



Optimising the performance of genetically engineered *Aedes aegypti*

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Abstract

The mosquito *Aedes aegypti* is the primary worldwide vector of dengue fever, of which there are an estimated 390 million infections annually. Traditional vector control methods have been unsuccessful in preventing *Ae. aegypti* from posing a serious threat to human health worldwide, and as a result increasing emphasis is being placed on new control technologies. One of these is RIDL® (Release of Insects carrying a Dominant Lethal), a modified form of the sterile insect technique. Released RIDL males of strain OX513A are genetically engineered to possess a repressible lethal construct. Upon mating with a wild female, the construct is inherited by the offspring, causing their death.

The RIDL strategy depends on the mass production and release of vigorous male insects, capable of competing with wild males to mate with wild females. **Chapter 2** investigates how rearing conditions can influence the size, asymmetry, quantity, and timing of OX513A mosquitoes produced. **Chapter 3** describes the design and testing of devices for the release of these adult males, and their implementation during the world's first release of transgenic mosquitoes. **Chapter 4** discusses how the modification of male size and nutritional status may affect subsequent performance, and how these changes to male vigour will be assessed in subsequent chapters. **Chapter 5** measures changes in competitiveness by examining longevity. Larger males lived longer, and longevity was increased by increasing the duration of the initial sugar feed. **Chapter 6** examines the potential improvements in male performance by measuring the flight capacity of OX513A males, using a purpose-built mosquito flight mill. Male size or nutritional status was not found to affect flight capacity, although light intensity was unexpectedly found to modulate the flight speed of the males. Finally, **Chapter 7** examines potential improvements in the mating performance of male OX513A *Ae. aegypti*. When OX513A males and wild-type males were placed in direct competition for virgin females, smaller OX513A males were found to have a significant mating advantage over larger wild-type males. The results of all three of these performance measures are discussed with relevance to field releases.

The results presented in this thesis improve current knowledge of how the performance of male mosquitoes can be effectively measured and improved. This is a vital part of ensuring the success of male-release vector control strategies such as RIDL.

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1 Introduction

1.1 *Aedes aegypti*

1.1.1 Ecology and Life Cycle



Figure 1-1. *Aedes aegypti* male in flight.
Image credit: D. Nimmo, Oxitec.

The ancestral form of the mosquito *Aedes aegypti* (Linnaeus) was a zoophilic African species, before subspeciation created an anthropophilic form, that has since spread throughout the tropical and subtropical regions of the world through the expansion of human trade routes (Brown et al., 2014). *Ae. aegypti* (Figure 1-1) is a peridomestic species, and is particularly abundant in populated areas such as towns and cities. Adults will reside in dark areas within a house, with the females taking blood meals from the occupants during the early morning or late afternoon. Although the females will take blood meals from other animals, human blood is preferred (Scott et al., 1993, 2000). Once mated and blood-fed, gravid females will deposit their eggs on wet or damp surfaces (Figure 1-2). The ancestral form primarily used water-retaining treeholes for this purpose (Moore et al., 2013), and while such oviposition behaviour still persists (Mangudo et al., 2015), the prevalent anthropophilic form has become well adapted to utilising artificial substitutes such as discarded tyres and containers. Eggs can resist desiccation for up to one year, and hatch when submerged in deoxygenated water. The larvae are aquatic and feed on the microbiota and detritus particles that are present in their immediate environment. Larvae develop through four instar stages, at a rate which is dependent on temperature (Beserra et al., 2006) and food supply, but which typically ranges from four to ten days (Womack, 1993). They then metamorphose into a pupal stage, as which they spend around two days. Finally, the imago ecloses from the pupa on the surface of the water.

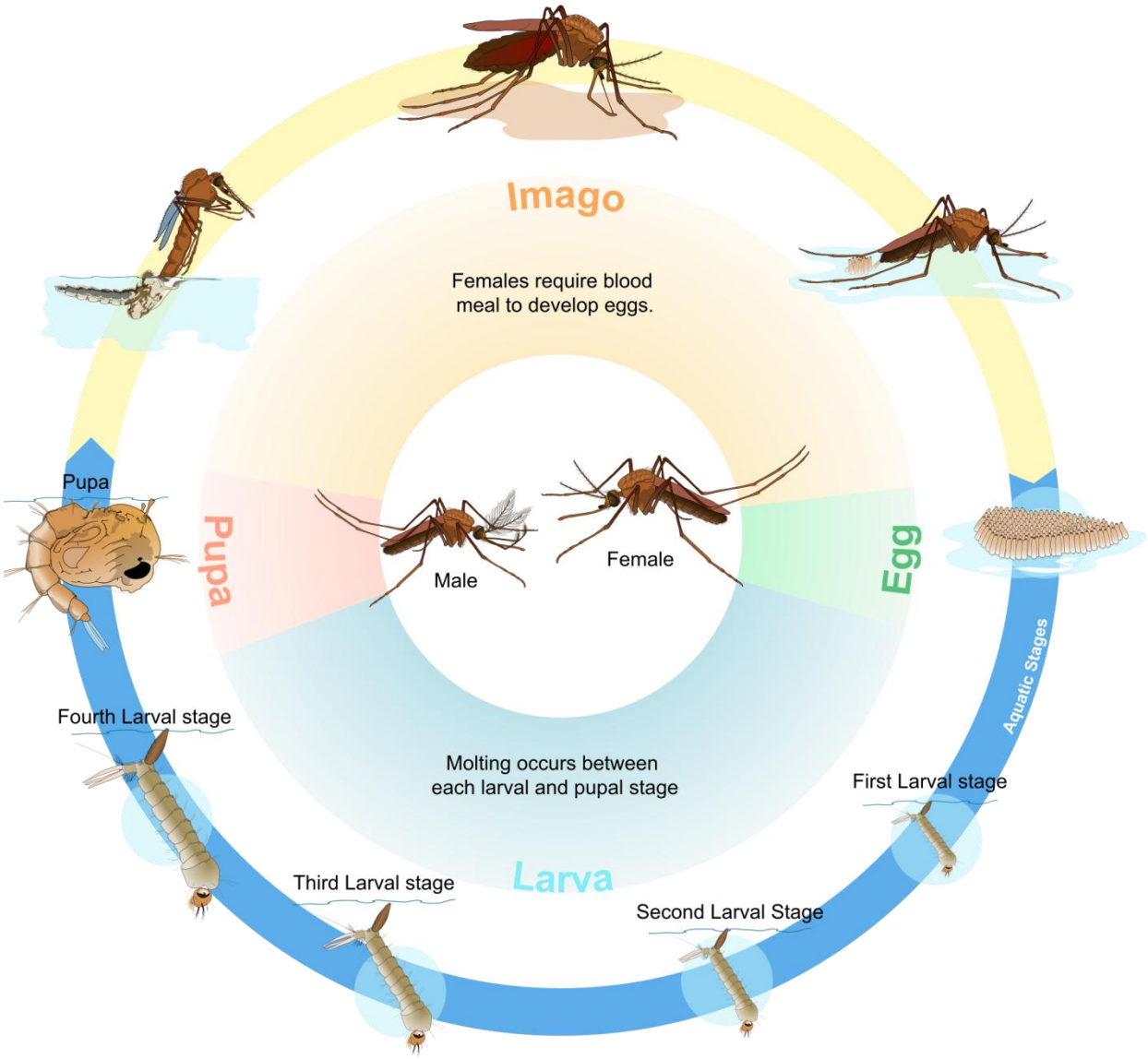


Figure 1-2. General mosquito life cycle. Image source: Wikimedia Commons.

1.1.2 As a Disease Vector

Ae. aegypti is the most important vector of both the dengue fever and yellow fever viruses, and is also responsible for carrying chikungunya. The ability of the eggs to lie dormant and resist desiccation means that the mosquito can act as a reservoir for these viruses, ensuring transmission from year to year.

Dengue is an acute febrile disease, caused by any one of four closely-related dengue viruses. Recovery from illness as a result of one of the viruses confers lifelong immunity from that serotype, but not from the three others. Symptoms range from mild to severe fever, as well as pain behind the eyes, rash, and muscle and joint pains (hence its other name 'breakbone fever'). A recent attempt to quantify the worldwide extent of dengue estimated that 390

million infections occur annually (Bhatt et al., 2013). A separate study estimated that there are 3.9 billion people at risk of infection worldwide (Brady et al., 2012). Within the mosquito, the virus incubates for 8-12 days, after which the mosquito is capable of transmitting the virus for the rest of its life. Vertical transmission from adult to offspring is also possible, though the extent and epidemiological significance of this, if any, is unclear (WHO, 2016b). There are several groups of potential dengue vaccines in preclinical and clinical development. The only one in current use is CYD-TDV, a live-attenuated tetravalent vaccine based on chimeric yellow fever-dengue virus. Phase III studies of this vaccine were carried out in Asia and Latin America, and reported approximately 60% efficacy of protection (Capeding et al., 2014; Villar et al., 2015). However, the level of protection varied by dengue serotype, age, and a patient's previous exposure to dengue. Particularly, in certain age groups which have not previously had exposure to dengue, vaccination can actually increase the likelihood of developing severe dengue in the long-term. As such, the World Health Organization currently recommends that the vaccine is only used in areas with the highest disease burdens (WHO, 2016a). The absence of a truly effective vaccine has resulted in dengue fever control strategies focussing on control of the vector, and bite prevention. Particular control efforts are being made in those developed countries where the mosquito is present (Figure 1-3) but where disease cases have yet to be reported, such as the U.S. state of Florida, and Northern Australia.

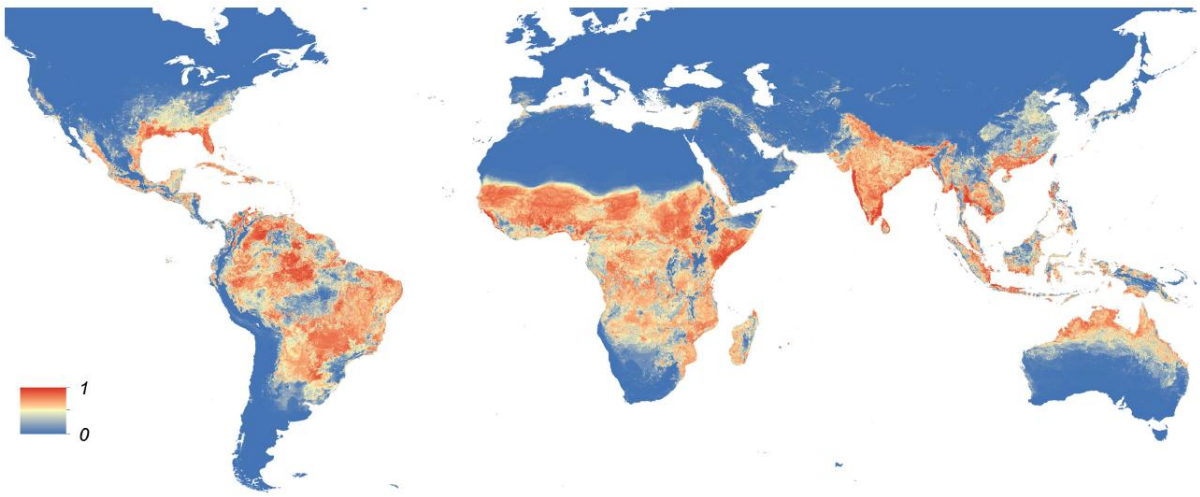


Figure 1-3. Predicted global distribution of *Ae. aegypti*. The map depicts probability of occurrence of *Ae. aegypti* from zero (blue) to 1 (red). Predictions were based on occurrence data combined with environmental variables. Figure modified from Kraemer et al. (2015).

Ae. aegypti is also a carrier of yellow fever, and every year there are 200,000 estimated cases resulting in 30,000 deaths. Its symptoms are flu-like, and in most cases disappear after 3-4 days. However, 15% of patients will enter a 'toxic phase' within 24 hours. Jaundice rapidly develops, bleeding from the ears, eyes, and mouth occurs, and kidney function can deteriorate. Fifty per cent of those patients in the toxic phase will die within 10-14 days. There is no specific treatment, although a vaccine exists that is fast-acting and long lasting. Large-scale vaccination programmes in various African countries have proven to be extremely effective. As with dengue, vertical transmission is also possible (WHO, 2016d).

Chikungunya is a viral disease, similar to dengue, although it is rarely fatal. Its similarity to dengue, and its endemicity to tropical Africa and Asia, often means it is misdiagnosed. Symptoms during the 2-4 day incubation period include fever, rash, and severe joint pain. There is no vaccine for chikungunya, and treatment usually comprises of anti-inflammatory drugs to treat the joint pain. Recorded outbreaks have occurred regularly over the last two centuries, and prevention is entirely dependent on vector control and taking steps to avoid being bitten (WHO, 2016c).

1.2 Chemical Control Methods

1.2.1 Larval Control

For container-breeding species such as *Ae. aegypti*, a large aspect of control is the removal of breeding sites, such as empty containers and discarded tyres. Preventing females from using standing water as a breeding site may reduce the local mosquito population, and can be achieved by simply tipping over containers, or in the case of larger water sources by the use of nylon netting (Maciel-de-Freitas and Lourenço-de-Oliveira, 2011) or monomolecular films (Ngrenngarmlert et al., 2016) to prevent larvae and pupae from breathing at the water's surface.

Chemical control has also been widely used. Temephos, an organophosphate, disrupts the central nervous system of insects through inhibition of acetylcholinesterase. Currently temephos is the most widely-used product for the control of *Ae. aegypti* larvae in areas such as Thailand, although resistance to it was recorded as early as 1960 (Fox and Garcia-Moll, 1961), and continues to be an issue (Polson et al., 2001; Ponlawat et al., 2005; Loke et al.,

2010; Bisset et al., 2011; Prophiro et al., 2011). In particular, a recent country-wide survey of temephos resistance in Brazil reported that resistance was widespread, and increasing in severity (Chediak et al., 2016). The increased availability of alternatives allowed the use of temephos to be phased out in some areas (Russell and Kay, 2008).

One such alternative is s-methoprene. Rather than being directly toxic, it is an insect growth regulator, more specifically an analogue of juvenile hormone (Gordon and Burford, 1984). The absence of juvenile hormone is required for the pupa to eclose into an adult, so treated pupae fail to eclose. s-Methoprene can be administered via embedding high concentrations in a slow-release briquette, ensuring the release of the chemical over many months at a low consistent dose (Kramer et al., 1993; Ross et al., 1994). However, similarly to temephos, resistance to s-methoprene has been recorded both in the laboratory (Brown and Brown, 1974; Marcombe et al., 2014) and in the field (Dame et al., 1998; Cornel et al., 2000).

Treatment of water with biological agents is also a widespread method of control. Most commonly used are bacterial formulations made up from *Bacillus thuringiensis* serovar *israelensis* (Bti), which has been in use for over 30 years (Lacey, 2007) since this soil-dwelling bacterium was first described and identified as toxic to mosquito larvae by Goldberg and Margalit (1977). Upon ingestion by the mosquito larva, toxins produced by the bacteria insert themselves into the epithelial cells of the midgut, forming pores and so lysing the cells, which results in the death of the larva (Bravo et al., 2007). As such, Bti is less effective when the feeding rate is reduced. This is the case in fourth instar larvae (which have a lower feeding rate) and pupae, which do not feed at all (Van Essen and Hembree, 1980; Mulla et al., 1990; Yousten et al., 1992). Feeding rate also decreases as water temperature decreases (Becker et al., 1992) as larval density increases (Becker et al., 1993) and as alternative food sources become more available (Mulla et al., 1990). Whilst Bti has become somewhat of a mainstay of larval control in many areas around the world, it is accepted that it only provides short-term control, as after 2-3 days toxin activity drops below effective levels (Karch et al., 1991).

