941 and AAEL015079). Ribosomal protein S7 primers (custom design) were used as an internal control to confirm cDNA presence. GFP served as a negative control and was synthesised using a plasmid template containing GFP (gift from D. Ellis).

To generate sufficient template, three 50 µL PCR reactions were performed for each gene. Each 50 µL reaction consisted of 25 µL HiFi polymerase, 5 µL of each forward and reverse primers, 2.5 µL of cDNA and 12.5 µL of dH<sub>2</sub>O. Amplification was performed on a Veriti 96-Well thermal cycler at the following conditions: 1 minute at 98 °C, followed by 40 cycles of 10 seconds at 98 °C, 10 seconds at 56 °C, and 30 seconds at 72 °C, and lastly a 2 minute extension time at 72 °C.

To verify the amplicon size, 5 µL of PCR product was run on a 1 % agarose gel at 90 V for 40 minutes. The remaining 145 µL of the pooled PCR reactions was then purified using the QiaQuick PCR purification kit (QIAGEN) to remove any off-target amplification products. To generate dsRNA 1 µg of purified PCR product was used as the template for *in vitro* transcription using the T7 high yield transcription kit (ThermoFisher). Two 20 µL reactions were carried out for each gene, incubated at 37 °C overnight to achieve sufficiently high concentrations of dsRNA. Both *in vitro* transcription reactions were then pooled and purified using the RNeasy kit (QIAGEN), eluting in 20 µL of RNase-free water (QIAGEN). dsRNA concentration was measured on a Nanodrop and 2 µL run on a 1 % agarose gel at 90 V for 40 minutes to check integrity. dsRNA was then split into 5 µL aliquots (to avoid repeated

freezing and thawing) and stored at -20 °C for up to 6 months. Immediately prior to microinjection the dsRNA was heated to 95 °C for 2 minutes and cooled back to 25 °C at a rate of 0.1 °C /s to anneal any single stranded RNAs (ssRNA).

**Table 4.1. Primers for PCR amplification of genes of interest plus GFP and positive control S7**

| Gene Name / ID                                | Forward Primer Sequence                            | Reverse Primer Sequence                                |
|-----------------------------------------------|----------------------------------------------------|--------------------------------------------------------|
| adam/<br>AAEL004881                           | TAATACGACTCACTATAGGG<br>GCCAAGAAGACCCGAGTGA        | TAATACGACTCACTATAGGGAC<br>GTCACGTTCTCGTAGACG           |
| rolling pebbles/<br>AAEL005908                | TAATACGACTCACTATAGGGT<br>CCGCCAATAATGCCACCTT       | TAATACGACTCACTATAGGGFA<br>TCTGGTGCTGTTCTCGCT           |
| gustatory receptor<br>Gr11/<br>AAEL011174     | TAATACGACTCACTATAGGGA<br>AGCTCATTGAAACCGTTGG       | TAATACGACTCACTATAGGGCC<br>CTTGTTGTATCTTGCTCCTTG        |
| AAEL019639                                    | TAATACGACTCACTATAGGGA<br>CCAAACGAACTCCGACCC        | TAATACGACTCACTATAGGGCG<br>TTGAACATCACCCAGTTGTC         |
| AAEL022941                                    | TAATACGACTCACTATAGGGC<br>GAACCGGAGAGTTGGTCAA       | TAATACGACTCACTATAGGGTC<br>ATTCCACGGATTACGGATCC         |
| pickpocket 315/<br>AAEL000863                 | TAATACGACTCACTATAGGGC<br>TTCGGAGGAACTTCGAAGCT      | TAATACGACTCACTATAGGGCA<br>AGCTTCCTCCGATAGCTTCT         |
| AAEL015079                                    | TAATACGACTCACTATAGGGC<br>GCCATCTACCTGTCAACCA       | TAATACGACTCACTATAGGGAT<br>CGCCAACAGGTAGAACGG           |
| GFP (Eggleston<br>and Adelman <sup>40</sup> ) | TAATACGACTCACTATAGGGA<br>TGGTGAGCAAGGGCGAGGA<br>GC | TAATACGACTCACTATAGGGAT<br>CTTCAACTTCACCTTGATGCCG<br>TT |
| S7                                            | ATGGTTTTTCGGATCAAGG                                | GGAATTCGAACGTAACGTCAC                                  |

## Microinjection of dsRNA

*Ae. aegypti* Liverpool eggs were hatched under a vacuum for 20 minutes. Newly emerged larvae were provided with 0.1 mg of ground fish food (Cichlid Gold [#04328], Hikari, Himeji City, Japan) and held in a 27 °C incubator overnight. First instar larvae were sorted into trays of 500 larvae / 1L water and provided fish food pellets *ad libitum*. Pupae were sorted by sex and allowed to emerge in separate cages. 0–2 day old male *Ae. aegypti* mosquitoes were anaesthetised on wet ice and dsRNA was injected into the thorax using the Nanoject II microinjector (Drummond

Scientific) and borosilicate glass capillary needles (0.2 µg in 60 nL per mosquito). Mosquitoes were allowed two days to recover post injection and provided with 10 % sucrose *ad libitum*.

### Quantitative real-time PCR (qRT-PCR)

qPCRs were performed to detect expression of target genes and evaluate knock down efficiency. Primers were designed for each of the seven target genes and the S7 internal control using Benchling. Primer efficiencies were first calculated to compare expression of target and housekeeping genes. Primer efficiencies were calculated in Excel using the average C<sub>q</sub> values and log<sub>10</sub> values for the cDNA dilution series. Primers all had an efficiency of 80-120 %. Melt-curves were also performed for all primer pairs to confirm the presence of just one amplicon.

cDNA template was synthesised from RNA extracted from a pool of 20 0-2 day old male mosquitoes as described above. Reaction mixtures totalling 10 µL were composed of 1 µL of male cDNA, 5 µL of PowerUp SYBR Green Master Mix (Life Technologies), 0.5 µL of each the forward and reverse primer (10 µM) and 3 µL of dH<sub>2</sub>O. Amplification was performed on the LightCycler 96 (Roche) using the standard cycling mode recommended for PowerUp SYBR Green Master Mix and primers with a T<sub>m</sub> ≥ 60 °C (50 °C for 2 minutes, 95 °C for 2 minutes, 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute, followed by a single cycle of melt curve 95 °C for 10 seconds, 65 °C for 1 minute and 97 °C for 1 second).

### qPCR Analysis

Relative gene expression was calculated for each treatment relative to the GFP control based on three biological replicates and two technical replicates using the Pfaffl method<sup>277</sup>:

$$Gene\ expression\ ratio = \frac{(E_{GOI})^{\Delta Ct\ GOI}}{(E_{HKG})^{\Delta Ct\ HKG}}$$

Where  $E$  is the primer efficiency, either of the gene of interest ( $GOI$ ) or the housekeeping gene ( $HKG$ ), and  $\Delta Ct$  is the control average  $Ct$  value minus the average  $Ct$  value.

**Table 4.2. Primers for qRT-PCR analysis of RNAi knockdown efficiency**

| Gene Name                                 | Forward Primer Sequence | Reverse Primer Sequence |
|-------------------------------------------|-------------------------|-------------------------|
| adam/<br>AAEL004881                       | GTTGCCCTGATCGTGGTGA     | TGTGCACTATGACGCCGTT     |
| rolling pebbles/<br>AAEL005908            | ACCACTGGACCGTGCAATT     | CTGCAGGCGATTTTTGCGA     |
| Gustatory receptor<br>GR11/<br>AAEL011174 | TACTGTCGTGTGCGAGTGAC    | ACGTACAGCATGACGAAGCA    |
| AAEL019639                                | CCGGTAAACTATACCAGGTCGG  | TCCGCTTGAGCGAGATCTTC    |
| AAEL022941                                | TAGTGGTGAATACGCTGTCGAAA | AGCGATGGCAGTTTCATTTTCG  |
| Pickpocket 315/<br>AAEL000863             | CCTCGGAACACTACGTCACC    | GATGCTGGCTATGAGATCGGT   |
| AAEL015079                                | GGATGCGAAGTTGGACGA      | TAGACTCCGTTCAAACGCCCG   |
| S7 (McFarlane et<br>al. <sup>41</sup> )   | CCAGGCTATCCTGGAGTTG     | CACGTGCTTGCCGGAGAAC     |

### Survival assay

To test whether knockdown of any of the genes of interest increased mortality, survival of injected male mosquitoes was recorded. Twenty 0-2 day old *Ae. aegypti* males were injected with dsRNA and held in 16 oz paper cups and provided with 10 % sucrose *ad libitum*. Mortality was recorded every other day from one day post-injection (dpi) until 30 dpi. Three replicates were completed per treatment.