1.2.2 Adult Control

The use of aerosolised insecticides is widespread, and can be split into two primary techniques: thermal fogging and ultra-low volume (ULV) spraying. Both typically administer the organophosphate insecticide malathion via vehicle-mounted units. Fogging tends to administer more insecticide, typically from a truck; whereas ULV spraying, as the name

suggests, administers far lower dosages of insecticide, typically from a plane and over a much wider area. There have been a number of comparative studies of thermal fogging and ULV spraying used against mosquitoes in an outdoor environment (Mount et al., 1968; Mount et al., 1970; Taylor and Schoof, 1971; Rathburn and Boike, 1972; Linley and Jordan, 1992; Britch et al., 2010). All six report fogging to be either of broadly equal or higher efficacy than ULV spraying. And yet, far more emphasis - both in research and in practice - has been placed on ULV spraying. Britch et al. (2010) point out that one of the main drivers of aerosol-based insect control are the US military, who depend on it especially in areas such as the Middle East. Since ULV spraying involves the release of less material into the environment, and is visually and aurally less conspicuous, preference is given to it over fogging.

A more localised and low-tech solution is indoor residual spraying (IRS). This involves the spray application of an insecticide (such as dichlorodiphenyltrichloroethane, DDT) to the indoor surfaces of a house, which then kills the mosquitoes on contact. As houses have to be entered and sprayed individually, the use of IRS can be labour-intensive; however it is currently a major component of malaria control (Raghavendra et al., 2011). Another simple tool is the use of bed nets impregnated with insecticide. Whilst these offer protection from nocturnal species such as the malaria carrier *Anopheles gambiae*, they are less effective against diurnal species such as *Ae. aegypti*. Furthermore, bed nets can be liable to misuse, for example for fishing (Minakawa et al., 2008).

The use of insecticides is a powerful and fast-acting tool to prevent disease transmission. However, even in the case of well-resourced vector control campaigns, current control methods are often not able to adequately control mosquitoes to below the levels required for disease transmission (Ooi et al., 2006; Egger et al., 2008). In addition, extensive use of chemical control can result in significant off-target ecological effects, for example the bioaccumulation of DDT in food chains. Also, as with larval control methods, the development of resistance is a perennial problem. For these reasons, now more than ever, attention is being given to non-chemical interventions.

1.3 Genetic Control Mechanisms

At its heart, the genetic control of insects comprises the introduction of one or more genetic traits into an insect population in order to eliminate it, reduce the fitness of its members, and/or reduce its impact in terms of public health (in the case of disease vectors) or

economic cost (in the case of agricultural pests). Several mechanisms have historically been used to artificially induce such gene flow into a wild population, outlined below.

1.3.1 The Sterile Insect Technique

The sterile insect technique (SIT) involves the mass production, sterilisation, and release of insects. Individuals can be sterilised by exposure to either gamma radiation (Davis et al., 1959) or a chemosterilant (Weidhaas, 1962), at either the pupal or adult stage. These treatments induce random chromosomal aberrations and dominant lethal mutations in the spermatozoa or unfertilised egg (Robinson, 2005). Once sterilised and released, males will seek out and mate with wild females, which produce non-viable eggs. If males are released in sufficient numbers over a sufficient period of time, reproduction can be significantly reduced over a number of insect generations, ultimately resulting in suppression or even eradication of the target population. Unlike the use of insecticides, the SIT is both environmentally non-polluting and highly species-specific. Furthermore, as the released males actively seek out females with which to mate, the SIT may be especially useful as a 'homing' solution to affect those parts of a wild population that would otherwise remain out of reach of conventional control measures.

The SIT is not a new concept, having been conceived by EF Knipling in 1937 (Knipling, 1960). By the 1950s, an SIT programme was underway in the USA against the New World screwworm (*Cochliomyia hominivorax*) (Lindquist, 1955), and as quickly as the early 1960s it had successfully eliminated this livestock parasite from the southeastern United States (Knipling, 1960, 1985). Over the following four decades the programme was successfully expanded to eliminate the pest from the entire of North and Central America (Van der Vloedt and Klassen, 1991), as well as a later outbreak in Libya (Lindquist et al., 1992). While control of the screwworm is the most successful and best-known example of the SIT, today a major target is the Mediterranean fruit fly (medfly) (*Ceratitis capitata*) (Hendrichs et al., 1995). There are more than 15 facilities around the world rearing medfly, including a massive operation at the El Pino facility in Guatemala which produces 15 billion flies per week. Other agricultural pests have also been successfully controlled using the SIT, including: melon fly (*Bactrocera cucurbitae*) (Kuba et al., 1996); various *Anastrepha* (tephritid fruit fly) species (Enkerlin, 2005); pink bollworm moth (*Pectinophora gossypiella*) (Staten et al., 1999); and codling moth (*Cydia pomonella*) (Myers et al., 1998). The SIT has also been used previously against the tsetse fly (*Glossina* spp.) (Dame et al., 1981; Vreysen et al., 2000) which transmits trypanosomiasis, also known as sleeping sickness.

Mosquitoes, being vectors of human disease, have been the subject of intense investigation with regard to the SIT. The earliest releases had mixed results. Releases of 4.6 million irradiated sterile male *Ae. aegypti* in Florida reduced the local population in some areas but not others (Morlan et al., 1962). The authors of that study suggested that had releases been performed closer together both temporally and spatially, a more consistent reduction in the wild population may have been observed. The following year, male *Ae. aegypti* sterilised by either irradiation or chemical means were compared in their ability to reduce the reproductive potential of caged populations. Although neither method prevented the sterilised males from mating outright, chemosterilized males were competitive with normal males, but irradiated males could only compete slightly, or - at the higher doses of radiation - not at all (Weidhaas and Schmidt, 1963). Similar findings were reported by Krishnamurthy et al. (1962). Irradiation is known to be generally damaging to mosquitoes, especially at the higher doses required to induce near-100% sterility (Sharma et al., 1978), although some species do seem to be more resilient than others (Helinski et al., 2006). Irradiation is more damaging when it is delivered at the pupal stage (Andreasen and Curtis, 2005), although irradiating the more fragile adults presents considerably more logistical problems and the resulting handling damage may offset any overall fitness improvement versus pupal irradiation.

The potential for employing the SIT against various *Anopheles* species - being the primary vectors of human malaria - has also been keenly pursued. Similarly to *Ae. aegypti*, the first releases of sterile *An. quadrimaculatus* failed to induce sterility in, or reduction of a wild population, again likely a result of insufficient numbers of males released per unit area (Weidhaas et al., 1962). Further investigation found that sterile males were able to mate with wild-caught females, but only if those females (having been caught) were re-released concurrently with the sterile males. Released sterile males were unable to be similarly successful with wild females that were already present in the natural environment, i.e. when the females needed to be sought out (Dame et al., 1964). Later, experiments in large semi-field cages, while largely eliminating the need for males to disperse to seek out females, encountered another problem. Lack of longevity (50% mortality 24 hours post-release, 93% mortality 48 hours post-release) meant that chemosterilised male *An. quadrimaculatus* were only able to inseminate 3% of available females, despite outnumbering wild-type males by up to 100 to 1 (Patterson et al., 1968). In each of these cases, it appeared that strain colonisation and maintenance in a laboratory over many years

had resulted in the degradation of the insects' ability to survive and perform natural behaviours in a 'wild' environment.

This issue was circumvented in the first successful use of the SIT against a mosquito species, a population of *Culex pipiens quinquefasciatus* on a small (⅓ km^2) island off the Florida coast. By using locally-collected eggs to establish the release colony, combined with total isolation of the target population and high ratios of sterile: wild males (as high as 100:1), the local population was near-eradicated within ten weeks (Patterson et al., 1970). The first successful suppression of an anopheline mosquito by the SIT was achieved soon after, in El Salvador. Again, the breeding colony was established immediately prior to the first releases, from wild females caught locally. Nearly 4.4 million chemosterilized *An. albimanus* were released over a 15 km^2 area during a period of five months, which reduced the wild population by 99%. By starting releases at a time of year when the population was at its lowest, the sterile males were able to prevent the annual population increase following wetter weather later in the year. Once releases had ceased, the wild population took four months to re-establish itself at its former level. (Breeland et al., 1974; Dame et al., 1974; Lofgren et al., 1974; Weidhaas et al., 1974).

The dual lessons of (i) establishing a breeding colony from locally-caught insects and (ii) starting releases prior to the seasonal increase of the local population, were incorporated into an attempt to control *Culex tarsalis* in California (Reisen et al., 1982). Over a period of eight weeks, 85,000 males were irradiated, marked with fluorescent powder, and released into a small semi-isolated canyon. However, while the males survived and dispersed well, they were uncompetitive with the wild males and so failed to prevent the vernal population increase. This was attributed to observed assortative mating - wild females mated less often with the irradiated males than with the wild males. In turn, this was attributed not to the irradiation process but rather to the process of laboratory colonization. While the breeding colony was established from 3,000 locally-caught females, by the time the release cohorts were produced the colony was in the F_9 generation (in a study of a different *Culex* species, assortative mating was reported after only three generations of colony maintenance; Reisen et al., 1980). Other possible factors for the lack of male competitiveness included severe pupal mortality (itself possibly a result of the 900 km round trip by road required to irradiate the pupae), which indicated potential sub-lethal damage in those males that did survive to be released (Reisen et al., 1982). Indeed, the

availability and proximity of irradiation sources, especially in the developing world, remains an issue (Helinski et al., 2008).

Although these first forays into the use of the SIT against mosquitoes certainly experienced a degree of failure, those programmes that were able to demonstrate control - in areas where conventional control had been left wanting - inspired a great deal of confidence and optimism in the ability of the SIT as a control medium for mosquitoes. Moreover, regardless of the specific experimental outcomes, these two decades of study constitute a foundation of knowledge that would be relied and built upon by technologies to follow. Of that work, the general principles of most relevance to the work in this thesis are[1]:

- the ability of the released males to mate with wild females, whilst in competition with wild males, is paramount to success (and their ability to do so in caged or semi-field conditions is unlikely to be indicative of their ability to perform similarly in the wild);
- sterilisation (chemosterilisation or irradiation) in of itself does not appear to overtly reduce male quality, rather the primary fitness penalty results from the treatment of males prior to release (namely: rearing at high density; separation of pupae from larvae; separation of male pupae from female pupae; eclosion and maintenance in release media; transport to release sites; and the handling associated with each of these stages);
- overflooding ratios (the proportion of released males to wild males) should be high and consistent, but equally are not a substitute for male quality;
- the effects of laboratory colonisation can reduce the effectiveness of a release cohort - maximising the genetic similarity between the released and wild males (by establishing the colony from locally-caught insects) can someway mitigate this (Benedict et al., 2009b);
- to achieve consistently high release numbers, full-scale production should be established in situ prior to the first releases, with known potential issues pre-empted as far as possible;
- releases should be initiated when the target population is seasonally low; and
- even a low degree of immigration of wild insects into the target area can nullify the controlling effect of sterile releases (Asman et al., 1981), and so a high degree of

[1] These and others were also thoroughly reviewed by Benedict and Robinson (2003).

isolation of the target population is desirable. For field trials this has been accomplished through careful selection of study sites, but in order to achieve wide-ranging control sterile-male strategies must be combined with conventional control and/or buffer zones around the release areas.

Despite the promise shown by the SIT, by the 1980s it had fallen out of favour as a method of mosquito control (although more recently it has been used with some success in pilot trials against the spread of *Ae. albopictus* into southern Europe (Bellini et al., 2007, 2013)). It was not until the emergence of new transgenic technologies (see Section 1.4) that there were renewed calls for sterile-male based methods to be revisited (Benedict and Robinson, 2003; Alphey et al., 2010; Black et al., 2011). Indeed, there has yet to be a sustained SIT programme targeting mosquitoes.

1.3.2 Cytoplasmic Incompatibility

The intracellular parasite *Wolbachia* infects a wide variety of insects, is maternally inherited, and confers on its host a reproductive advantage in order to propagate itself. It does so by modifying its host's gametes, such that if an uninfected female is fertilised by an infected male, the offspring will be non-viable. However, when an infected female is mated by an infected male, she will produce viable offspring. As this so-called 'reproductive incompatibility' is caused by *Wolbachia* which reside in the cells' cytoplasm, the effect is termed cytoplasmic incompatibility (CI). CI was demonstrated in the field to be an effective way to control *Culex pipiens fatigans* (Laven, 1967), before the *Wolbachia* parasite was later identified as the cause of CI (Yen and Barr, 1971).

As CI caused by *Wolbachia* confers a reproductive advantage on its host, the parasite is able to drive itself through a population, as has been observed in (amongst other species) *Drosophila simulans* (Turelli and Hoffmann, 1991; Sluder et al., 1999). This is particularly promising from a control perspective, given that when the *Wolbachia* Mel (*w*Mel) strain is transinfected into *Ae. aegypti*, it actually blocks transmission of dengue (serotype 2), as well as reducing the lifespan of the insect (Walker et al., 2011). The authors of that study were able to show that the strain approached fixation within a semi-field population within a few generations, and so full-field trials were conducted in Australia, with similar results (Hoffmann et al., 2011). Further monitoring showed that the infection has since remained stable in the wild population (Hoffmann et al., 2014), and so the use of CI to drive *Wolbachia* into a wild population remains a very promising avenue of exploration.

Additionally, a related *Wolbachia* strain, *w*MelPop, drastically increases egg mortality in *Ae. aegypti* and so has been proposed as a means of eliminating egg banks that would otherwise be highly resistant to desiccation (Ritchie et al., 2015).

1.3.3 Chromosomal Translocations

Following the successful SIT campaign against *An. albimanus* in El Salvador, the next step was to attempt similar control over a larger area. However, the rearing methods used in the first trial were relatively inefficient at removing females (capable of transmitting malaria) from the release cohort, such that 14% of the released insects had been female (Dame et al., 1974). Therefore, the next priority was to establish a system for the production of much larger quantities of male pupae, with as low a proportion of females as possible. Fortunately, by this time the technique of chromosomal translocation had been successfully implemented to create a 'genetic sexing strain' of *An. albimanus*. As opposed to the random chromosomal breaks generated by irradiation or chemosterilants, chromosomal translocations are targeted and specific. By linking a gene that confers resistance to propoxur (an insecticide) to the Y-chromosome by means of a radiation-induced translocation, and suppressing further recombination, 99.7% of the females were susceptible to propoxur, with zero male loss (Kaiser et al., 1978; Seawright et al., 1978). As the system was effective at all life stages, the females could be eliminated at the egg stage, effectively doubling the rate of male production from the same rearing facilities. The result was the production of over a million pupae per day, of which up to 99.9% were male (Bailey et al., 1980). With the use of the new strain, significant population suppression was again achieved, although this was in major part a result of dramatic scaling-down of the treatment area. Complete control was not achieved; this was attributed to immigration by wild insects, despite the use of barrier zones (Dame et al., 1981).

Similar genetic sexing systems using chromosomal translocation were developed at around the same time for other species: *Ae. aegypti* (McDonald and Rai, 1970); *An. gambiae* (Curtis et al., 1976); the housefly (*Musca domestica*) (McDonald, 1971); and the Australian sheep blowfly (*Lucilia cuprina*) (Foster and Maddern, 1979). In the case of the latter, although the system was progressed to field trial stage (Foster et al., 1985; Vogt et al., 1985), it failed to induce the desired population reduction. The primary reasons for this failure were threefold: (i) mating competiveness of the released males was only approximately one-third that of the wild males; (ii) instability of the chromosomal translocation itself (which has also been observed in medfly (Robinson et al., 1999)); and (iii) immigration of wild individuals

into the treated population. While the latter is a common feature in all genetic control methods, the first two are a direct consequence of the irradiation needed to generate the necessary targeted chromosomal rearrangements. While this system was largely deemed to be a failure, the principle of inducible or repressible lethality would later be revisited by the RIDL system.

1.4 The RIDL® System

By the year 2000, insect transgenesis and recombinant DNA methods had progressed to the point where a specifically designed control mechanism could be directly inserted into an insect's genome, rather than relying on inducing chromosomal aberrations. A new system, based on the repressible expression of a dominant lethal gene (Heinrich and Scott, 2000), was named RIDL® (Release of Insects carrying a Dominant Lethal) and was first developed in *Drosophila melanogaster* (Thomas et al., 2000) before being adapted for use in *Ae. aegypti* (Phuc et al., 2007). Transformed individuals contain a dominant lethal gene, expression of which is repressible by tetracycline. Tetracycline, although functionally absent in the insect's natural environment (Curtis et al., 2015), can be added to the diet of the insect during mass production, repressing lethality in the release cohorts. The offspring from matings between released RIDL males and wild females inherit the lethal construct, and in the absence of tetracycline, those offspring die before they eclose.

Transgenic line OX513A - the focus of investigation in this thesis - was developed in *Ae. aegypti* by Phuc et al. (2007). The method of lethality of the OX513A construct and its repressibility by tetracycline is shown in Figure 1-4. This line is described as a 'late-acting bi-sex lethal' because it is lethal to both sexes equally and lethality occurs late in the pre-adult life stages, either at the fourth larval instar or pupal stage.

The late-acting lethality is an important benefit to the RIDL system, as follows. By their nature, populations of agricultural insect pests tend to exist in habitats with a non-limiting quantity of food. Their populations are often regulated by seasonal change or human intervention. By contrast, populations of insect disease vectors are subject to strong density-dependent effects, where the population is limited by resources. The population size able to be supported by the available resources is known as the 'carrying capacity' of

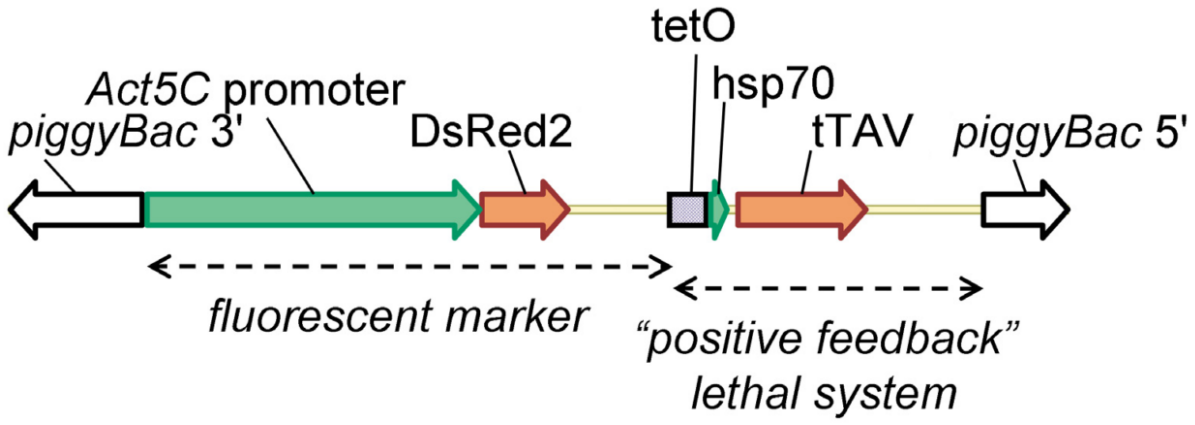


Figure 1-4. Structure of the OX513A transposon. The construct (8.4 kb) was inserted into the insect's genome by means of the *piggyBac* system, using standard egg micro-injection methods (Jasinskiene et al., 1998). The Act5C promoter controls expression of DsRed2, ensuring that transgenics and their offspring can be identified by red fluorescence. The remainder of the construct comprises a positive feedback element, as follows. tTAV is a transcriptional activator, which is under the control of its own regulatory element and binding site, tetO, as well as the hsp70 minimal promoter. In the absence of tetracycline, the minimal promoter drives production of a small quantity of the tTAV protein, which binds to tetO. This further upregulates the expression of tTAV, resulting in a positive feedback loop. High quantities of tTAV are toxic (tTAV contains a VP16 domain which interacts with many of the cell's generic transcriptional factors meaning they are not available for vital processes elsewhere; this is known as 'transcriptional squelching' (Lin et al., 2007)). In this way, the upscaling of tTAV production causes lethality at the late-larval or pupal stages, and so the construct is said to be 'late-acting'. However, in the presence of tetracycline the tTAV is bound by the tetracycline and so is not available to bind to tetO, preventing the initialisation of the positive feedback loop of tTAV production. Thus in the presence of tetracycline there is only low, basal expression of tTAV, which is non-toxic to the insect. Figure modified from Phuc et al. (2007).

that ecosystem, and populations which are changed about this number (e.g. by sudden cull or immigration) will tend to return to this equilibrium. In the case of mosquitoes, the females are very fecund, but resources (such as larval food or suitable oviposition sites) are insufficient to support the maximum potential number of offspring through to adulthood. Indeed, so limiting are these environmental factors that many-fold reductions of the population's reproductive rate can have no impact at all on total population size (Dye, 1984). This fact can result in mosquito populations being 'resistant' to traditional SIT, which induces death at the embryonic stage. Although matings by irradiated or chemosterilised males will prevent a portion of eggs from hatching, those offspring that do hatch into larvae (being the product of wholly-wild matings) will be subject to a lesser degree of density-dependent competition and mortality. This can reduce or entirely offset the population-reducing effect of the sterile matings, or possibly even result in a net population increase (Rogers and Randolph 1984, and Figure 1-5, panel A).

In contrast to the SIT (where irradiation or chemosterilisation induces random dominant lethal mutations in the gametes, causing embryonic death), RIDL strain OX513A has been specifically engineered to cause late-acting lethality, at the final larval or pupal stage. As a result, the offspring of the sterile males will survive long enough to maintain the density-dependent pressure on the non-RIDL offspring that will go on to eclose into the next generation of breeding adults. This effect was mathematically modelled by Phuc et al. (2007) using estimates of mosquito population parameters obtained from field studies of *Ae. aegypti* (Figure 1-5). Late-acting lethality improves the efficacy of a control programme as a whole, by (i) suppressing the population more quickly, and (ii) reducing the overflooding ratios required to achieve eradication. Furthermore, as the strength of the density-dependent effects increases, so do the benefits of late-acting lethality.

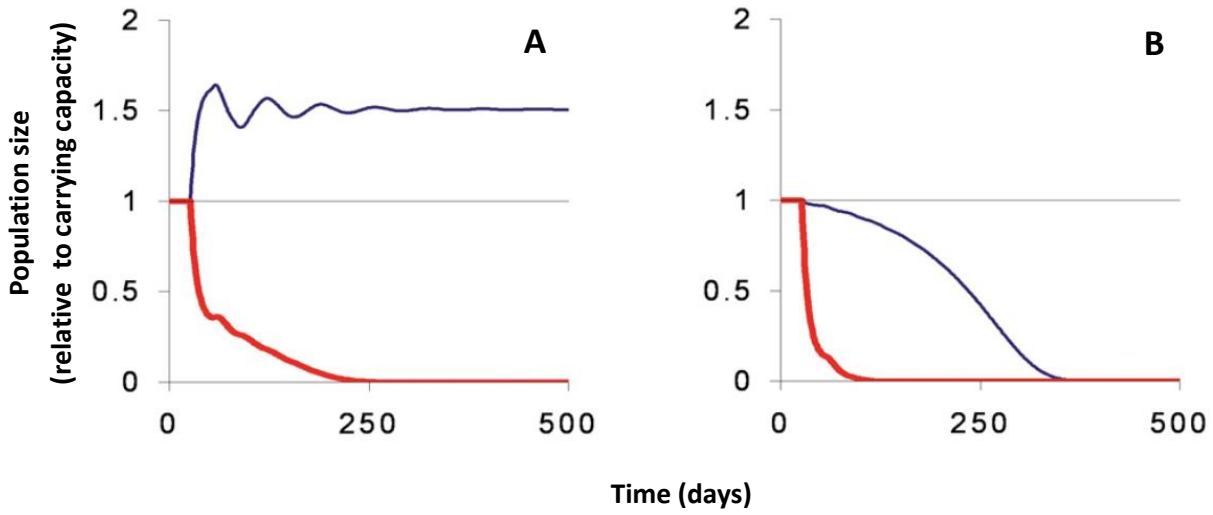


Figure 1-5. Beneficial effect of late-acting lethality on control of a density-dependent *Ae. aegypti* population. The blue line represents traditional SIT (early-acting lethality); the red line represents RIDL control (late-acting lethality). The grey line at y=1 represents the carrying capacity of the environment, at which level the population is assumed to remain if there is no other control intervention. Changes in population size following control intervention are expressed relative to the carrying capacity. (A) At a low release ratio (2:1 sterile: wild males), the SIT can actually increase the size of the population (due to a reduction in density-dependent mortality), while RIDL will eventually eradicate it. (B) At a higher release ratio (7.5:1 sterile: wild males), the SIT will eradicate the population, although not as quickly as RIDL. Figure modified from Phuc et al. (2007).

The model used to generate Figure 1-5 assumes that the OX513A construct is 100% penetrant, that is, in the absence of tetracycline 100% of the heterozygote offspring that inherit the construct will die. In fact, OX513A is 95-97% penetrant. The authors therefore

expanded their model to include 'leakiness' of the construct at the observed 3-5% level, and found that it would not materially reduce the effectiveness of RIDL releases (Phuc et al., 2007). The same principle has separately be shown to be true for incomplete sterility in conventional SIT releases (Barclay, 2001).

There are possible fitness penalties directly arising from transgenesis. The insertion of a foreign gene - such as the RIDL transgene - could reduce the fitness of the individual either by insertional mutagenesis (disruption of the native genes) or via the negative effects of any transgene products (Marrelli et al., 2006). The latter is especially pertinent to OX513A, where the primary function of the transgene is (under certain conditions) to continually express a toxic protein (tTAV) with the aim of killing the insect. Although this system is designed to be repressed by ingestion of dietary tetracycline at the larval stage, any failure of this repression system - even if not directly lethal - would likely negatively impact fitness of the insect. This effect may be exacerbated by the fact that the Act5C promoter in the OX513A transgene results in ubiquitous (whole-body) expression; expression limited to a specific region or organ is thought less likely to result in a fitness cost (Moreira et al., 2004). Furthermore, individuals bred for release are homozygous for the transgene, which was achieved by a process of inbreeding the offspring of the originally transformed individuals. If the transgene was inserted close to a recessive negative mutation with a deleterious effect, then making the strain homozygous for the transgene will also result in the recessive gene being made homozygous. This 'hitchhiking' effect can result in inbreeding depression (Marrelli et al., 2006; Koenraadt et al., 2010).

1.5 Mass Rearing and Release

Although RIDL has removed the need for sterilisation via irradiation or chemosterilants, it is modelled on traditional SIT and as such there is still a requirement for very large numbers of competitive males to be produced and released, with the purpose of competing with wild males for female matings. Therefore one of the challenges faced by a RIDL-based control approach is how to produce the maximum number of insects given limited resources, without compromising the vigour of the insects produced. The mass rearing of mosquitoes as a field of study developed alongside the earliest uses of the SIT (Trembley, 1944), and in many instances practices remain largely unchanged to this day (Benedict et al., 2009b).

Greater numbers can be produced by rearing at higher densities, but this can be detrimental to insect performance, in a number of ways. Rearing larvae at higher densities is known to result in delayed and less synchronous pupation, and produce smaller individuals (Hawley, 1985; Lyimo et al., 1992; Lounibos et al., 1993), which reduces their longevity (Reisen et al., 1984) and fecundity (Gomulski, 1985; Ponlawat and Harrington, 2007). In the absence of a genetic sexing system such as that used in El Salvador (Kaiser et al., 1978; Seawright et al., 1978) male and female mosquitoes are reared together up until the pupal stage. However, the release of female adults alongside the males is highly undesirable. There is high potential for the males to simply mate the females they were released with rather than seeking out wild females, which would reduce the overall efficacy of the programme. In addition, any females released may contribute to increased biting rates or risk of disease transmission, which raises both regulatory and ethical concerns. Therefore, in releases of OX513A, female pupae are mechanically separated from the males and removed from the release cohort.

Mosquito pupae posses a strong, waxy cuticle (Christophers, 1960) which facilitates handling to a greater extent than larvae, and also enables size separation of the larger females from the smaller males. The principles and techniques used in the process of mechanical sex separation have changed little since the earliest use of the SIT in mosquitoes. Several types of system exist. The first involves trapping the pupae in a gap of diminishing size between two fixed glass plates. Being larger, the females are trapped higher up while the smaller males fall further down, and so can be washed through separately from the females. This system was first described by Fay and Morlan (1959), and subsequently used in the large-scale SIT programme against *An. albimanus* in El Salvador (Dame et al., 1974; Lowe et al., 1980; Savage et al., 1980). However, the degree of separation was relatively poor, with 14% of the male release cohorts comprising females (Dame et al., 1974). An improved design (Focks, 1980) is still commercially available (John W. Hock Company, Gainesville, FL, USA) and was used to produce the male release cohorts for releases of OX513A in Brazil (Carvalho et al., 2014, 2015). A separate style of device is based on a sluiceway segmented by plates with gaps of precise size. As the male-female pupal mix is washed down the sluiceway, the largest pupae are stopped by the first plate, while smaller pupae continue further down. Devices of this sort have been used to sort *Culex pipiens* (Gerberg et al., 1969) and *Ae. aegypti* (McCray, 1961; Fay et al., 1963). However, as the plates are of fixed construction they are limited in their ability to adapt to

the variation in pupal size which results from changes to larval rearing conditions and/or harvesting schedules. A new proprietary method, with can be adjusted in response to changes in pupal dimensions, was used to separate male and female OX513A pupae during releases in the Cayman Islands (Harris et al., 2011, 2012) and Panama (Gorman et al., 2016). This method was also used throughout the experiments described in Chapter 3 of this thesis, and a device operating under a similar principle is in use as part of the ongoing SIT campaign against *Ae. albopictus* in Italy (Bellini et al., 2007). However, while an adjustable sex-separator can be used in a wider range of rearing regimens, the calibration of such devices can be problematic. When reared at higher densities, the size ranges of male and female pupae can overlap considerably. Therefore in order to remove >99% of the females, only the smallest males can be permitted to pass through the mechanism. This greatly reduces the total yield of male pupae (e.g. 40% by Ansari et al., 1977; and 15-25% by Bellini et al., 2007). In addition, it is possible that the final release cohort, comprising only the smallest males, may suffer a performance penalty (the effect of male size on performance is more fully investigated in Chapters 5-7). It is also possible that the selection of only the smallest males for release leads to assortative mating in the field, in which wild females are selected to prefer larger males (Papathanos et al., 2009).

The pupae of *Ae. aegypti*, like the larvae, require access to atmospheric oxygen (Barbosa and Peters, 1972). They are naturally buoyant, and breathe from the surface via two respiratory trumpets (Christophers, 1960). When disturbed, they will dive below the surface and stay there for some minutes. However, when compared to the larvae, the pupae are less tolerant of being prevented from accessing the surface. After a period of forced submersion the oxygen in their tissues becomes used up and they lose their buoyancy, at which point getting back to the surface is all but impossible (Christophers, 1960). For this reason, when storing pupae care must be taken to ensure there is adequate surface area for all the pupae to have access to the water's surface. In addition, adequate space must be available for the pupae to eclose. An emerging adult takes up considerably more space than a pupa (Figure 1-6), and during and immediately after emergence is quite vulnerable, as its exoskeleton takes time to harden (Clements, 1992). Whilst pupae do not all eclose at exactly the same time, when space is limiting a considerable number will not successfully eclose into adults.

Adult mosquitoes require space in which to rest. Most work has been directed at the resting habits of female mosquitoes; either with relevance to their choice of resting site in the wild or within a house, or in the laboratory regarding their preference before and after bloodfeeding, mating and oviposition. Less research has been conducted on males in the laboratory. Establishing optimal packing densities is important for maximising the output of a release programme based on the resources available. Certainly, each adult male requires a certain surface area on which to rest, but also important is the texture of the resting surface and its orientation in space. The associated energy costs of being stored in sub-optimal environments may severely affect the performance of the males once they are released.



Figure 1-6. Eclosion of *Ae. aegypti* from pupa to adult. Image credit: D. Nimmo, Oxitec.

Keeping the adults at high densities may also produce selection pressure on certain behaviours. For example, artificial photoperiods in the laboratory, entrained over generations, may cause behaviours like feeding and swarming to be out of phase with those of the wild type insects (Baker et al., 1980). A historical example is the efforts to control melon fly (*Bactrocera cucurbitae*) in Japan. A full twenty years into the SIT programme, female melon flies began to exhibit assortative mating and preferentially mated with wild-

type males, which greatly reduced the efficacy of control in the area, and resulted in the spread of a female genotype that was 'behaviourally resistant' to the SIT (Hibino and Iwahashi, 1989, 1991; Koyama et al., 2004). In a similar way, mass reared males may rapidly become adapted to mating with similarly lab-reared females, to the point where their ability to mate with wild-type females is diminished (Reisen, 2003).