### Insemination capacity

To determine if intrathoracic dsRNA injection influenced the ability of a male to mate a female or multiple females, insemination capacity was measured. Insemination capacity was only assessed for genes for which expression could be significantly

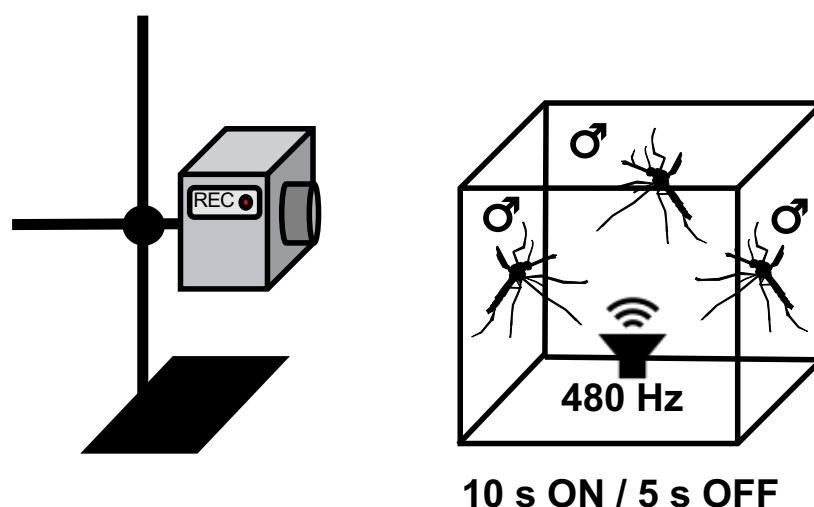
depleted and for which survival was not reduced in the first seven days following injection. dsRNA-injected males (2 dpi) were held singly in 16 oz cups with six virgin females and provided 10 % sucrose solution *ad libitum*. After 24 hours all females were collected, anaesthetised on wet ice and their spermathecae dissected in 1 x phosphate buffered saline (PBS) and examined under a brightfield microscope to determine insemination status. The insemination capacity of around 60 males per gene were assessed over three replicates.

### **Single male-female mating interactions**

To capture any change in the ability of the dsRNA knockdown males to locate and orient to females, the outcome of single male-female interactions was assessed. Male mosquitoes 2 dpi were held singly in 19 cm<sup>3</sup> perspex cages with 10 % sucrose solution provided *ad libitum*. Sucrose was removed from the cages at 0800 on the day of the phonotactic assay. All experiments were performed between 0900 and 1200. A single female was added to each cage using a mouth aspirator and the number of mating attempts, defined as physical contact between the male and female, was recorded. The time at which the first mating attempt was recorded was used to calculate the contact latency, and whether the pair formed a mating copula (defined by genital contact between the male and female) was recorded (Y/N). For those pairs that formed a mating copula, the length of time for which there was genital contact between the male and female was used to calculate copula duration. The trial was halted after four minutes, or after a mating copula was formed, whichever was sooner. For those trials where the male and female formed a mating copula, the female was removed and the spermatheca dissected in 1 x PBS and examined under a brightfield microscope to confirm that insemination had occurred. All data was recorded by the observer at the time of the assay. The experiment was repeated three times, with 20 males for each treatment in the first replicate, 30 males for each treatment in the second replicate, and 29 dsGFP males and 30 dsPPK315 males in the third replicate (159 trials total; 79 dsGFP and 80 dsPPK315).

### **Phonotactic response and activity assay**

To assess any effect of gene knockdown on the ability of males to respond to female flight tones, the phonotactic response was measured. Male *Ae. aegypti* mosquitoes show reliable phonotaxis towards pure tones within the range of frequencies produced by females<sup>110,111,278</sup>. Male mosquitoes 2 dpi were held in 16 oz cups with 10 % sucrose solution provided *ad libitum*. Sucrose was removed from the cups at 0800 on the day of the phonotactic assay. All experiments were performed between 0900 and 1200. Three males from each treatment were aspirated into a 19 cm<sup>3</sup> plexiglass cage containing a single earphone speaker (Figure 4.2). The males were allowed one minute to acclimatise to the cage before a 480 Hz tone (as in Cator and Zanti<sup>134</sup>) was played through the speaker at maximum volume (~100 dB) in bouts of ten seconds, with five second intervals between each bout, for a total of two minutes. Due to the difficulty in observing multiple males in this assay, all data was recorded by watching video playback of each two-minute trial. The video was recorded using a GoPro HERO4 camera mounted on a clamp. Videos were used to record the timing of each contact any one of the males made with the speaker during the trial. A contact was defined by male legs touching the speaker. Whether or not any one male physically contacted the speaker was recorded (Y/N), and the time until physical contact was made with the speaker was used to calculate contact latency. The proportion of the two-minute trial for which any one male was in flight was determined by watching the video recordings and manually calculating activity time.



**Figure 4.2.** Diagram of the phonotactic response and activity assay set up.

## Statistical analysis

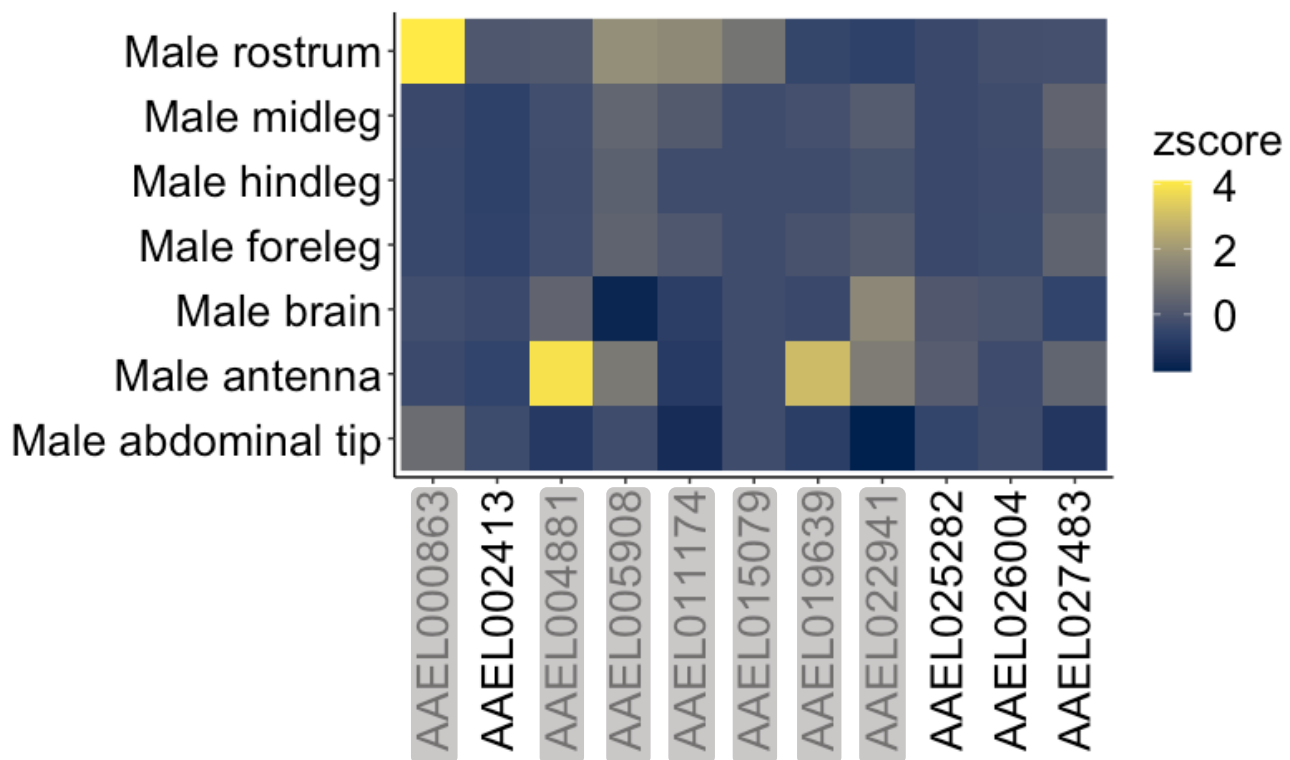
All analyses were conducted in R (version 4.0.3)<sup>279</sup>. Independent *t*-tests were used to determine if injection with dsRNA resulted in a significant reduction in target gene expression relative to the control. A log-rank test was used to determine if there was a difference in survival between each test gene and the control pre- and post- seven days following dsRNA injection. Whether males were able to achieve a mating in 24 hours (Y/N) was analysed with a binomial Generalised Linear Model (GLM) (package 'afex') with knockdown gene as predictor and mating status as response variable, with replicate included as a random factor. Number of females mated out of a maximum of six in 24 hours was analysed with GLM (Poisson distribution) with gene knockdown as treatment and number of females mated as the response variable with replicate included as a random factor.