1.6 Final Introductory Remarks and Aims of the Thesis

Efforts to reduce the incidence of dengue fever in humans focus on the control of its primary vector, the mosquito *Ae. aegypti*. Recently, promising genetic techniques to control mosquito populations - such as RIDL - have emerged. However, their implementation depends on the successful deployment of engineered males into the field, and on the ability of those males to secure matings with wild females. Male mosquitoes can be hampered in their ability to perform this, their primary function, by a host of factors including: deleterious effects of the transgene and/or the process of transformation and laboratory colonisation; rearing, handling, containment, and release at high densities; and laboratory adaptation preventing the adequate expression of survival, dispersal, and mate-seeking behaviours. In order to ensure the success of any future *Ae. aegypti* suppression programme based on a genetic control mechanism, the fitness burden borne by the released males must be both quantified and mitigated to the maximum extent possible.

This thesis contributes to that goal as follows. Chapter 2 investigates how larval rearing conditions can influence the size, asymmetry, quantity, and timing of pupae and adults that are produced by mass rearing methods. Each of these measures provides insight into the pressure that mass rearing exerts on the quality of the final released product. These experiments also serve to investigate, calibrate and standardise appropriate rearing regimens for experiments in later chapters. Chapter 3 details applied experiments (firstly in the laboratory and then in the field) investigating the creation and optimisation of physical release techniques, including the causes of and solutions to various causes of mortality in release devices. The issue of applicability of laboratory results to field studies is also explored.

The remaining Chapters 4-7 directly examine the competitiveness of males reared and released under conditions explored in Chapters 2 and 3, in combination with modifications to the nutritional status of the males. Specifically, the effects of (i) male size, (ii) quality of sugar diet, and (iii) length of diet provision on three key fitness measures are investigated. Chapter 5 uses one of the simplest measures of insect vigour - longevity - to assess the potential impact on overall male performance. Chapter 6 uses miniature flight mills to examine the link between nutritional status and a male mosquito's ability to disperse. Finally, Chapter 7 directly measures males' ability to inseminate females, and their ability to do so while in direct competition with wild-type males.

There is currently a shortfall in knowledge of male mosquito ecology, to the extent that it is a limiting factor for the progress of genetics-based control strategies (Ferguson et al., 2005, 2010). The experiments presented in this thesis will go some way to quantifying the potential performance of male genetically engineered mosquitoes.

2 Mosquito Rearing and Methods Development

2.1 Introduction

This chapter contains four parts. Section 2.2 (General Methods) outlines those methods that remained unchanged throughout the experiments presented in this thesis. Sections 2.3-2.5 detail experiments that were undertaken to better understand the process of larval rearing, and how changes in larval rearing conditions affected the number, size, asymmetry, and timing of the pupae and adults that were produced.

2.2 General Methods

2.2.1 Strain Background

The strain under investigation in this thesis was *Ae. aegypti* strain OX513A. It is a late-acting bi-sex lethal strain, as described in Section 1.4. OX513A was originally transformed in the Rockefeller WT strain of *Ae. aegypti* in 2002. In 2006, it was introgressed into a 'Latin' genetic background (freshly field-collected from Chiapas, Mexico) by five generations of back-crossing, after which it was made homozygous. Since being made homozygous, it has been maintained in laboratory conditions for over 120 generations.

All experiments in this thesis use the 'Latin' OX513A strain as described above, apart from the mating competitiveness experiments (Section 7.3), which additionally uses the Latin WT strain as a competitor to OX513A.

2.2.2 Mosquito Husbandry

Gravid *Ae. aegypti* females will readily lay their eggs onto a damp substrate (such as a moist filter paper), which can then be dried out and stored for several months prior to hatching. To hatch *Ae. aegypti* eggs, they were submerged in deionised water and placed under vacuum for between 1 and 16 hours. This reduces the levels of dissolved oxygen in the water; in the wild this would be indicative of water containing decomposing particulate organic matter and bacteria on which the larvae could feed (Judson, 1960; Ponnusamy et al., 2011). The first instar larvae were then removed from the vacuum and concentrated into a volume of 500 ml. This volume was mixed to a uniform suspension with a magnetic stirrer, and five 1 ml samples were taken. The number of larvae in each of these samples was counted, and the average used to calculate the volume of water that would contain a specific desired number of larvae (for example 3,000), which could be set up into a rearing tray. This volume was aliquoted into a plastic tray of internal dimensions 33.3 x 23.1 x 4.8

cm, to which was added 1 l of tap water. This gave a water depth of ≈ 1.4 cm. All experiments required that the mosquitoes be reared 'on tet' (i.e. in the presence of tetracycline, essential for the survival of OX513A larvae to adulthood), so tetracycline solution was added to give a concentration in the rearing water of 30 µg ml^{-1}.

Typically larvae were hatched overnight, being put under vacuum in the evening of Day 0. On the morning of Day 1 they were aliquoted as described above. Also on Day 1, the first dose of larval food was given. Larvae were fed on 'tetramin' (TetraMin® Ornamental Fish Flakes, by Tetra GmbH, Germany); the daily amounts are given in the Experiment 'Production from Rearing Trays' (Section 2.5), which established the standardised regimen used throughout the experiments in this thesis. Although every effort was made to adhere to this regimen whenever possible, occasionally ad-hoc modification was required. For example, slightly elevated temperatures can increase evaporation (in which case water levels were topped up), or increase the rate of pupation (which lowered the requirement for food on later days as there were less larvae remaining).

Larval rearing trays were kept in a controlled environment chamber (insectary) at 28±2 °C and 70±20% RH, with a 12:12 light/dark photoperiod. Pupation generally occurred on Days 7-9; pupae were manually 'picked' from trays with a 2.5 ml plastic pastette, and if required were manually sexed by examination of their terminal segments.

2.2.3 Wing Length Measurements

For experiments where wing length measurements were taken, individuals were either allowed to die naturally or were frozen, before having both wings dissected from the body and mounted onto glass microscope slides using 50% ethanol. Slides were sealed with clear tape and wings photographed in front of a graticule. Measurements were made from the digital photographs using ImageJ software (National Institutes of Health, USA). Wing length was measured from the notch (immediately distal to the alula) to the end of the R_{4+5} vein, not including the wing fringes (Figure 2-1). When possible both wings were dissected and measured, and the average of the two lengths was used for that individual. This also meant that an asymmetry analysis (Section 2.4) could be carried out on all collected wing length data.

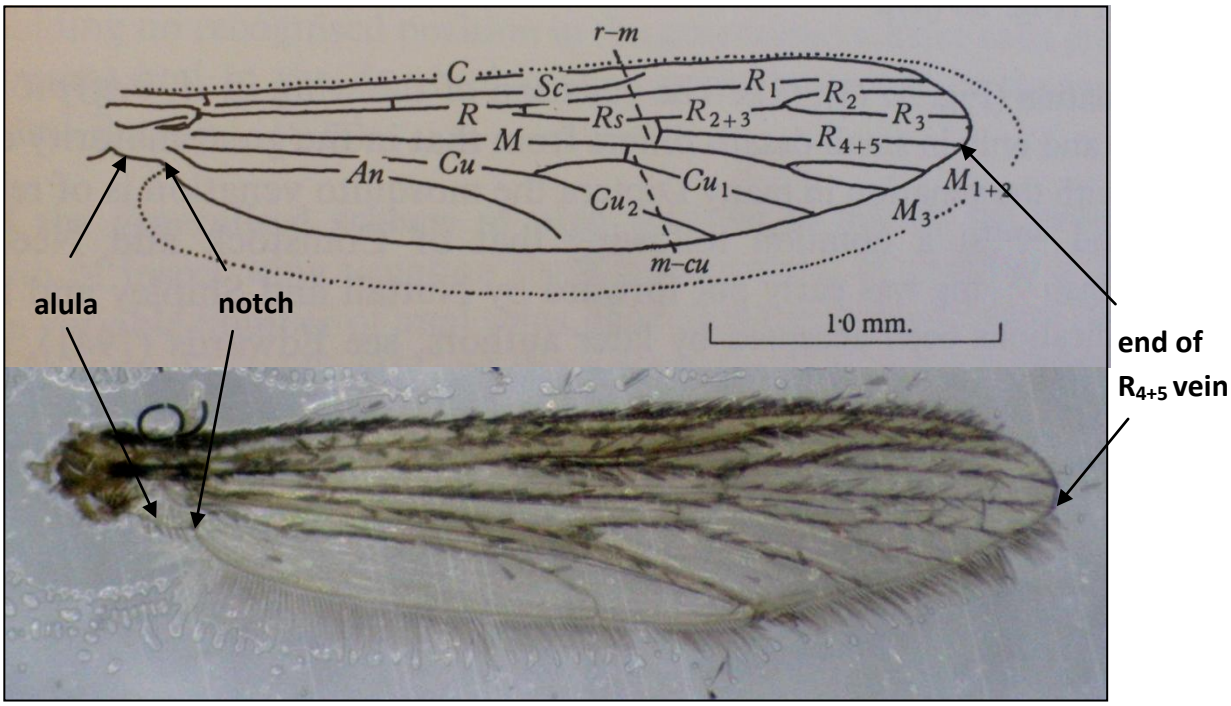


Figure 2-1. Reference points for wing length measurements. 'Wing length' was defined as the length in millimetres between the notch and the very end of the R_{4+5} vein (excluding the wing fringes). Upper portion of the figure modified from Christophers (1960).

2.2.4 Data Processing

For statistical analysis and graphical outputs, three software packages were used: Microsoft® Excel® 2007 (Microsoft Corporation, Redmond, WA, USA), Minitab® 16 (Minitab Inc., State College, PA, USA), and R version 3.3.1 (R Core Team, 2016).

2.3 Pupal Size as a Proxy for Adult Size

2.3.1 Introduction

The ability to quantify insect size is an essential tool when attempting to understand insect vigour. The most commonly-used method of quantifying mosquito body size is by the use of wing length measurements as a proxy (Nasci, 1990), as described previously (Section 2.2.3). However, this is time-consuming and destroys the individual. Furthermore if only one wing is dissected the representativeness of the measurement may potentially be compromised by the effects of wing asymmetry (see Section 2.4, Fluctuating Asymmetry). An alternative solution proposed by Koenraadt (2008) is to use the size of the pupa as a means of assessing subsequent adult size. Instead of individually dissecting, mounting, and photographing wings, pupae can be photographed quickly in batches, without the need to destroy the insects or rear separate batches especially for dissection. Pupal size - measured as either the width or length of the cephalothorax - was found to be a good predictor of adult size in WT *Ae. aegypti* females, but less so in males (Koenraadt, 2008). This experiment was designed to see which (if either) of these two measurements - pupal width and pupal length - are good predictors of adult wing length, specifically in male and female OX513A *Ae. aegypti*. In the event that good predictors were found, these could be used instead of wing length.

2.3.2 Materials and Methods

In order to obtain a range of sizes, larvae were reared at three different densities: 0.3, 1, and 3 larvae ml^{-1}, all on the same restricted diet (similar to that in Table 2-1), but amounts were proportionally less). Individual pupae were photographed under a microscope and against a graticule, with first lateral (Figure 2-2, panel A) and dorsal (panel B) sides face-on to the camera. Brief chilling on ice reduced movement while photographing. Pupae were allowed to eclose individually, before being killed and having their wings dissected and measured as described in General Methods (Section 2.2.3). In total 53 males and 62 females were measured.

Statistical Analyses

Not all data were normally distributed, therefore correlation coefficients were calculated on ranked data (Spearman rank-order method); this is also a more conservative test. Correlation is reported as an r_s value, indicating the strength of the association. r_s values of -1 indicate a perfect negative correlation, 0 no correlation, and 1 a perfect positive correlation. Each r_s value has an associated p-value indicating its significance.

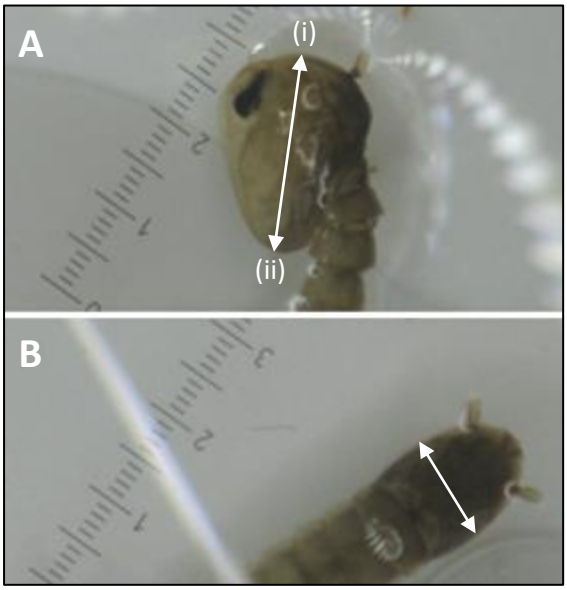


Figure 2-2. Measuring pupal length and width. (A) Pupae were rolled onto their side, and the length of the cephalothorax was measured from the uppermost (i) to the lowermost (ii) point on the scutum. (B) Pupae were suspended in a drop of water, and width was measured at the widest point of the cephalothorax, as estimated by eye.

2.3.3 Results and Discussion

Pupal width and length were both strong predictors of wing length, for males and females, with all r_s values >0.85. Both male predictors were stronger than both female predictors, with male pupal length being the strongest predictor overall (r_s = 0.946).

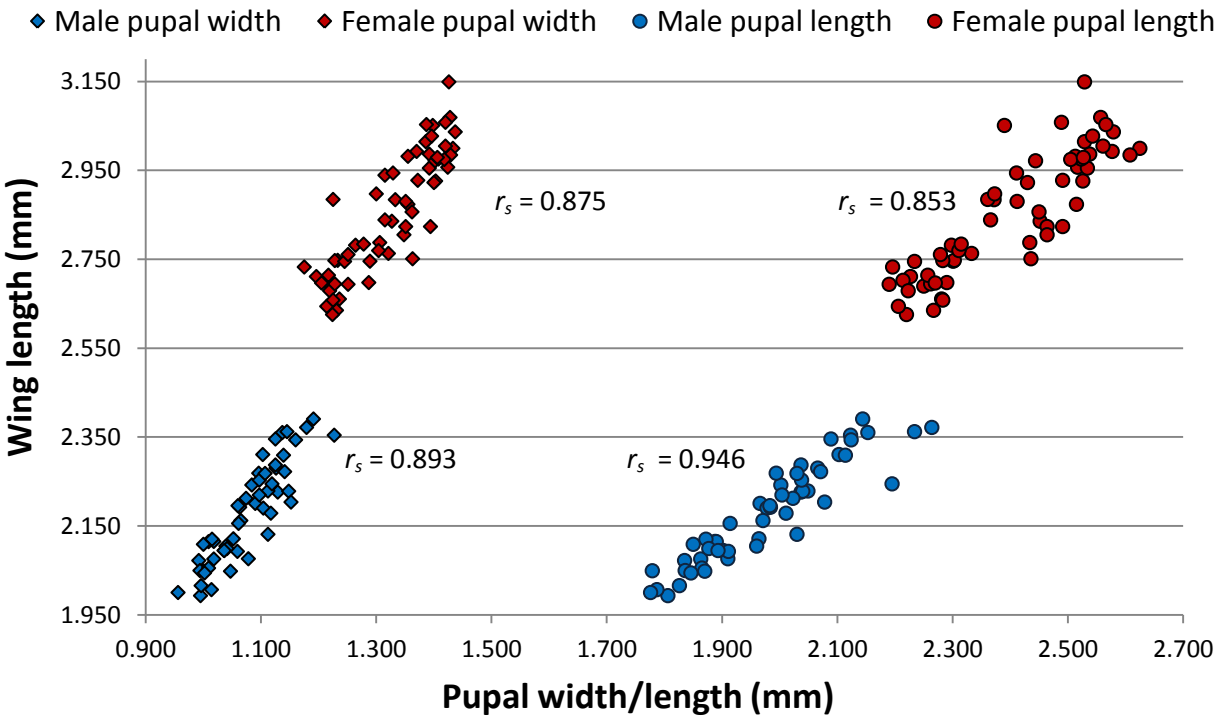


Figure 2-3. Pupal width (diamonds) and pupal length (circles), of both males (blue) and females (red), plotted against wing length. For each correlation, the r_s value is given, and p<0.0001 in all cases. n=53 for males and n=62 for females.

The results of the current study were broadly in agreement with those of Koenraadt (2008), who found female pupal measurements (both width and length) to be good predictors of adult female wing length, with correlation statistics approaching 0.9[2]. However, the predictive strength of the male pupal measurements was far greater than had previously been shown: r_s=0.89 and r_s=0.95 for pupal width and length, compared to R^2=0.55 and R^2=0.66 found by Koenraadt (2008). Also contrasting is the work of Steinwascher (1982), who similarly reported the strength of association between pupal and adult mass to be stronger in females than in males. The former author suggested that the lower association between pupal and adult size in males may be an inherent aspect of male biology. The fact that the present results showed a consistently high association between male pupal and adult size puts this in doubt. However, it is possible that the differing findings were a result of differences between the two mosquito strains used. The mosquitoes used in the study by Koenraadt (2008) were second-generation offspring of a field-caught strain; as such their recent larval rearing history would likely be more heterogeneous in terms of larval rearing conditions (food availability, density-dependent competition, temperature, and other environmental conditions). By contrast the OX513A strain used in the current study had been maintained in a laboratory for approximately 120 generations, and reared intensively under consistent densities and environmental conditions. Although both studies were conducted in similar and controlled environments and on similar numbers of insects, the lack of historical laboratory adaptation in the field-caught strain might have been the cause of the wider range of sizes observed by Koenraadt (2008) (approximately double the size of the range in the present study), and so perhaps also the lesser strength of the predictive measurements.

Given the strength of pupal measurements as predictors of adult size (especially in males, of more relevance to male-release programmes), they showed promise as a first stage routine quality control measure. For example, if during a mass rearing programme pupal lengths were observed to deviate from a defined acceptable range, further investigations could be made into the performance potential of those insects. Defining that range is a question addressed by subsequent chapters in this thesis, i.e. how will the size of a male *Ae. aegypti* affect its ability to survive, fly, and mate with wild-type females? Measuring pupae is far less time consuming than mounting and measuring wings, and so achieving sufficient

[2] Parametric (R^2) and non-parametric (r_s) correlation coefficients should not be compared statistically; however, it is acceptable to compare then informally for the purpose of discussion.

sample sizes to detect a deviation from an acceptable range would be much more practicable on a routine basis.

However, there are also practical disadvantages to using pupal measurements rather than wing length measurements as a proxy for adult size. The first is that the window in which pupal measurements can be made is much shorter, as mosquitoes only spend ≈2 days as pupae before eclosing into adults. By contrast wing length measurements can be made any time during or after the adult lifespan. The second disadvantage is that it is impossible to photograph large groups of pupae and still be able to know which measurement corresponds to which individual. Neither of these issues would likely apply to a mass production facility, where samples of males would be sacrificed in order to provide a quality estimate for the rest of the batch. However, the experiments described in this thesis required measurements specific to individuals, and so wing lengths were found to be a more practical measure.

2.3.4 Conclusions and Further Work

Within the context of a mass rearing programme, with no requirement of knowledge of specific individuals, pupal measurements would provide an excellent alternative to the more labour intensive wing length measurements as a means of routine assessment of insect quality. This is especially true of a male-release strategy such as RIDL, as the predictive power of pupal measures was greatest in males. However, questions remain over whether the strength of pupal measures as predictors is consistent over a range of environmental conditions. For example, the ambient temperature shortly after emergence has been demonstrated to affect the final wing length of adult *Aedes* mosquitoes (van den Heuvel, 1963), and changes in rearing temperature can result in different pupal sizes (Mohammed and Chadee, 2011). Repeating the experiment presented here with carefully-regulated rearing temperatures (for example by using water baths set to temperatures within the range 20-35°C) would likely expose any temperature-related changes to the correlation between pupal and adult size. Any such environment-related changes would not be an issue as part of a mass rearing programme - once rearing protocols are established, the presumed consistency of rearing conditions would allow this technique to be a very useful routine measure of insect quality.

2.4 Fluctuating Asymmetry

2.4.1 Introduction

In organisms that exhibit bilateral symmetry, there are three kinds of asymmetry. *Directional asymmetry* is the consistent bias towards development on one particular side of the body compared to the other; flatfish are a good example (Hubbs and Hubbs, 1945). In this case the right-minus-left (R - L) differences of a population are normally distributed, about a mean that is significantly higher or lower (depending on the direction) than zero. *Antisymmetry* is similar, but the directional bias is not consistent; for example in male fiddler crabs, development of the large signalling claw occurs with approximately equal frequency on either side (Davis, 1978). In this case, R - L values are bimodally distributed, with two peaks, one either side of zero. Finally, there is *fluctuating asymmetry* (FA), displayed by mosquitoes (amongst many other organisms) and which has no directionality at all; the R - L differences are normally distributed about a mean of zero.

FA has been a target of study for decades, as it is seen as a quantifiable measure of so-called 'developmental noise'. This term can be used loosely to describe the suite of minor environmentally-derived perturbations that conspire to pull a developmental process away from the perfect expression of genotypes into a three-dimensional organism. The outcome in the absence of these effects - perfect bilateral symmetry - provides an excellent baseline by which to measure them. Generally speaking, higher levels of heterozygosity appear to provide a 'buffer' against such perturbations, resulting in lower FA in more heterozygous individuals and populations (Palmer and Strobeck, 1986).

Wing length is commonly used as a measure of mosquito size (Nasci, 1990), and the fact that each insect has one wing either side of its bilateral divide means that it also provides a convenient feature by which to measure FA. This experiment used the wing length data accrued during the other experiments performed as part of this thesis. As such, wing length measurements were from mosquitoes of varying sex, strain and rearing density (although the majority of the measurements were from OX513A males). It might be anticipated that the smallest mosquitoes (those reared at the highest densities) would have had the most exposure to more developmental stress at the larval stage, and so might exhibit higher FA. Therefore, the aim of the experiment was to investigate whether there was a relationship between mean wing length and FA.

2.4.2 Materials and Methods

Wings were measured according to the methods described in 2.2.3. During wing dissections no record was kept of which wing was left and right, so FA was defined as the positive value of 'Wing 1 - Wing 2' (in place of R - L), where Wing 1 and Wing 2 are determined arbitrarily. Using this index of FA was recommended in a review of FA by Palmer and Strobeck (1986). However, a possible disadvantage of using an index that measures FA absolutely (i.e. in millimetres) rather than proportionally is that it does not account for differences in the size of the feature being measured (the wing). For example, a larger wing might be more asymmetrical than a smaller one in absolute terms, but proportional to its size it might be less asymmetrical. As such, males and females were analysed separately due to their inherently different wing lengths. In total there were complete measurements (both wings) for 913 males and 174 females[3].

FA values were correlated with mean wing length values (the average size of an individual's two wings). Both were normally distributed, so a Pearson product-moment correlation was used. Correlation is reported as an r value, indicating the strength of the association; r values of -1 indicate a perfect negative correlation, 0 no correlation, and 1 a perfect positive correlation.

[3] The software used to measure wing lengths returned values with three significant figures (e.g. 1.923 mm). To test the true accuracy of the measurements, a single set of ten repeated measurements was taken from the same pair of wings. From these ten measurements, FA ranged from 0.000 mm to 0.012 mm, with a mean of 0.0045 mm. The width of the 95% confidence interval around this mean was approximately 0.005 mm (0.00446 mm). Given that the true accuracy was less than that implied by measurements made to the nearest 0.001 mm, a secondary analysis was conducted where all individuals with FA ≤ 0.005 mm were excluded prior to analysis, leaving complete data for 659 males and 149 females. Excluding these individuals made very little difference to the correlation statistics reported and so the results are not presented.

2.4.3 Results and Discussion

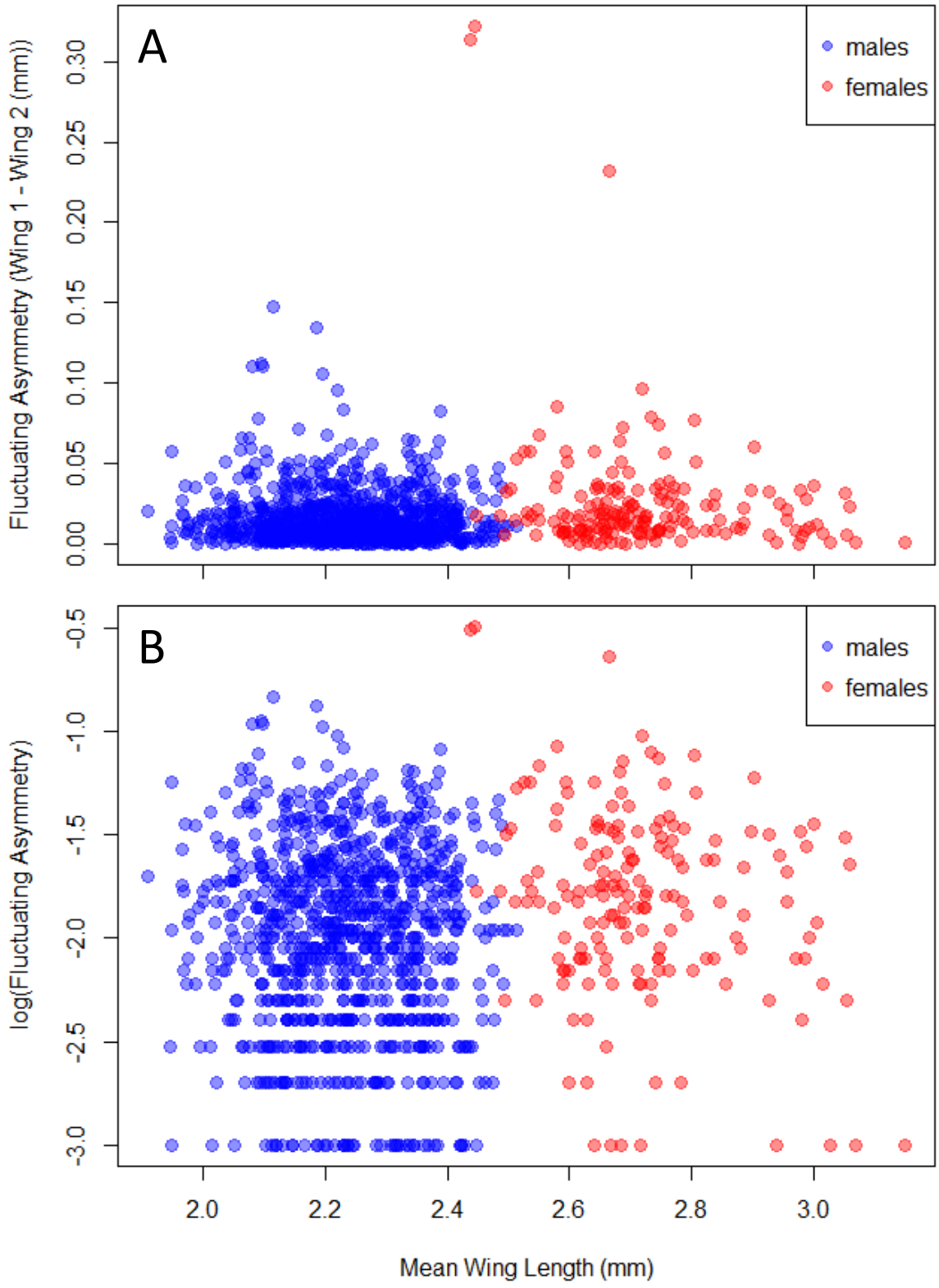


Figure 2-4. Fluctuating asymmetry of *Ae. aegypti* as a function of mean wing length. (A) shows absolute FA plotted against mean wing length, n=913 for males and n=174 for females. (B) shows the same data plotted on a log scale (excluding those individuals where FA was exactly zero (n=28)).

Male and female OX513A *Ae. aegypti* are distinct in size, as evidenced here by the almost complete lack of overlap in size ranges. There was no correlation between mean wing length and asymmetry in either males or females (r= -0.054 and -0.233, respectively). Although the correlation statistic appears to be much higher in females, a large part of this was due to 3 outliers (visible in Figure 2-4, panel A), which were smaller individuals with much higher asymmetry. The impact of these outliers was increased by the relatively smaller size of the female dataset. Removal of these 3 data points reduced the female statistic from r= -0.233 to r= -0.116. The wings from one of these outlying females can be seen in Figure 2-5. Correlation values were also calculated for subgroups within males and females (OX513A and WT strains; and 1, 3, and 5 larvae ml^{-1} rearing densities); no correlation was found in any of these subgroups.

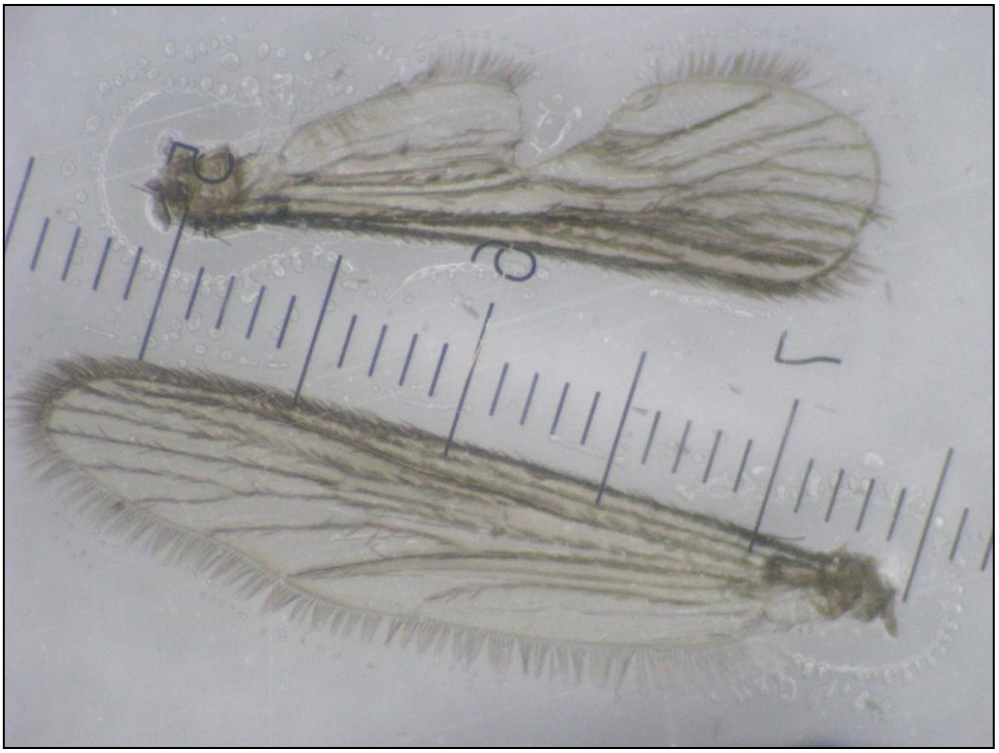


Figure 2-5. Asymmetry of *Ae. aegypti* wings. Both of these wings were from the same individual, but a morphological abnormality in one wing (the upper one in this picture) resulted in a high degree of asymmetry. Such extreme instances of asymmetry were very rare.

As is typical in FA, the majority of FA values were very low: 70.3% had FA values of less than 0.02 mm; or put another way, 76.4% of individuals had FA of less than 1% of their mean wing length. When the overall male and female data were logged (Figure 2-4, panel B), these lower values could be more easily visualised. The appearance of data points grouped into horizontal lines was a result of the measurement resolution becoming limiting.

2.4.4 Conclusions

Fluctuating asymmetry is a field of study in its own right. This was not an experiment designed specifically to investigate FA, rather a secondary use for the considerable quantity of wing length data gathered over the course of the other experiments in this thesis. As a result, the data came from individuals from a wide variety of rearing conditions, which - while helpful for generating a dataset with a range of sizes - may have also added in other sources of variation that made any existing correlation between mean wing length and FA harder to detect.