GLMs were also used to detect an effect of gene knockdown on key behaviours. For single male-female mating interactions the response variable was 'contact latency', 'copulation duration' or 'number of mating attempts', with replicate as a random factor. To assess the effect of gene knockdown on the phonotactic response a GLM was fit with a binomial distribution, the response variable was the outcome of the trial (i.e. whether contact made with the speaker playing female flight tones, Y/N) with replicate included as a random effect. The effects of gene knockdown on other aspects of behaviour as part of the phonotactic response assay were also analysed using a GLM with activity time (the amount of the trial spent in flight) or contact latency (time until contact was made with the speaker) as the response variable, with replicate included as a random factor.

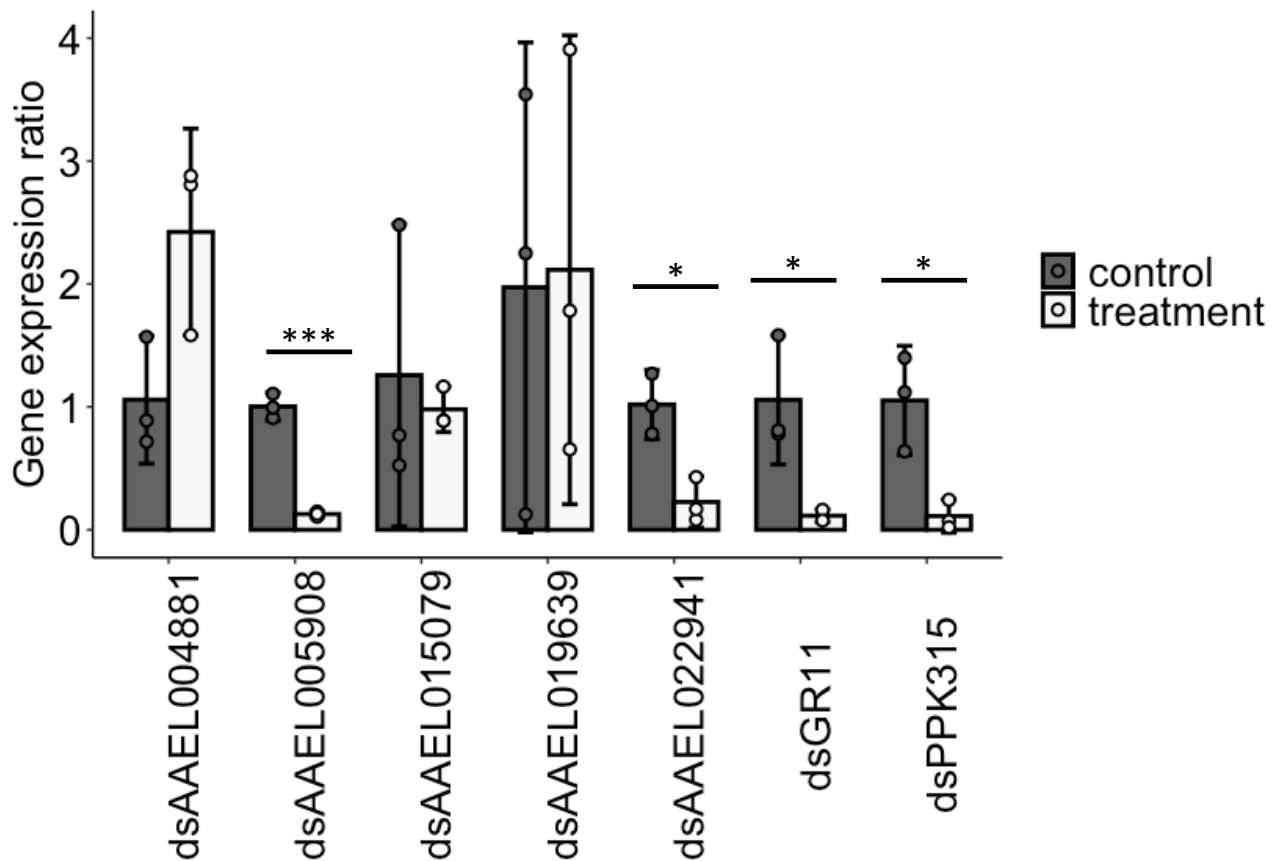
## Results

The results from **Chapter 3** identified 11 genes that colocalised with 12 candidate high-impact (null) variants (Supplementary Table 1). Previously published expression data from *Ae. aegypti*<sup>276</sup> indicated that seven of the 11 genes showed relatively high expression in adult male tissues (Figure 4.3). These seven genes were selected for RNAi-mediated gene silencing in order to test their role in male mating-related phenotypes.

Four of the seven genes were effectively silenced two days following intrathoracic injection with 500 ng of dsRNA (*gustatory receptor11*, *pickpocket315*, AAEL022941 and AAEL005908) and were available to screen for effects on male mating success (Figure 4.4).



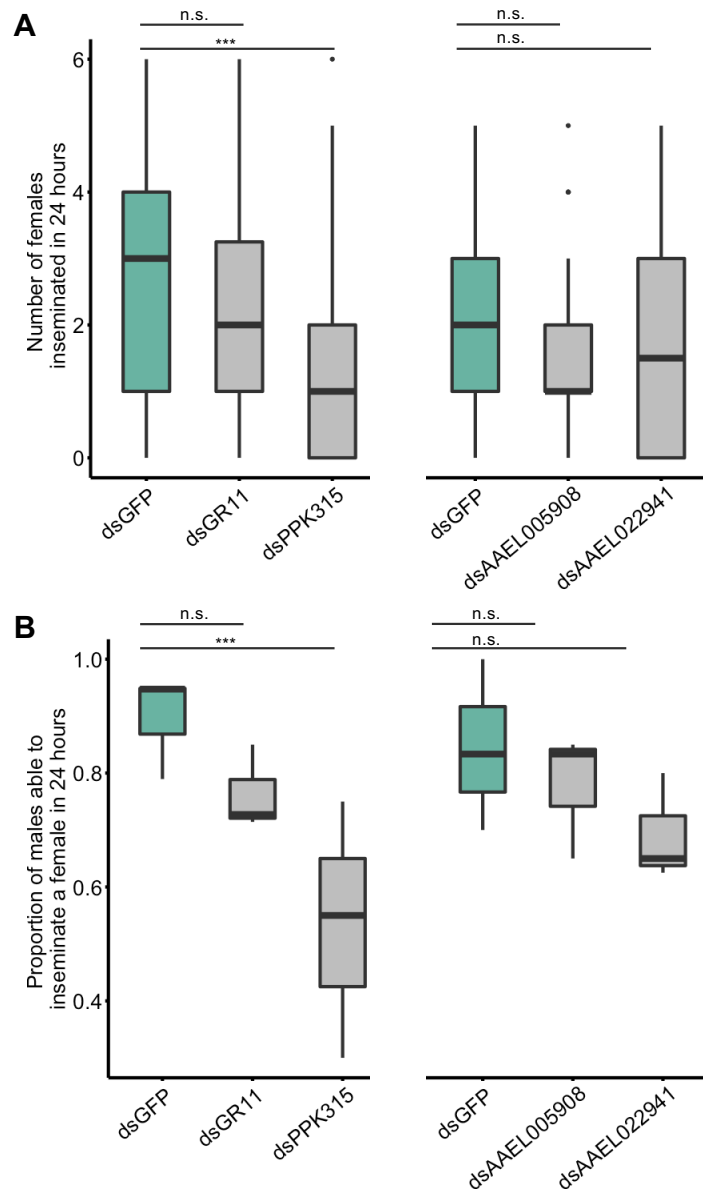
**Figure 4.3. Tissue-specific expression of high functional impact candidate SNPs.** Z scores by gene for transcriptomic data from adult male tissue samples sequenced by Matthews et al.<sup>276</sup>. The seven genes (highlighted in grey) with the highest expression levels in any of the adult tissues were selected for RNAi-mediated gene silencing.



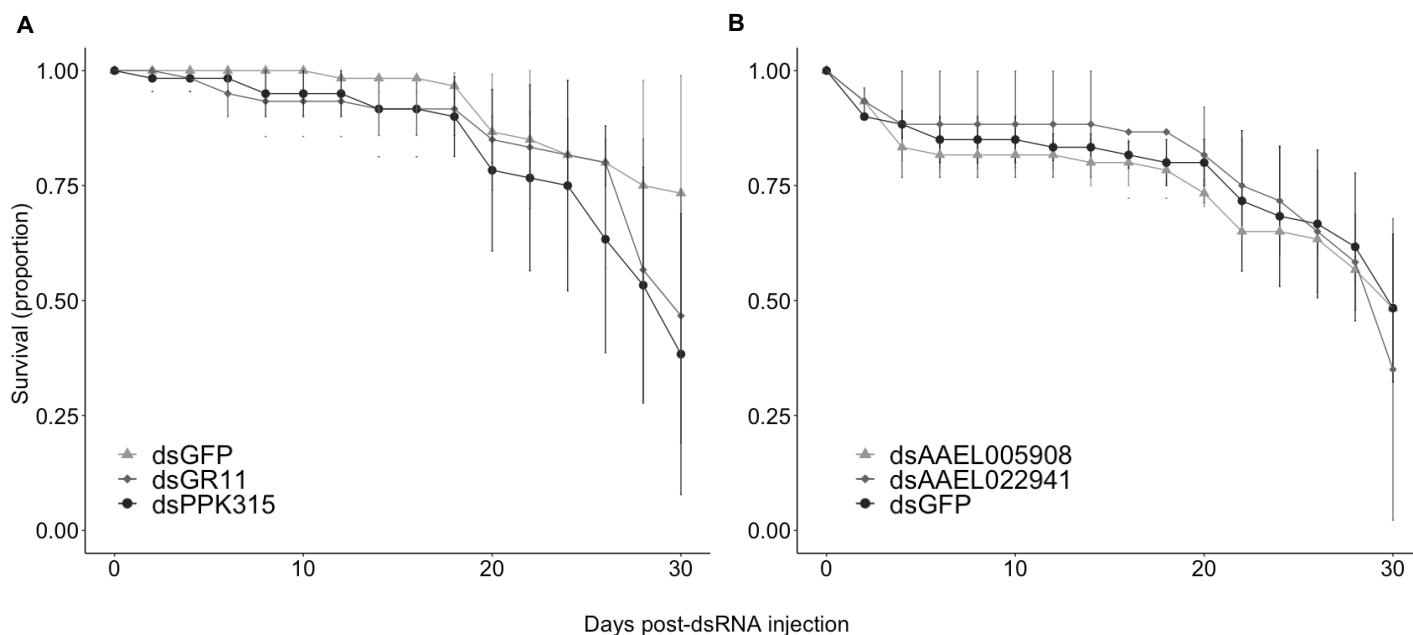
**Figure 4.4. Efficacy of candidate gene silencing using RNAi.** Gene expression ratio of all seven genes targeted with intrathoracic injection of double-stranded RNA (dsRNA) with respect to a dsGFP control. Bars show mean and two standard errors of gene expression ratio of the gene of interest relative to ribosomal S7 housekeeping gene from three biological replicates (each replicate is plotted as a circle in line with the bars) of 20 whole male mosquitoes. P-values less than 0.05 are marked with one star (\*), p-values less than 0.01 are marked with two stars (\*\*), and p-values less than 0.001 are marked with three stars (\*\*\*).

To measure insemination capacity of males with reduced target gene expression, two days following dsRNA injection virgin male mosquitoes were held singly with six virgin females and allowed to mate for 24 hours. The reproductive tracts of females were dissected to determine the number of females that individual males were able to successfully inseminate in this period. Males with reduced *pickpocket315* (dsPPK315) expression inseminated significantly fewer females relative to the dsGFP control (Figure 4.5A; GLM: p-value < 0.001). Strikingly, while 89 % of control males were able to mate in the 24-hour period, only 55 % of the males with depleted *ppk315* expression were able to achieve a single successful mating (Figure 4.5B;

GLM:  $p$ -value < 0.001). This was not the result of a general deleterious effect of *ppk315* knockdown in these males, as I did not observe any significant decrease in survival associated with knockdown of *ppk315* expression in the first seven days post injection (Figure 4.6). Knockdown of *gustatory receptor 11*, AAEL022941, and AAEL005908 did not affect insemination capacity (Figure 4.5A and B; GLM with replicate as factor:  $p$ -value = 0.38,  $p$ -value = 0.70,  $p$ -value = 0.13, respectively).

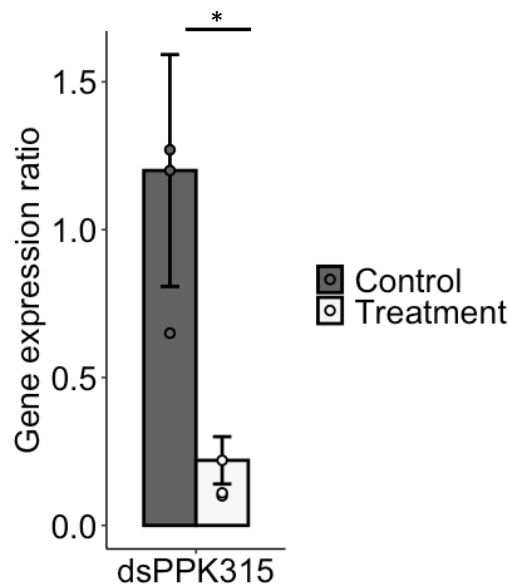


**Figure 4.5. Functional assessment of male insemination capacity following RNAi.** (A) Boxplot showing the mean number of females a male was able to inseminate (of a maximum possible six) over 24 hours, two days post-injection with double-stranded RNA (dsRNA) targeting four high-confidence candidate genes and a GFP control. (B) Boxplot showing the proportion of males from each treatment that were able to inseminate at least one female during the 24 hour assay. \*\*\* indicates a significant difference between the treatment and its control using a GLM at a  $p$ -value < 0.001.



**Figure 4.6. Male survival following RNA-mediated gene silencing of candidate genes.** Proportion of males surviving measured every two days for 30 days post injection with double-stranded RNA (dsRNA) targeting one control gene (GFP) and 4 target genes (GR11, PPK315, AAEL005908, AAEL022941). (A) Survival of *gr11* and *ppk315* relative to a GFP control. (B) Survival of AAEL005908 and AAEL022941 relative to a GFP control. Points indicate mean and standard error of proportion of surviving males from three independent biological replicates, each consisting of 20 males.

After establishing a mating-related phenotype in males with reduced expression of *ppk315*, subsequent experiments were performed using the low-generation Thai strain of *Ae. aegypti*. Firstly, the knockdown efficiency of *ppk315* was confirmed for this strain, using the same volume and concentration of dsRNA that had been used to silence candidate genes in the Liverpool strain. Expression of *ppk315* was significantly reduced in males of the Thai genetic background (Figure 4.7; two sample *t*-test:  $t = 4.4883$ ,  $df = 4$ ,  $p\text{-value} = 0.01092$ ).