It was hypothesised that smaller individuals, generally the result of rearing at higher densities, might be subject to greater developmental stress and so exhibit higher FA. Although the correlations for males and females were weak, the correlation statistics were both less than zero, indicating that smaller individuals did have slightly higher FA. It is suggested that this was largely due to the small number of more highly asymmetrical individuals (those with FA greater than approximately 0.05 mm), which in both sexes appeared to occur slightly more often towards the lower end of the size range (Figure 2-4, panel A). An increase in the percentage occurrence of these highly asymmetrical individuals could be an indicator of a developmentally stressed rearing population.

2.5 Production from Rearing Trays

2.5.1 Introduction

The mass production of insects is one of the major challenges faced by suppression programmes, which require the release of large numbers of insects. In the wild, *Ae. aegypti* larvae live in standing water, including man-made sources such as blocked gutters, water butts, and used tyres. Within the context of rearing for laboratory experiments, the basic 'unit' of rearing is a tray containing 1 litre of water, into which newly-hatched first instar larvae are volumetrically aliquoted. This was also the case during mass rearing for the Cayman Islands suppression trial in 2010 (see Section 3.5). The number of larvae reared per tray (expressed as larvae ml^{-1}) is an extremely important variable within a mass rearing programme; even a small increase in rearing density can result in a much greater production output when multiplied over hundreds of rearing containers. However, one of the costs of increasing the rearing density is a reduction in the average size of the produced mosquitoes. The impact of this size reduction on the vigour and performance of the insects will be explored in detail in subsequent chapters.

This experiment was chiefly concerned with understanding how rearing density changed the pupae produced by rearing trays, in terms of (i) their absolute numbers (daily, and in total), and (ii) their individual size. Measuring daily production is especially important, as typically an entire male release cohort is harvested as pupae on a single day. This means that for each density it is important to identify on which day peak male pupation occurs, and how many pupae are produced on that day. Males that pupate on other days will likely not be harvested and so will not contribute to suppression[4]. Similarly, identification of the day of peak female pupation is also important; if the male and female peaks are sufficiently overlapping this can make it much more difficult to size-separate the male and female pupae, resulting in a reduction in both the number and size of males released[5]. As well as these applications to mass rearing, the results of this experiment informed (i) the rearing schedule for other experiments in this thesis, and (ii) the extent of size differences between males reared at different densities.

[4] These males may still be usefully used for egg production and/or ancillary experimentation.
[5] See the discussion of pupal sex separation beginning on page 24.

The experiment aimed to answer the following questions:

1. For each larval rearing density, how many pupae were present on any given rearing day, and which was the peak day of pupal production?
2. How did larval rearing density affect the total number of pupae produced by a rearing tray?
3. Did the larval densities and feeding regimens used in this experiment produce pupae of distinct size classes?

2.5.2 Materials and Methods

Mass reared OX513A mosquitoes are typically reared at 3 larvae ml^{-1}. Therefore larval densities of 1, 3, and 5 larvae ml^{-1} were used to cover a range around this value. These densities equate to 1000, 3000, and 5000 larvae per 1 l tray (n=4, 3, and 3; respectively). Eggs were hatched on Day 0, and first instar larvae aliquoted volumetrically (see General Methods, Section 2.2.2) into trays on Day 1. Pupae were picked and sexed daily; once all trays had had that day's pupae removed, they were all fed the appropriate amount for that day. All trays were fed identical amounts up to and including Day 6 (Table 2-1). Relative to each other, and as expected, food was consumed very quickly in the 5 larvae ml^{-1} trays, less quickly in the 3 larvae ml^{-1} trays, and slowly in the 1 larva ml^{-1} trays. Therefore, the food availability at each density could be respectively described as insufficient, sufficient, and ample. By Day 7 (for 1 larva ml^{-1} trays) and Day 11 (for 3 and 5 larvae ml^{-1}), the majority of larvae had pupated, and so feeding was reduced to prevent overfeeding and spoiling of the trays.

Table 2-1. Larval feeding regimen for larvae reared at 1, 3, and 5 larvae ml^{-1}. Values are grammes of food provided per rearing tray per day. This regimen was based on one that had previously been designed for trays of 3,000 larvae in a 1 l volume (Oxitec internal data). Therefore, males at higher and lower densities than this would respectively experience a shortage or abundance of food, which would result in differently-sized pupae. For the lowest density (1 ml^{-1}), the majority of pupation had occurred by Day 7, therefore after this day the amount of food was reduced to prevent over-feeding and spoilage of the rearing trays (red text).

Day	Larval Density (ml^{-1})		
	1	3	5
0	Eggs hatched	Eggs hatched	Eggs hatched
1	0.18	0.18	0.18
2	0.00	0.00	0.00
3	0.48	0.48	0.48
4	0.96	0.96	0.96
5	0.96*	0.96*	0.96*
6	0.96	0.96	0.96
7	0.00	0.48	0.48
8	0.00	0.48	0.48
9	0.00	0.24	0.24
10	0.00	0.24	0.24
11	0.24	0.24	0.24

*500 ml tap water added to each rearing tray in order to counteract the effects of evaporation.

2.5.3 Results and Discussion

Total Pupation

Table 2-2. Total pupae produced (±sd) for trays reared at 1, 3, and 5 larvae ml^{-1} (n=4, 3, and 3, respectively).

Larval Density (ml^{-1})	Number of Larvae in 1 litre	Total Pupae Produced		
		Males	Females	Total
1	1,000	527 (±27)	538 (±20)	1,065 (±35)
3	3,000	1,607 (±12)	1,274 (±38)	2,881 (±46)
5	5,000	2,046 (±77)	2,004 (±244)	4,051 (±303)

Table 2-2 gives the total numbers of pupae produced by trays of each density. These figures are most useful in the context of mass rearing for egg production (rather than release), where the entire pupal production of a tray can be used. Here, as density increases the total pupal production falls increasingly short of the number of larvae aliquoted into the tray. For trays of 1,000 and 3,000 larvae, the number of pupae produced was close to those values (deviating by +6.5(±3.5)% and -4.0(±1.5)%, respectively). However, for trays of 5,000 larvae, the number of pupae produced was 19.0(±6.1)% less than expected. This could be a density-dependent effect, where higher densities cause increasing larval death. This is especially likely in this experiment, where the highest-density trays had a deliberately restricted feeding regimen in order to produce pupae of smaller size. Larval death is difficult to detect (especially during the early instars when larvae are smaller and harder to see), as dead larvae are cannibalised.

Alternatively, the problem may lie in the volumetric aliquoting technique (see Section 2.2.2). First instar larvae were stirred to uniform suspension in a 500 ml beaker, as five 1 ml samples were taken and the number of larvae in each sample counted. Once the overall number of larvae per ml in the beaker had been estimated from these samples, the necessary volumes were then aliquoted from the beaker (also whilst being stirred) to the rearing trays. The five samples were taken using a standard auto pipette with a 1 ml tip, whereas the aliquots (requiring larger volumes) were taken using a battery-powered pipette controller with 25 ml tip. The aliquot pipette had a wider aperture and lower suction pressure than the sample pipette, and so it is possible that the aliquot pipette drew in larvae

at a lesser rate than the sample pipette. This would result in insufficient numbers of larvae per rearing tray. Such an effect would be exacerbated at higher larval densities, and so it is possible that there is an upper maximum beyond which the aliquoting lost its accuracy.

Daily Pupation

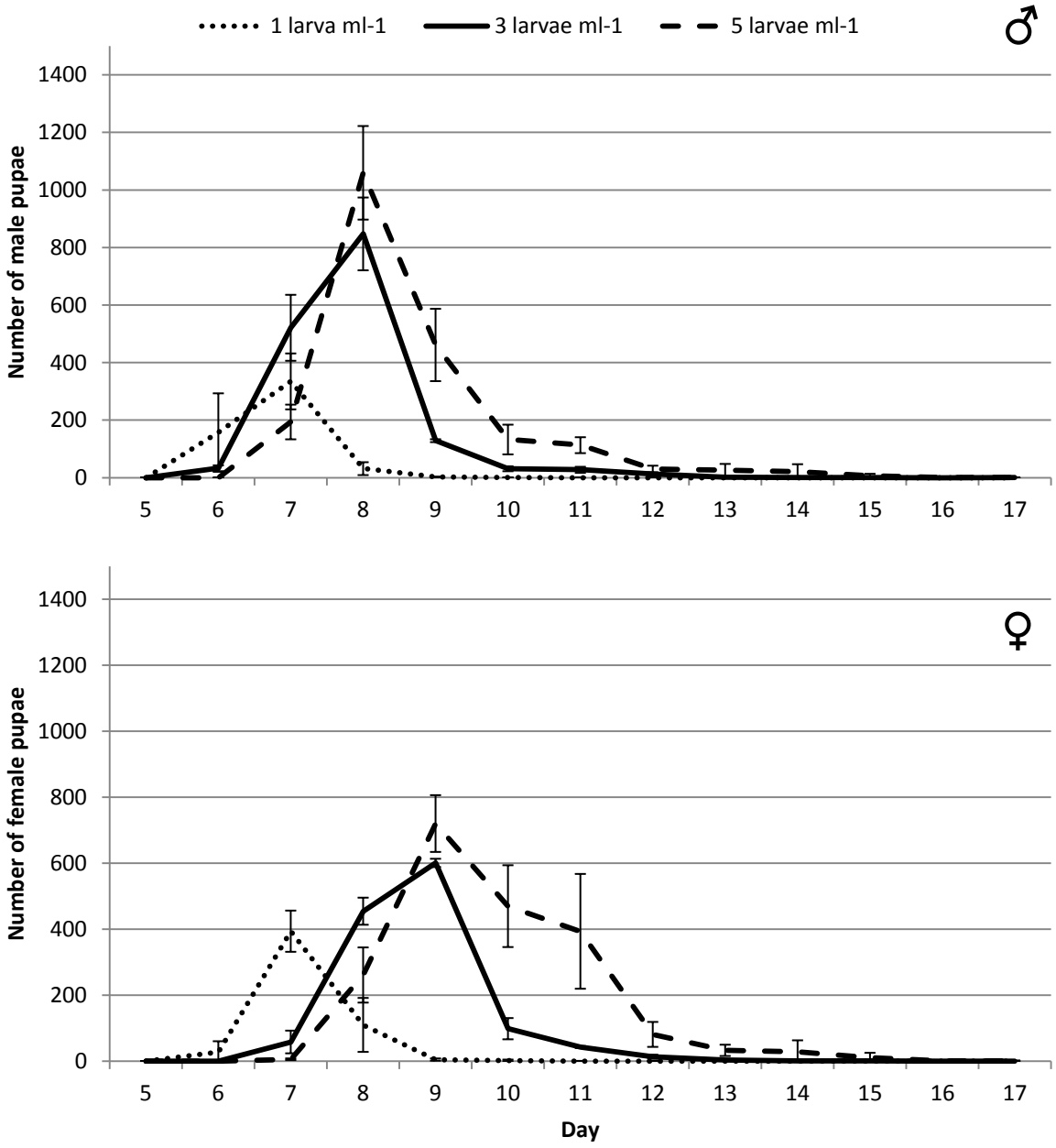


Figure 2-6. Daily pupation of OX513A *Ae. aegypti* males and females, reared at three densities. Eggs were hatched and larvae aliquoted into trays on day zero. Error bars = sd, n=4 (for 1 larvae ml^{-1}), and n=3 (for 3 and 5 larvae ml^{-1}).

Across all trays, pupae were produced on each of days 6-17, although most pupation occurred on days 7-11 (Figure 2-6). For males, the peak day of pupation was Day 7 (when reared at 1 larva ml^{-1}) and Day 8 (when reared at 3 and 5 larvae ml^{-1}). Peak female pupation also occurred on Day 7 at 1 larva ml^{-1}, but at 3 and 5 larvae ml^{-1} peak pupation did not occur until Day 9, a day later than in the equivalent males. Males are known to spend less time developing as larvae and so eclose earlier than females (Christophers, 1960; Clements, 1992). This is also helpful during mass production, where the aim is to have a pupal cohort consisting of only males; this is easier to achieve when the pupae are already mostly male at the point of harvesting. In addition, there was not a great deal of variation around the number of pupae produced each day. The ability to rear precise numbers of insects reduces the need to overcompensate in order to guarantee sufficient numbers for experimental purposes. As such there is less waste of associated labour, rearing space, and eggs.

Peak-Day Production

Mass-reared males to be released as part of the same cohort are usually harvested from the rearing trays over the course of a single day; ideally this is the day on which most male pupae are produced. With this in mind, future experiments will use males harvested on a single day, and so knowledge of (i) on which day the pupation peak occurs, and (ii) how many pupae can be expected on that day, is important for the purposes of experimental planning.

Table 2-3. Peak-day production of male pupae reared at three different densities. Day 7 was the peak day of production for males reared at 1 larva ml^{-1}; Day 8 was the peak day for males reared at 3 and 5 larvae ml^{-1}. Values are given ±sd, rounded to the nearest whole value.

	Larval Density (ml^{-1})		
Peak-day male pupae produced:	**1**	**3**	**5**
1. Number of individuals:	335(±97)	848(±127)	1060(±163)
2. As a % of total males:	64.1(±20.4)%	52.8(±8.0)%	51.9(±8.9)%

As might be expected, the larval density had a significant effect on the absolute numbers of pupae produced on the peak days (F=30.5, p=0.00035, one-way ANOVA), with more peak-day pupae produced in the trays containing more larvae (Table 2-3, row 1). However, while this difference was significant between 1 and 3 larvae ml^{-1} (p=0.0029) and 1 and 5 larvae ml^{-1} (p=0.00036), the difference was not significant between 3 and 5 larvae ml^{-1} (p=0.17, all Tukey HSD post hoc test). When peak-day pupal production was expressed as a proportion of total males produced on all days (Table 2-3, row 2), there was no significant effect of larval density (F=0.76, p=0.50, one-way ANOVA).

There was also an associated significant decrease in wing length with increasing larval rearing density. For each density, 30 peak-day males had wing length measurements taken. The mean wing lengths for each rearing density were all significantly different (p<0.001, 1 way ANOVA with Tukey HSD post-hoc tests), with males reared at 1 larva ml^{-1} being especially larger than the other two densities (Figure 2-7).

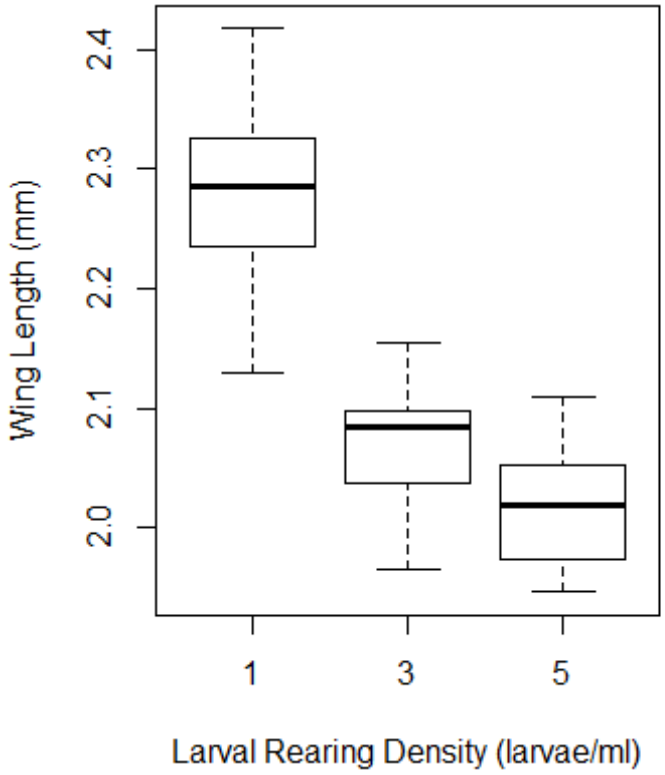


Figure 2-7. Wing lengths of males from each larval rearing density, n=30. Measured males were from days on which pupation peaked. All three densities resulted in males of statistically distinct sizes.