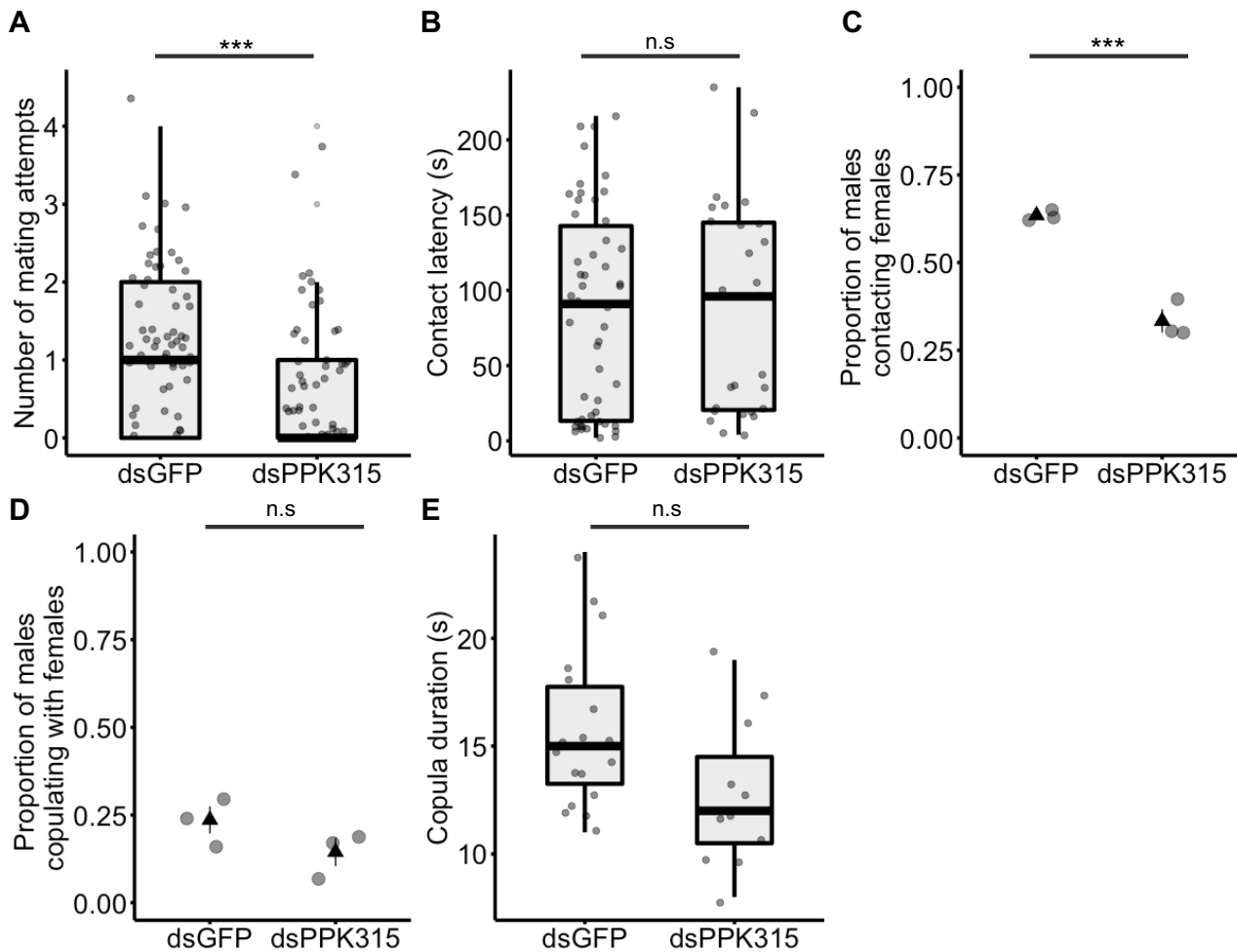


**Figure 4.7. Efficacy of *pickpocket315* gene silencing in Thai genetic background *Ae. aegypti* males.** Gene expression ratio of dsPPK315 with respect to a dsGFP control. Bars show mean and two standard errors of gene expression ratio of gene of interest, *pickpocket 315* (AAEL000863) relative to ribosomal S7 housekeeping gene from three biological replicates (individual replicates plotted as dots) of 20 whole male mosquitoes. P-values less than 0.05 are marked with one star (\*).

The results of the single male-female interactions showed that males with depleted expression of *ppk315* made significantly fewer mating attempts than control males (Figure 4.8A; dsGFP mean =  $1 \pm 0.110$  SE, dsPPK315 mean =  $0.488 \pm 0.091$  SE, GLM with replicate as factor: p-value < 0.001). Of those males that did contact / attempt to mate with a female, there was no difference in the time it took to make contact (Figure 4.8B; GLM with replicate as a factor: p-value = 0.055), though there was a significant effect of replicate 3 (p-value < 0.001). Overall, a smaller proportion of males with reduced *ppk315* expression contacted a female (mean proportion = 0.635, SD = 0.015) compared to the control treatment (mean proportion = 0.333, SD = 0.058) (Figure 4.8C; binomial GLM with replicate as factor: p-value < 0.001)

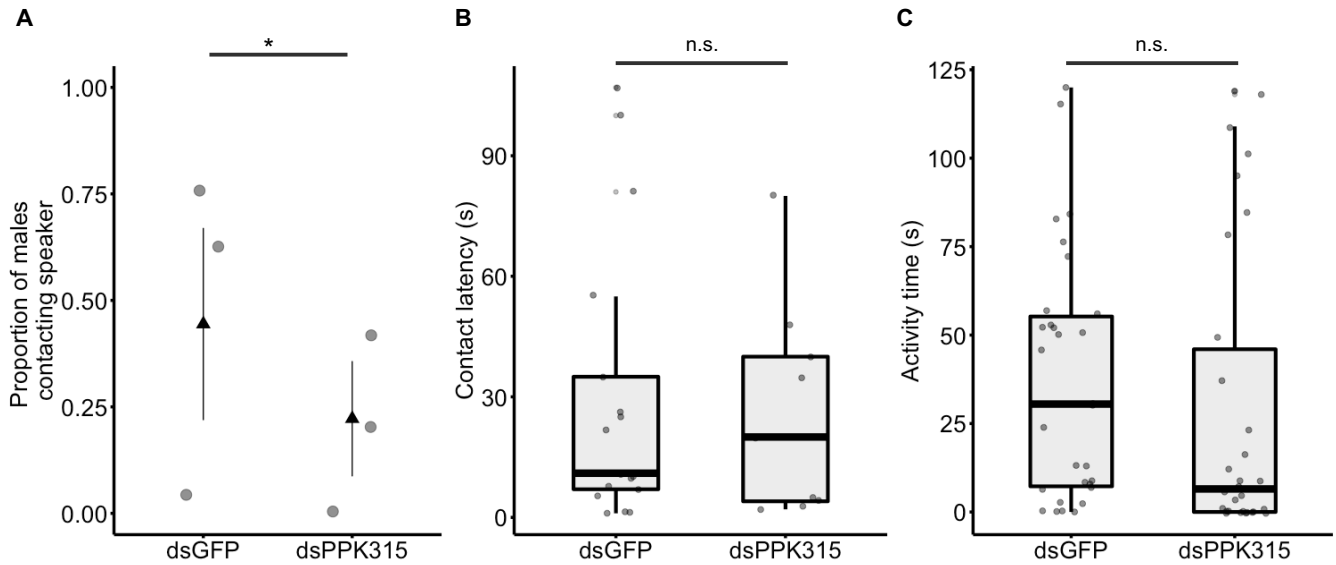
Despite the differences in mating attempts, there was no difference in the proportion of males from each treatment that successfully mated with a female (Figure 4.8D; binomial GLM with replicate as factor: p-value = 0.1433), with an average of 23.6 % (SD = 6.68 %) of dsGFP males mating with a female and 14.4 % (SD = 6.94 %) of dsPPK315 males mating with a female. There was also no significant difference in

the duration of any copulas formed (Figure 8E; GLM with replicate as factor:  $p$ -value = 0.0729).



**Figure 4.8. Single male-female mating interactions following RNAi targeting high-confidence candidate gene, *pickpocket 315* (dsPPK315) and control gene GFP (dsGFP).** Three replicates were performed in total. For the dsGFP treatment there were  $n = 20$ ,  $n = 30$ , and  $n = 29$  males assayed for replicates 1, 2 and 3, respectively. For the dsPPK315 treatment there were  $n = 20$ ,  $n = 30$ , and  $n = 30$  males assayed for replicates 1, 2 and 3, respectively. (A) Box plot showing the number of mating attempts made in the four-minute trial. A mating attempt was defined as any incidence of contact between the male and female, regardless of outcome. Data from individual males shown as grey circles. (B) Box plot showing time to first contact between the male and female. Data from individual males shown as grey circles. Only males that physically contacted a female are plotted. For the dsGFP treatment the sample sizes were  $n = 13$ ,  $n = 19$ , and  $n = 18$  for replicates 1, 2 and 3, respectively. For the dsPPK315 treatment the sample sizes were  $n = 6$ ,  $n = 9$ , and  $n = 12$  for replicates 1, 2 and 3, respectively. (C) Dot plot showing proportion of males that contacted females for each individual experimental replicate, and overall mean from three replicates (black triangles) with standard deviation. (D) Dot plot showing proportion of males that formed a successful copula with a female for each individual experimental replicate, and overall mean from three replicates (black triangles) with standard deviation. (E) Box plot showing the duration of any successful copulas formed during the four-minute trial. Data

from individual males shown as grey circles. Only data for males that formed a mating copula are plotted. For the dsGFP treatment the sample sizes were  $n = 6$ ,  $n = 5$ , and  $n = 7$  for replicates 1, 2 and 3, respectively. For the dsPPK315 treatment the sample sizes were  $n = 4$ ,  $n = 5$ , and  $n = 2$  for replicates 1, 2 and 3, respectively. For all figure panels dots have been spread out using the `geom_jitter` function for ease of visualisation. Three stars (\*\*\*) indicates a significant difference between the dsPPK315 test treatment and GFP control treatment using a generalised linear model (GLM) at a  $p$ -value  $< 0.001$ .



**Figure 4.9. Male phonotactic response to artificial female flight tones following RNAi targeting high-confidence candidate gene, *pickpocket 315* (dsPPK315) and control gene GFP (dsGFP).** Three replicates were performed in total. For both the dsGFP and the dsPPK315 treatments there were  $n = 10$ ,  $n = 15$ , and  $n = 5$  males assayed for replicates 1, 2 and 3, respectively. (A) Proportion of males contacting speaker. Each point is the proportion of males contacting speaker from an individual replicate. The triangles depict the mean of the three replicates, and bars are standard deviation from the mean. (B) Boxplot showing the latency to speaker contact, with only males that contacted the speaker included. For the dsGFP treatment the sample sizes were  $n = 6$  and  $n = 11$  for replicates 1, 2, respectively, whilst no observations were recorded for replicate 3. For the dsPPK315 treatment the sample sizes were  $n = 2$  and  $n = 7$  for replicates 1 and 2, respectively, whilst no observations were recorded for replicate 3. Dots are the contact latency times for individual males. (C) Boxplot showing the number of seconds that any one male in a cage was in flight during the assay. Dots are the amount of time (in seconds) spent in flight for individual cages. One star (\*) indicates a significant difference between the *pickpocket315* test treatment and GFP control treatment using a generalised linear model (GLM) at a  $p$ -value  $< 0.05$ .

The results of the phonotaxis assay showed that cages of males with reduced expression of *ppk315* were less likely to contact the speaker playing artificial female

flight tones (Figure 4.9A; binomial GLM with replicate as factor: p-value = 0.0241). Interestingly however, if a male did contact the speaker, there was no significant difference in the time taken to make contact (Figure 4.9B; GLM with replicate as factor: p-value = 0.779). The lack of significance in this result may be due to a very small sample size given that only 9 cages (of a possible 30) in the dsPPK315 treatment resulted in speaker contact as opposed to the 17 in the dsGFP control cages. Finally, there was no significant difference in the amount of time spent in flight between the control and test treatments (Figure 4.9C; GLM with replicate as factor: p-value = 0.425).

## Discussion

The identification of genes involved in *Ae. aegypti* male mating success has major benefits for maintaining mating competitiveness in laboratory-reared colonies intended for field release, as well as for advancing our understanding of male mating biology in this medically important species. Several phenotypic traits have previously been associated with male mating success (or proxies thereof) in mosquitoes (e.g. body size<sup>146</sup>, sugar feeding<sup>280</sup> and acoustic signalling<sup>117</sup>). However, little is known about the genetic basis of these traits, nor if they are truly under sexual selection, rather than merely correlated with mating success. In **Chapter 3** (and in Wyer, Cator and Hollis<sup>281</sup>), I identified several genes that evolved rapidly in the presence or absence of sexual selection, indicating that they are likely under sexual selection in *Ae. aegypti*. However, causation cannot be applied to these genes without functional validation. In this study, I used a reverse genetics approach to silence a subset of the candidate genes identified in **Chapter 3** and found that knockdown of *ppk315* had a significant impact on male mating behaviour. Males with depleted expression of *ppk315* showed a reduced ability to inseminate females, were less likely to contact females, made fewer attempts to mate with females, and showed a slightly reduced phonotactic response. These same males exhibited no differences in survival or in a simple measure of flight activity, suggesting that the expression of *ppk315* affects male traits specific to mating performance. These results represent the first phenotypic characterisation of the *ppk315* gene in *Ae. aegypti* and demonstrate the

successful validation of an evolve and resequence approach using reverse genetics in this species.

The transient silencing of the *ppk315* gene resulted in males inseminating fewer females than controls (Figure 4.5A). Moreover, *ppk315*-depleted males were less likely to inseminate a female at all (Figure 4.5B). This result should be considered within the selective environment that it was carried out in. That is to say that when test males neither needed to overcome male competition for mates or female choice, and were within close range of a female, that they still were not able to mate as many females as the control males. It is known that in these non-competitive laboratory settings where males are provided many virgin females to mate with, males can inseminate multiple females<sup>63,94,96,98,123,282</sup>.

Males with reduced expression of *gustatory receptor 11 (gr11)*, AAEL005908 and AAEL022941 appeared to be equally likely to mate a female as control males and to mate a similar number of females (Figure 4.5A and B). The absence of a detectable effect of these three genes on insemination capacity in this study does not necessarily indicate that they have no role in male mating success (and were therefore false positives in the screen in **Chapter 3**). It may be that knockdown of these genes instead had effects that were not captured by the insemination assay. Candidate gene *gr11*, for instance, is an orthologue of the *Drosophila melanogaster* gene *Gr64a*, which is a sugar gustatory receptor involved in feeding motivation<sup>283</sup>. Sugar feeding is important for fuelling mosquito flight<sup>284–286</sup> and has been linked to successful mating<sup>287,288</sup>, so this gene could conceivably be under sexual selection in males. It also might explain why no phenotype was observed in the insemination assay, where it may not have been energetically demanding for males to mate females in the absence of competitors, and where the small container may have prohibited characteristic swarming flight. However, for practical reasons, the behavioural experiments that followed could only be performed on one test gene, and so those genes that did not show an effect in the insemination capacity experiment were not assayed further. Encouragingly, knockdown of *gr11*, AAEL022941 and AAEL005908 expression did not significantly affect male survival in the seven days following dsRNA injection, and so experiments will be able to use different assays to detect the effect of these uncharacterised genes on male mating

phenotypes. In the remainder of this discussion, I will focus on *ppk315* and its effect on male mating behaviour.

*Pickpocket* (*ppk*) genes belong to a superfamily of Degenerin/ENaC channel genes that encode cation channel subunit receptors of a wide variety of stimuli. Previous research, mostly in *Drosophila*, has implicated *ppk* genes in a range of processes such as nociception<sup>289,290</sup>, larval locomotory behaviour<sup>291,292</sup> and negative regulation of feeding behaviour<sup>293</sup>. Furthermore, evidence from multiple studies supports the involvement of *ppk* genes in mating. For instance, *ppk23* and *ppk29* are crucial for inhibiting male courtship towards other males and promoting courtship towards females<sup>294</sup>, with *ppk23* involved in the male response to female aphrodisiac pheromone 7,11-heptacosadiene<sup>295</sup>. In *Ae. aegypti*, however, the role of *ppk* genes is not well understood. *Ae. aegypti* is predicted to have 31 *ppk* genes distributed across the genome<sup>222</sup>, and while transcriptomic data have described their tissue-specific expression<sup>222,276</sup>, functional characterisation has been performed for only one of these genes. Specifically, a *ppk301* genetic mutant that was found to be partially responsible for egg-laying behaviours, with *ppk301*-expressing sensory neurons responding to water and high salt levels<sup>296</sup>.