The relative similarity of the sizes of males reared at 3 and 5 larvae ml^{-1} pointed towards the 5 ml^{-1} trays having fewer larvae than the intended 5,000 (this was also evidenced by the total pupation being closer to 4,000 than 5,000; Table 2-2). This could be due to density-dependent larval death occurring in the 5 ml^{-1} trays. In addition, it indicates that the majority of death probably occurred early in development, when the larvae are less resistant to starvation (Chambers and Klowden, 1990; Clements, 1992). Density-dependent larval mortality has been shown in the field to occur primarily at the earlier larval stages (Southwood et al., 1972). Had the mortality occurred at a late-larval stage, the surviving larvae would have spent most of their development time at or near the intended 5 ml^{-1} density, likely reducing their average size relative to the 3 ml^{-1} males below what was actually observed. However, it is still impossible to rule out reduced aliquoting accuracy at higher densities as a cause of the observed similarity of body size of these two densities.

2.5.4 Conclusions and Future Work

This experiment successfully answered all three questions that were asked: (i) for each larval rearing density, data were collected for the number of pupae produced on each day, and the peak day of production for each was identified; (ii) increasing larval density was found to increase total and peak pupal production, although not always significantly; and (iii) all three rearing densities produced pupae that subsequently emerged into adults of significantly different wing length.

The three larval rearing densities chosen did not vary equally from each other; rather, 3 and 5 larvae ml^{-1} produced similar outcomes. They shared a peak day of pupation, the quantity of males produced on that peak day was similar (in terms of absolute quantity, and as a proportion of their respective totals), and the sizes of the males (whilst significantly different) were more similar than those of males reared at 1 larva ml^{-1}. This information was useful not only for the other experiments in this thesis, but also generally regarding the quantification of the exact insect products of larval rearing under different conditions. Finally, the adapted feeding regimen shown in Table 2-1 (while not formally assessed) did not result in rearing trays that were obviously over- or under-fed on any day, and pupation within each rearing density was consistent (in terms of both timing and numbers produced). As such, the feeding regimen was used in all future experiments.

At higher larval rearing densities, the total numbers of pupae produced were less than expected. It is difficult to determine whether this was a result of density-dependent

mortality, an inaccuracy of the aliquoting technique that was exacerbated by higher densities, or a combination of the two. If this experiment were to be repeated (perhaps for another RIDL strain), it would be prudent to perform a separate analysis of the accuracy of the aliquoting technique, by aliquoting trays of various densities, and then immediately performing manual counts of the first instar larvae.

Also worth bearing in mind is that the comparison of wing lengths in this experiment was not strictly speaking like-for-like; the measured 1 ml^{-1} males pupated on Day 7, as opposed to Day 8 for 3 ml^{-1} and 5 ml^{-1}. While it is highly doubtful that this increased the size distinction between 1 ml^{-1} and the other two densities, it would be interesting to conduct a full analysis of if and how within-density insect size (both pupal size and wing length) varies as a function of day of pupation. For example, as later-pupating individuals spend longer feeding as larvae, it follows that they may also be larger. This would mean that a mass rearing system that only harvests pupae once, and harvests them early, could be selecting against larger individual size, which would be likely to affect male fitness overall. In this experiment, of primary interest were those males pupating only on peak days, as those would be the individuals used in future experiments. Conducting a full investigation of how size varies with density and day of pupation would be considerably more time-consuming.

Extra wing length measurements might also have been useful in terms of collecting information on the females pupating on those days of peak male pupation. Having very similar-sized males and females (which can occur when rearing at higher densities; N. Naish, personal communication) can be problematic when mass rearing males for release. Male pupae are smaller than females, and so size separation is achieved by sieve-based mechanical sorting. When male and female sizes overlap, the selectivity of the sieving increases to ensure no females contaminate the male release cohort. This results in fewer males passing through the sieves, and those that do are of a smaller size. This could have implications on the efficacy of a suppression programme; male size is known to affect longevity, dispersal, and mating performance. Chapter 4 introduces this issue, which is then investigated fully in Chapters 5-7.

3 Field Release Experiments

3.1 Introduction

The first field releases of OX513A were performed in late 2009 on Grand Cayman, in the Cayman Islands; these were the first releases of genetically engineered mosquitoes anywhere in the world (Harris et al., 2011). The releases were small scale (≈18,000 total males released over four weeks), with the purpose of the trial being to assess whether OX513A *Ae. aegypti* males were capable of mating with WT females in an open-field environment. This was indeed shown to be the case; a proportion of eggs collected from the release area and hatched in the laboratory produced fluorescent larvae, indicating that they were the progeny of OX513A males. Subsequent PCR analysis confirmed these larvae were the result of successful mating between the released OX513A males and WT females (i.e. ruling out the possibility of the progeny being from OX513A males and inadvertently co-released OX513A females). Following this positive result, a larger-scale trial was planned at the same location, starting in April 2010, with the aim of demonstrating suppression of a local population of *Ae. aegypti* by means of OX513A male releases.

The 2009 trial had answered in the affirmative the question over whether or not OX513A males were capable of finding and mating with WT females. Nonetheless, significant questions remained regarding the competitiveness of those males, and how it might be improved. However, with the unique and sudden opportunity to attempt a suppression trial, investigating such questions (see Chapters 5-7) was delayed in favour of preparing for the suppression trial in 2010. The author's role was to design and laboratory-test suitable mosquito release devices, before implementing the design in the suppression trial.

3.1.1 Pupal Release

During the period c.1950-1980, numerous attempts were made to demonstrate the effectiveness of 'classical' SIT (discussed in Section 1.3.1) for the control of mosquito populations. During this time, workers in this field often released their sterile males at the pupal stage; male pupae were harvested, sterilised, transported to release sites, and then left in containers to eclose in the field. There are a number of advantages to this approach. Pupae take up significantly less factory space than adults, and so the overall output of a production facility can be increased by forgoing the requirement to maintain the insects as

adults prior to their release (Lowe et al., 1980). Pupae are also more robust than adults, and for the purposes of transportation can be chilled, kept damp rather than fully immersed, and/or packed at relatively high densities without significant ill effect. However, pupal releases also have a number of key disadvantages. An unprotected pot densely packed with mosquito pupae presents an extremely attractive prospect to predators such as wild fowl or even ants, which can quickly decimate a single release point (Bailey et al., 1979; author's anecdotal observation during test pupal releases as part of the study described by Harris et al., 2012). Vandalism or theft by locals, and adverse weather conditions may all also effect successful emergence and dispersal from the pupal release point (Lowe et al., 1975, 1980; Dickerson et al., 2012). Accurately estimating the level of failed emergence and dispersal can be more difficult using pupal releases, as the act of release happens gradually and out of sight of project workers. Furthermore, *Ae. aegypti* males that do successfully eclose and disperse from a pupal release site are incapable of breeding until they are 15-24 hours old (Roth, 1948; Klowden, 1990). Given that the average life expectancy of released OX513A males is only around two days (Lacroix et al., 2012; Neira et al., 2014; Carvalho et al., 2015; Winskill et al., 2015), the risk of death during this period of sexual maturation is significant. Nor can these males simply 'sit tight' while they sexually mature; their teneral energy reserves cannot sustain more than a token level of activity and so the males are required to add to these in order to accomplish any significant dispersal or mating behaviours (Foster, 1995). Such initial sugar-feeding behaviour may be mutually exclusive to mate-seeking (Haeger, 1960; Nayar and Sauerman, 1975; Nayar, 1981; Magnarelli, 1983; Haramis and Foster, 1990; Smith and Gadawski, 1994; Foster, 1995). The high mortality risk faced by the pupal and early-adult stages led to the expression of a strong preference for adult-only release by those researchers releasing chemosterilised *Culex pipiens* in Delhi, India (Sharma et al., 1976).

These disadvantages of pupal release were also variously experienced during the smaller-scale 2009 Cayman Islands trial, which had used only pupal releases of male OX513A. As a result, for the 2010 trial the decision was made to primarily release male OX513A at the adult stage. To accomplish this, a new form of release device (RD) was required. The design, testing, and optimisation of such a device are the subject of this chapter.

3.1.2 Adult Release

In the laboratory, as far as possible the insects must be allowed room to rest, fly, feed, mate, and oviposit. As such, laboratory cages are often cubical in shape, with relatively little

resting surfaces but considerable volume in which to exhibit these behaviours. For the purposes of field releases these priorities change; only males are released, so space for blood-feeding, oviposition, and mating are not required. However, adult males do require a surface on which to rest. Crucially, the thousands of insects required for each release must be transportable from the rearing facility to the release site, and so vehicle space will often be limiting. Therefore the surface area: volume ratio of an RD must be far greater than that of a laboratory cage.

Two suppression programmes targeting *An. albimanus* were carried out in El Salvador in the 1970s (Lofgren et al., 1974; Dame et al., 1981). At its peak, the second programme was producing 1 million male pupae per day (Lowe et al., 1980), and so considerable effort went into improving adult releases. With genetic technologies now offering a new alternative to classical SIT, these efforts provide a valuable resource for informing release optimisation today. In El Salvador, the male release cohorts were eclosed, sexually matured, and sugar-fed in relatively spacious holding cages. Following which, the males were immobilised with cold and then transferred volumetrically to release devices. However, the process of transferring males between cage types is space- and labour-intensive, and the delicate bodies of the adults may easily be damaged when packed together in this manner. By contrast, pupae can be harvested from rearing trays and counted volumetrically with relative speed and precision, and without excessively increasing subsequent mortality (Oxitec internal data).

Therefore, for the purposes of the upcoming suppression trial in the Cayman Islands it was deemed highly desirable to create a single device that would encompass all the stages of eclosion, sexual maturation, sugar-feeding (if required), transport to the field, and release. One result of this approach was that such a device would need to accommodate both aquatic and non-aquatic life stages (pupae and adults), each of which have specific requirements. However, it was felt that this potential issue was worth tackling in order to avoid the need to transfer adult males from one cage type to another prior to release.

Each RD would need to be able to house six hundred[6] OX513A males from eclosion to release, and do so without the insects sustaining significant lethal or sub-lethal damage that would impinge on their subsequent competitiveness in the field. In El Salvador, the release

[6] This figure was calculated by Oxitec field trial managers using data and experience from previous trials, taking into account factors such as the required OX513A: WT release ratio, the size of the release area, and the spread of release points.

devices took a variety of forms, such as: large plywood boxes (Lowe et al., 1980); expanded foam ice chests (Lowe et al., 1975, 1980); foam drinking cups (Smittle and Patterson, 1974; Lowe et al., 1975, 1980); and 'flat' release cages, split into two halves that opened about a hinge to 180°, like a book (Bailey et al., 1979). However, none of these were required to house both pupae and adults. Based on this and other requirements specific to the upcoming trial, two starting points for the design of the RDs were selected for design and development: a 'cup' design and a 'flat cage' design. Rather than creating these from scratch, prototypes were based on ordinary disposable coffee cups and hard plastic stationery folders, respectively. The merits of each of these designs as RDs were investigated as described below.

3.2 Release Device Prototype 1 - Coffee Cup

Disposable paper coffee cups (F. Bender Ltd., UK) were chosen as a starting point for designing an RD, as they were considered to be of approximately appropriate size for 600 adult males, in terms of both surface area and volume available to pupae and adults (Table 3-1). In addition, coffee cups stack very efficiently for easier transportation, are generally available globally, are reasonably cheap and lightweight, and are easy to dispose of. Drinking cups of one kind or another have previously been used to transport adults of various species to field release sites (Smittle and Patterson, 1974; Lowe et al., 1975, 1980; Helinski et al., 2008). Figure 3-1 shows the initial prototype of an RD based on a coffee cup.

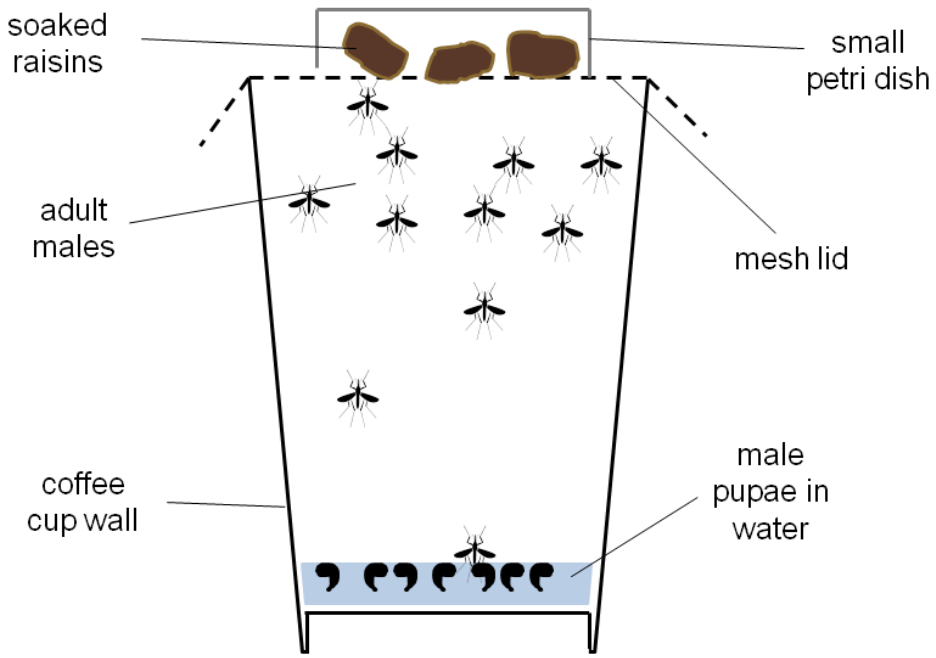


Figure 3-1. Diagram of an RD for adult males, based on a coffee cup. Six hundred male pupae were placed (in water 1-2 cm deep) into the bottom of the cup. The adults then eclosed and were free to fly within the cup, or rest on the walls or the mesh lid, which was held in place by the rim of the cup's original lid. A sugar source (soaked raisins or otherwise) was provided via the mesh lid, and was kept moist by the petri dish, which also served to protect against ants. After eclosion, a small puncture was made in the bottom of the cup, allowing the water and pupal exuviae to drain out, but not large enough to allow the escape of adults. The 'dry' cup was then able to be transported to the field without fear of water damaging the adults in the case of instability or accidental inversion (the intention is always to transport the RDs in the pictured orientation as far as possible). Once at the release point, the mesh lid could easily be removed to allow the adults to disperse. Each RD was single-use.

A series of experiments was then conducted to determine the practicality of the system shown in Figure 3-1. In each case pupae were introduced into the RD, and after a period of several days (to allow eclosion and sexual maturation) the RD was opened inside a cage. The RD's suitability for purpose was assessed by counting the number of males that were able to successfully 'disperse' from the RD. At the end of the experiment, males that were found anywhere outside the RD were counted as 'dispersed'.

3.2.1 Coffee Cup Release Devices I - Preliminary Experiment

Materials and Methods

OX513A larvae were reared at a density of 3 larvae ml^{-1}, according to the methods described in General Methods (Section 2.2.2). In addition, pupae were processed using several methods that were also under testing for use in the upcoming suppression trial. Pupae were separated from larvae by means of cold water separation: the larval-pupal mix was immersed in cold (≈13°C) tap water, which caused the pupae to float and the larvae to sink (as described more fully by Weathersby (1963) and Hazard (1967)). Pupae were then skimmed off the top. The elimination of the final larvae from these pupae was achieved by forcing them through a slit of fixed width that allowed the passage of larvae, but not the pupae, which were retained. To then separate the male pupae (used in the experiments) from the females (discarded), the pupae were all passed through a proprietary device which allowed the smaller male pupae to pass through. This final cohort of male pupae was checked under the microscope to ensure it was in fact 100% comprised of males. This level of mechanical handling had the potential to reduce the overall competitiveness of the adult males, and was not strictly necessary for these experiments as the numbers of insects required was not that large. However, such techniques would certainly be required during the suppression trial itself, and so it was prudent that the insects used in these experiments suffer the same potential performance penalties.