Knockdown of *ppk315* expression in male *Ae. aegypti* not only affected insemination capacity, but it reduced the number of mating attempts (defined as any instance of physical contact between a male and female) by males in single male-female mating interactions. *Ppk315* males made less than half as many mating attempts as control males and were less likely to contact females overall (Figure 4.8A and 4.8C). This apparent lack of persistence in males might be explained by a reduced ability to orient to females, such that they are less likely to make physical contact, suggesting impaired locomotor activity. Locomotor performance is particularly important in mating systems characterised by scramble competition where males must actively search for females<sup>297</sup>. Indeed, sexual selection on locomotor performance has been documented in many invertebrates<sup>298–300</sup>. For example, high locomotor activity has been shown to be associated with male mating success in the red flour beetle, *Tribolium castaneum*<sup>301</sup>. Although I did not observe any difference in activity (the amount of time spent in flight) between treatments in the phonotactic assay (Figure 4.9C), this measurement didn't capture any information on flight ability or manoeuvrability, which are likely very important for intercepting females. Male *Ae.*

*aegypti* “patrolling” patterns of swarming involve flying in tight loops, followed by rapid acceleration towards nearby (~10 cm) females<sup>88,302</sup>. There have been multiple accounts of evasive flight in female *Ae. aegypti* as part of their cache of behaviours to evade courting males<sup>88,96,110,117,134,184</sup>, and work by Aldersley and Cator<sup>88</sup> showed that 6 % of mating interactions are terminated by females prior to contact. Interestingly, a study in the common chironomid midge, *Chironomus plumosus*, which also mates in aerial swarms characterised by scramble competition, showed that males with aerobic superiority had increased mating success<sup>300</sup>. Further work should seek to determine how reduced expression of *ppk315* affects the ability of males to contact females.

Alternatively, the lack of mating attempts in *ppk315* males may suggest a problem with locating the female within the cage, suggesting a possible role of *ppk315* in the perception of important female signals. It is well documented that at close range male *Ae. aegypti* detect and orient to females via their wing beat frequencies<sup>110,111,278</sup>. The reduced ability of males to contact females (Figure 4.8A and C) and decreased likelihood of responding to a speaker playing female flight tones (Figure 4.9A) makes it reasonable to suggest that *ppk315* may have some effect on male auditory function and warrants further investigation. Also, it cannot be ruled out that reduced expression of *ppk315* affected the ability of males to perceive or produce cues of a different sensory modality that may be important for locating and orienting to females, and that were not assayed in this experiment. Indeed, for many insects both visual and olfactory cues are key components of mate recognition<sup>303–306</sup> and courtship behaviours<sup>307</sup>.

Although the focus of this chapter was on male mating phenotypes, It should also be considered that both sexes were under selection in the experimental evolution regimes described in **Chapter 3** that generated the rationale for this work<sup>132</sup>. As such, high confidence candidate genes may also have mating-related phenotypes in female mosquitoes, and so further investigation into how these candidates affect females is necessary.

Though these behavioural assays have provided some useful insight into the potential role of *ppk315* in male mating behaviour, I can only speculate about the precise function of this gene. Further, there are some inherent limitations associated

with using RNAi to reduce gene expression which should be considered when interpreting these results. Firstly, RNAi-mediated knockdown of gene expression does not eliminate all mRNA, and so results in only partial loss of gene function. Secondly, RNAi may also cause some off-target effects, silencing non-target mRNAs<sup>308</sup>. As such, the phenotypes reported in this experiment may be explained by the effect of RNAi on mRNA levels of other genes in the genome. Future work using CRISPR-based methods could be used to knock out high confidence candidate genes.

To further understand the role of *ppk315*, future work should seek to identify the precise timing and location of *ppk315* gene expression, using qPCR or RNA-sequencing. Although previous transcriptomic studies have identified *ppk315* expression in adult males<sup>276</sup>, it is not known how expression changes over the course of the adult stage, for example, before, during and after mating<sup>309</sup>. Whilst *ppk315* is likely expressed in the rostrum and abdominal tip<sup>276</sup>, key sites of sensory perception, the precise number and location of *ppk315*-expressing cells is unknown. Methods such as fluorescent in-situ hybridisation (FISH) could be used in a fine-scale analysis of spatial expression.

## Conclusion

Identifying genes under sexual selection in mosquitoes is essential for ensuring the fitness of males destined for field release, and for identifying new targets for mosquito control. In this chapter I have performed a behavioural characterisation of *ppk315*, a gene that showed an evolutionary response to sexual selection in the previous chapter. This gene is not only expressed in adult male tissues, but in key sites of sensory perception. Knockdown of this gene is possible using RNAi, and males with reduced expression of *ppk315* display an altered mating phenotype. *Ppk315* knockdown males make fewer attempts to mate with females, are less successful in making physical contact with females, and are less likely to respond to female flight tones. Despite this exciting result, inherent weaknesses associated with this method of interrupting gene expression necessitate further work to develop a genetic *ppk315* mutant.

## Chapter 5

### The Genetic Basis of Male Mating Success in the Yellow Fever

#### Mosquito, *Aedes aegypti*:

#### Summary and Future Directions

##### Summary of principal findings

In this thesis I provide evidence to suggest that when male *Ae. aegypti* mosquitoes are subject to sexual selection in the form of mate competition and female choice, mating success is unequal. The skew in mating success is such that most males do not mate a single female in competition, despite the capacity to mate multiple times on multiple occasions. While variation in competitive mating success between males is significantly associated with siblings, a small but non-significant proportion of this variation is due to additive genetic variation.

I demonstrated that sexual selection has a pervasive effect on the *Ae. aegypti* genome, and that sexual selection is important for retaining genetic similarity to field populations. I found that the major and rapid changes in allele frequencies in those populations that had evolved in the absence of sexual selection are most likely due to the action of genetic drift.

I also showed that chemosensory genes evolve rapidly and consistently in response to the removal of sexual selection, and that a previously uncharacterised gene, *ppk315*, not only showed an evolutionary response to the removal of sexual selection but has a male mating phenotype. My behavioural experiments demonstrated that males with depleted expression of *ppk315* showed a reduced capacity to inseminate females in the absence competition, were less likely to attempt to mate females in one-on-one mating interactions and had a reduced phonotactic response.

Together, these results highlight the importance of sexual selection in *Ae. aegypti* at both the phenotypic and genomic level. Below I expand on several key findings and suggest some important avenues for future research.

## **When all pre-copulatory mechanisms of sexual selection are present most males do not mate**

To design and implement effective reproductive control strategies for *Ae. aegypti* mosquitoes, it is crucial to determine the mating competitiveness of males destined for field release. The success of these strategies hinges on the likelihood of a successful mating between a released male and a wild-type female, which determines the efficacy with which desirable traits such as pathogen resistance or sterility will spread throughout a target population. Insemination capacity, the number of females inseminated by a male during a given time period, is commonly used to quantify male mating success, likely due to the ease with which it can be measured. Several studies use insemination capacity to infer mating success of males held in equal, or even female-biased sex ratios<sup>94,310</sup>. Given that there is ample evidence to suggest that mosquito mating swarms are heavily male-biased<sup>63,84,86,91</sup>, which theory suggests will impose intense sexual selection<sup>311</sup>, I would argue that insemination capacity is a highly generalised metric with little grounding in *Ae. aegypti* mating behaviour, that may overestimate the potential for male mating success.

In **Chapter 2** I demonstrate that in the presence of both pre-copulatory mechanisms of sexual selection, female choice and mate competition, most males within the swarm do not mate a single female, and the minority of males mate multiple times. Though males may have the capacity to inseminate multiple females, when sexual selection is imposed, this metric is of little consequence.

It is still important to consider that this experiment was performed on laboratory populations of *Ae. aegypti*, and that other selection pressures affecting wild populations, such as predation<sup>312</sup> or locating energy sources<sup>313</sup>, may affect mating success further. Aldersley et al.<sup>131</sup> demonstrated that mating success can vary depending on the conditions in which it is measured, and that laboratory measures of mating success can overestimate performance. It would be of applied relevance to determine if variation in performance in the competitive mating success assay does indeed correlate with performance in field or semi-field settings.

## **The potential effects of environment on the low heritability of male mating success**

Studies of full-sibs have been used to estimate broad-sense heritability ( $H^2$ ) for a range of mating and non-mating traits for many organisms<sup>171,190–192</sup>. However, as discussed in **Chapter 2**, estimates of  $H^2$  are likely to overestimate the amount of genetic variation responsible for variation in the trait of interest, due to the often-significant potential bias of shared environment in full-sib study designs. By measuring male mating success in both full-sib families and in parent-offspring pairs, I showed that the estimate for broad sense heritability of male mating success ( $H^2 = 0.694$ ) does not reflect that of the estimate for narrow sense heritability ( $h^2 = 0.2$ ). The discrepancy between these two estimates is most parsimoniously explained by the inflating effects of a shared environment and the potential contribution of genetic dominance to the full-sib estimate of  $H^2$ . This inconsistency between heritability measures generated by different study designs has been recorded before<sup>205</sup>, but I would argue that  $h^2$  estimates should be generated alongside  $H^2$  estimates to prevent erroneous or misleading conclusions being drawn, particularly for traits with applied importance for mosquito and therefore disease control.

The significant potential contribution of environmental effects to male mating success implied by the relatively high  $H^2$  values generated by the full sib experiment in **Chapter 2** is an interesting and important avenue for further investigation. Though recent work has suggested that larval diet does not have a significant impact on male mating success<sup>131,153</sup>, to my knowledge, few other studies have investigated the effect of environment on male mating success whilst imposing mate competition and female choice mechanisms of sexual selection. The identification of environmental parameters that affect male mating success would not only increase our understanding of mating biology in this important disease vector but could also be used to inform and improve mass-rearing protocols.

## **Predicted effective population size is affected by sexual selection**

Effective population size ( $N_e$ ) is a central concept in population genetics that determines the effect of genetic drift on a population<sup>314</sup>. The results from **Chapter 3** that used allele frequency trajectory data to indirectly estimate  $N_e$  in replicate

populations evolved with and without sexual selection showed that populations evolved under enforced monogamy had reduced  $N_e$ . This result could be interpreted as suggesting that fewer breeding individuals contributed to each generation, or that fewer offspring were produced by females in the no sexual selection regime.

If the number of individuals contributing to each population was indeed different between the two treatments, then this may have affected the ability of the populations to respond to the experimental evolution regime<sup>315</sup>. This possibility was investigated by measuring both the likelihood of a female being mated and the number of offspring produced under each selection regime. Though there was no difference in either of these two measures between experimental regimes (Table 3.1), this experiment was carried out after the populations had been experimentally evolved. It is also technically possible that females may have been mated multiple times in those populations evolved in the presence of sexual selection. Females are generally thought to be monoandrous<sup>63</sup>, though evidence for polyandry in field and semi-field studies of *Ae. aegypti* suggests that it does happen, albeit infrequently<sup>105,106</sup>. Therefore, the possibility that lower  $N_e$  was driven by variation in breeding individuals or offspring number cannot be unequivocally ruled out. Experiments are needed to determine if mate competition increases the likelihood of polyandry in *Ae. aegypti*.

One possible alternative explanation for the reduced estimates of  $N_e$  in the no sexual selection populations is that removing sexual selection resulted in a sweeping change in net selection—that is, an environment where sexual selection has been eliminated might just as easily be considered one in which a new kind of selection has been imposed. In this scenario, favourable alleles in the new selective environment would be dragged to new frequency optima (along with hitchhiking variants)<sup>316</sup>, a phenomenon also known as a ‘selective sweep’<sup>317</sup>, resulting in reduced estimates of  $N_e$ . However, there is little evidence of consistent changes in allele frequency between replicates of the no sexual selection lines that would support this (Figure 3.1).

Instead, the reduction in estimated  $N_e$  following the relaxation of sexual selection likely reflects the evolution of populations evolving in unique or arbitrary directions, with alleles rapidly changing and fixing consistent with the action of genetic drift. This

explanation is supported by the relatively high levels of genomic differentiation between replicate populations of the no sexual selection regime (Figure 3.2) and the observed reduction in nucleotide diversity in the no sexual selection regime (Figure 3.4).

Irrespective of the mechanisms underlying the significant genomic changes associated with the relaxation of sexual selection, this work clearly demonstrates that sexual selection is a major evolutionary force in *Ae. aegypti* mating systems. If sexual selection is not allowed to act then both the mating phenotype<sup>132</sup>, and the genomic underpinnings of the phenotype<sup>281</sup> change rapidly; mosquitoes must “use it or lose it”<sup>318</sup>. These findings should be of significant interest to those involved in the reproductive control of mosquitoes and suggest that mass-rearing facilities should subject mosquitoes to sexual selection to retain genetic similarity to ancestral populations and to ensure mating competitiveness of males destined for field release.

The short evolutionary time scale in which male mating success is reduced<sup>132</sup>, and in which associated genomic changes occur<sup>281</sup>, suggests that traits associated with male mating success may be costly to maintain or express<sup>77</sup>. Whilst the fitness benefits of increased mating success are clear, future work should seek to identify and quantify the costs of traits that contribute to male mating success.

### **Evidence of a genomic signature of sexual selection and further molecular and function characterisation of candidates**

The identification of parallel changes in allele frequencies across replicate evolved populations is clear evidence of a genomic signature of sexual selection. This finding is further supported by the confirmation of a mating related phenotype in one of the high-confidence candidate genes identified in the screen. Males with reduced expression of this gene, *ppk315*, were able to inseminate fewer females in the absence of competition (Figure 4.5), made fewer attempts to mate in one-on-one mating interactions (Figure 4.8), and displayed a reduced phonotactic response to female flight tones (Figure 4.9). In future work I plan to repeat the experiments in **Chapter 4** and perform additional behavioural assays using an *Ae. aegypti* knockout line in which the *ppk315* coding sequence has been disrupted. Additionally, to

understand the spatial expression profile of *ppk315* it would be beneficial to generate a reporter line. This could be achieved by engineering the *ppk315* regulatory elements to drive a fluorescent reporter protein, which would show the specific cells that *ppk315* is expressed in. Even further, to gain an understanding of *ppk315* localisation within the cell, the *ppk315* coding sequence could be tagged with a fluorescent protein.

A greater understanding of the role of *ppk315*, and other putative sexually selected genes identified in the screen, in male mating success will not only provide us with an insight into the genetic basis of sexual selection in mosquitoes but may also provide novel targets for mosquito control.

### **Concluding remarks**

For over a century, evolutionary biologists have been fascinated by sexual selection, and it is only recently that we have begun to gain an understanding of how this selective force operates in mosquitoes<sup>81</sup>. My thesis demonstrates that not all males have equal mating success, and that genetics may play a significant role in determining whether a particular male might mate. The rapid and widespread genomic changes that occur after the removal of mate competition and female choice provide compelling evidence of sexual selection's major impact on *Ae. aegypti*. While previous research has largely focused on the role of acoustic interactions in mate choice<sup>87,117,213</sup>, the "evolve and resequence" approach used here presents evidence that other sensory modalities such as chemosensation may also play a part in mosquito mating behaviour.

Male mating behaviour is a severely understudied topic<sup>130,319</sup>. The genetic basis of male mating behaviour, in particular, has received very little attention. Given that the ability of modified, mass-released males to mate with wild females is critical to the effectiveness of next generation mosquito control tools, this topic is more relevant than ever.

## Additional files

**Supplementary Table 1. Location of high impact variants that showed large and consistent changes in allele frequency in response to opposing sexual selection regimes.** Information obtained from Ensembl Metazoa (release 56)<sup>320</sup>

| Variant locus | Gene       | Description                     |
|---------------|------------|---------------------------------|
| 1:101172041   | AAEL002413 | sphingomyelin phosphodiesterase |
| 1:22976847    | AAEL015079 | NA                              |
| 1:247387355   | AAEL019639 | NA                              |
| 1:287707302   | AAEL004881 | adam                            |
| 1:287707464   | AAEL004881 | adam                            |
| 1:42351105    | AAEL026004 | NA                              |
| 2:182341811   | AAEL011174 | gustatory receptor Gr11         |
| 2:402934915   | AAEL027483 | NA                              |
| 2:78739129    | AAEL022941 | NA                              |
| 3:173436976   | AAEL005908 | rolling pebbles                 |
| 3:317027071   | AAEL025282 | NA                              |
| 3:333863283   | AAEL000863 | pickpocket 315                  |

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