Male pupae were harvested from rearing trays, processed as described above, and counted into RDs. They day on which this was performed was designated Day 0. Two sizes of cup were tested (medium and large; Table 3-1), and three quantities of male pupae (150, 300, 600). On Day 4 the RDs were drained of the pupal eclosion water and opened inside 15 x 15 x 15 cm cages. The next day cages were frozen and the males counted. Successfully-eclosed males were classed as either 'dispersed' (found outside the RD), or 'non-dispersed' (remained inside the RD). All remaining males were classed as 'non-viable', and were not

counted directly but rather by subtracting the other two classes from the total number of pupae introduced on Day 0. As this was a preliminary experiment, and the numbers of replicates were both low and variable, no statistical tests were performed on the data.

Table 3-1. Number of males per unit space inside the two tested sizes of coffee cup RD. Values are based on having 600 males per RD.

	16 oz (medium) cup	20.5 oz (large) cup
pupae per cm^2 water	23.51	21.95
adults per cm^2 resting space	1.69	1.44
adults per cm^3 air volume	1.10	0.90

Results and Discussion

Of the three categories, only non-viable males (orange bars, Figure 3-2) would definitely be lost to a release effort; these mostly consisted of males that died before or during emergence. Males that successfully eclosed but did not choose to disperse from the opened RD are anticipated to be carrying some kind of fitness penalty, possibly sustained during the few days spent in the RD. Nonetheless during a real release these would still contribute to numbers released, as they would be forcibly ejected from the RD.

Across all four treatment combinations of RD size and adult density, mortality ranged from 33.6% to 46.7%. Furthermore, mortality did not decrease as expected when male density was reduced, even by a factor of 4. Regardless of density, a 40% loss of male output is unacceptably high, especially when considering the sub-lethal damage that the surviving 60% would likely be carrying.

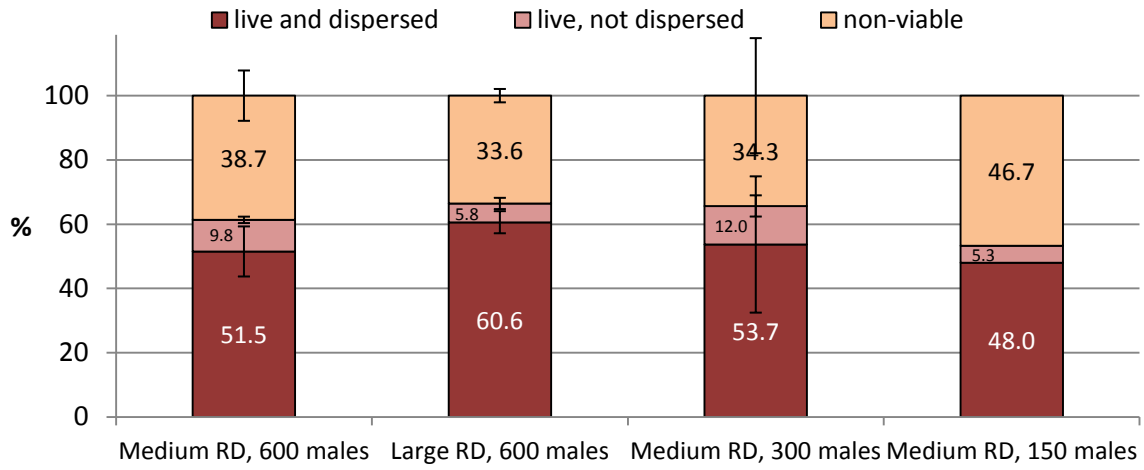


Figure 3-2. Fate of pupae from a range of RDs. Error bars = sd, n = (L-R): 3, 3, 2, 1.

Anecdotally, it seemed that most mortality occurred as a result of emerged males drowning on the surface of the water they had emerged from. Draining the water at an earlier stage would mitigate this problem, but would also likely result in a significant loss of uneclosed pupae. Therefore, the next step was to engineer a method that would provide a protective barrier between successfully-emerged adults and the water's surface. Furthermore, the next experiment would also make a more thorough assessment of the 'non-viable' category of males, by performing direct counts of losses through live pupae, dead pupae, semi-emerged adults, and adults that died on the surface of the water.

3.2.2 Coffee Cup Release Devices II - Testing Different Inserts

Materials and Methods

Three kinds of 'inserts' were tested in this experiment (Figure 3-3), all designed to reduce mortality by forming a barrier between emerged adult males and the surface of the water:

1. *'RD with poly chips'* - Approximately 5 polystyrene packing chips (Flo-Pak Super Green 'foam peanuts', 1 x 3 x 1.5 cm), were broken in half and added to the RDs after the pupae. The chips floated on the surface of the water, and so did take up a reasonable portion of the water's surface available for eclosion. However their large particle size ensured sufficient space for the emerged males to crawl upwards through into the main chamber of the RD. Poly chips coated with fluorescent powder had previously been used in a very similar fashion to dye-mark mosquitoes as they eclosed (Oxitec internal experiment).
2. *'RD with poly cup'* - a small polystyrene food packaging cup (Dart® product 4J4), when upturned, fitted snugly into the bottom of the RD. By cutting a hole in the bottom of the poly cup, the adults could progress into the main chamber of the RD.
3. *'RD with paddle'* - masonite (or similar) strips are used worldwide as oviposition substrates in ovitraps. Although they did not prevent access to the water's surface as completely as the two proposed solutions above, they are cheap and widely available.

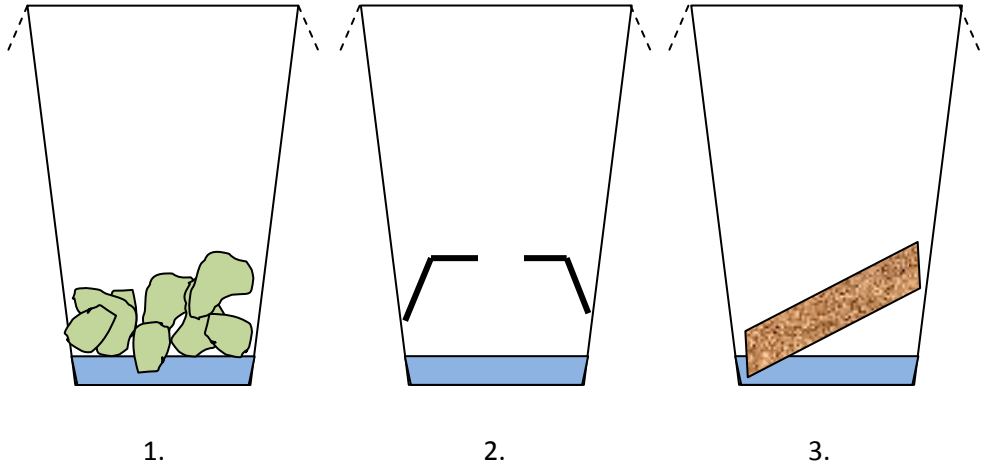


Figure 3-3. Modifications (inserts) to a basic coffee cup RD. (1) *'RD with poly chips'*, (2) *'RD with poly cup'*, and (3) *'RD with paddle'*.

In addition, two control treatments were set up:

4. *'No insert'* - unmodified RDs (Figure 3-1) with 300 male pupae.
5. *'Large WB in cage'* - large weigh boat (12 x 12 x 1.5 cm) from which adults eclose into a 15 x 15 x 15 cm cage. Mosquitoes are not limited by surface area for eclosion, air volume for flying adults, or surface area for resting adults. These conditions serve as 'optimal'.

All five treatments were set up in triplicate. The number of males in all treatments was reduced to 300 (from 600 in the previous experiment; Section 3.2.1), in order to prevent possible density-dependent mortality masking any potential beneficial effects of the inserts. Only medium-sized RDs were used, as the difference in space available to the insects was similar to that in large RDs, and medium RDs were more readily available. As in the previous experiment, male and female pupae were separated by size, but in this instance only a sample were checked for female contamination[7] (during a suppression trial manually checking and removing all females by hand is impossible). Finally, to reduce the time taken to count males into RDs of 300 males each, male pupae were dried on blotting paper and

[7] The first sample had 4 females in 1,052 pupae (0.38%), and the second had 0 females in 362 pupae (0%). This was deemed to be below an acceptable limit of 1%.

aliquoted by weight[8]. The timeline of RD set-up, draining and release was identical to the previous experiment.

Statistical Analysis

Individual mosquitoes were categorised based on their status at the end of the experiment, with each category comprising a percentage of the total. These categories were not always easily distinguishable (for example a 'semi-emerged' adult could potentially have been confused with an adult that successfully eclosed, but then later died on the water's surface). Therefore, for the purposes of statistical analysis, categories in which adults were capable of dispersal and mating (specifically: adults live on water; adults live on RD walls; and adults live and dispersed) were summed to get 'total survival' (red colours, Figure 3-4). Similarly, those individuals that would not contribute to a release programme (live pupae; dead pupae; semi-emerged adults; and adults dead on water) were summed together to get 'total mortality' (blue and white colours). Neither of these groups was found to be normally distributed, even following log or arcsine transformation. Therefore, a Kruskal-Wallis test was used to compare 'total survival' and 'total mortality' across all treatments, followed by a pairwise comparison test[9] to identify differences between pairs of treatments (Siegel and Castellan, 1988).

Results and Discussion

All three RDs with inserts (three right-hand bars, Figure 3-4) had extremely high total survival (left-to-right: 92.2%, 94.8%, and 89.8%). As a result it was expected that their survival would be significantly higher than the control RD ('no insert'), which in the previous experiment had displayed only 65.7% survival. However this time survival in the control RD was much higher (83.8%), lower than - but still comparable with - the three RDs with inserts. The increased survival observed in the control in this experiment versus the equivalent treatment in the previous experiment was put down to a combination of (i) refinement of experimental set-up processes in order to minimise damage to the pupae (for example, the proprietary pupal sex-separator has potential to cause damage if not used carefully), and (ii) increasing the number of replicates from two to three (which reduced the overall reduction in mean survival caused by a single replicate with lower survival).

[8] Three groups of exactly 50 males were weighed (0.0998g, 0.1002g, and 0.1009g, mean = 0.1003g). Multiplying this value gives a weight for 300 pupae as 0.6018g. This weight (or as close to it as possible) of pupae were aliquoted into each replicate RD.

[9] R function `kruskalmc` within package *pgirmess*.

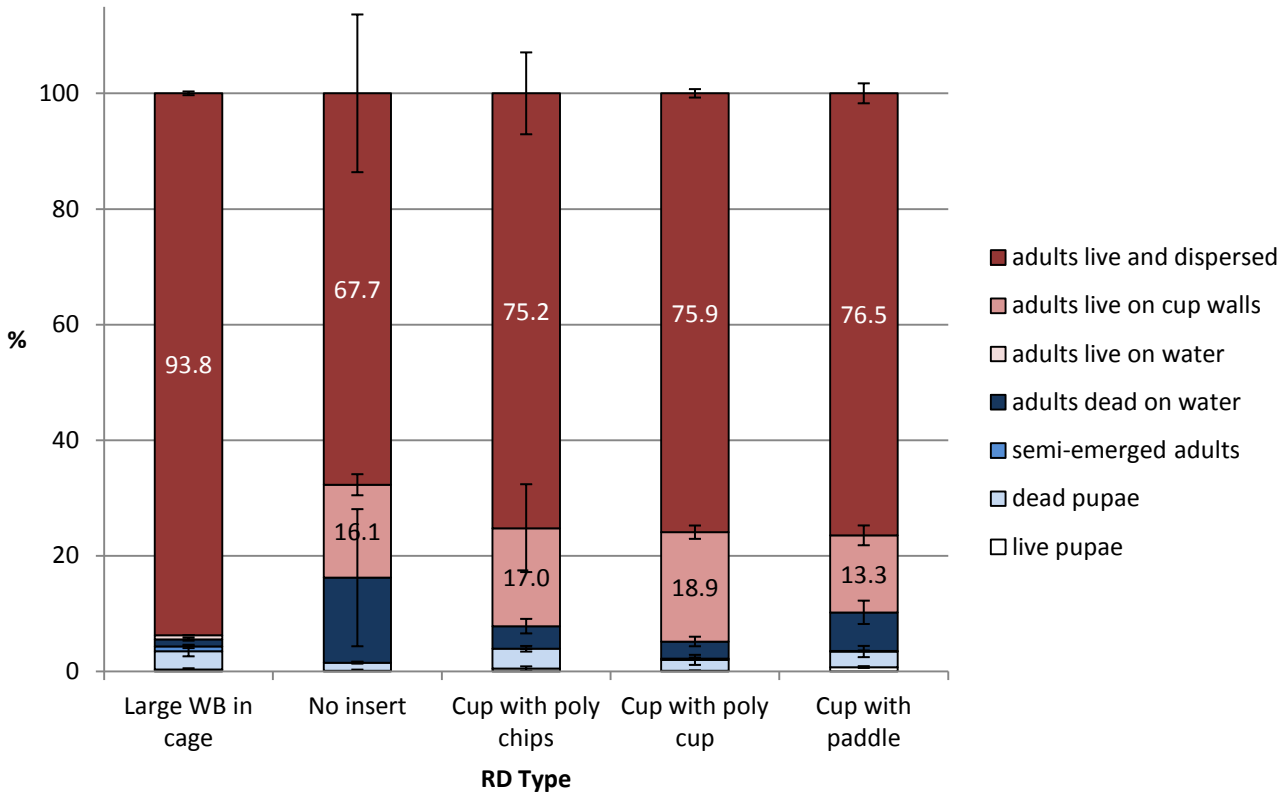


Figure 3-4. Fate of pupae across a range of RD types. All treatments were set up with ≈300 male pupae (295±13). Error bars =sd, n=3. Categories shown in red are those which would successfully disperse if released in the field, those in blue and white would not.

The Kruskal-Wallis test indicated that there were significant differences between treatments (χ^2=9.833, p=0.043). However, the subsequent pairwise comparison tests did not find any significant differences between any treatments[10]. Overall this was an extremely positive result, with all RDs with inserts showing high and consistent survival that was statistically indistinguishable from the 'ideal conditions' control (*'large WB in cage'*).

The number of pupae per cm^2 of water in the present experiment (300 males per RD) was 11.8[11]. When *An. albimanus* pupae were eclosed at various densities from petri dishes into cages, approximately the same number of pupae per cm^2 resulted in 87.2% successful emergence (Bailey et al., 1979). The treatments with inserts in the present study met or exceeded those values, despite the resultant adults eclosing into more constrained

[10] As an alternative analysis, an outlying value was excluded from the 'no insert' treatment, which conferred approximate normality on the dataset. A subsequent ANOVA also found that there were no significant differences in total survival (F=3.16, p=0.07, one-way ANOVA).

[11] Greater in the 'cup with poly chips', due to the occupation of some of the water's surface area by the poly chips.

surroundings and an additional period spent in the RDs after emergence. However, introducing inserts to the RDs did not result in a significant improvement in survival as compared to controls, as the control RDs had much higher survival than expected based on the results of the previous experiment. However, survival in the control RDs was more variable (both within this experiment, and considering the variation in its performance between this experiment and the previous one). Within this experiment, this was due to a single replicate having a higher proportion of 'adults dead on water' - precisely the problem that the inserts were introduced to deal with. Therefore the introduction of inserts was deemed a worthwhile addition. Of the three insert types, the 'poly cup' RD showed the highest absolute mean survival (actually slightly higher than the 'ideal' control). Furthermore, the poly cup insert does not intrude on the pupal eclosion surface (unlike the poly chips), and unlike both other insert types is fixed in position and so is least likely to present a hazard to the adult males during transportation. Therefore the use of poly cup inserts in the RDs was taken forward to the next stage of refinement.

3.2.3 Coffee Cup Release Devices III - Optimising Poly Cup Insert

The previous experiment (Section 3.2.2) showed that excellent (89.8-94.8%) survival could be achieved in RDs with poly cup inserts, with 300 male pupae in each. The next stage was to perform experiments at various densities with this design of RD, in order to establish the threshold density above which survival would become adversely affected. It is worth reiterating that even those insects that survive to disperse may be carrying sub-lethal damage as a result of being kept under high densities; at this stage no attempt was made to quantify this.

Materials and Methods

Densities of 600 and 800 males per RD were chosen as tests of the maximum capacity of the RDs. Also tested was an improvement to the poly cup design; by trimming the top from the poly cup before fitting inside the release device, it can be made to fit snugly right against the surface of the water, freeing up more space in the main chamber of the release device. Poly cups modified in this way are referred to as 'cut'; unmodified are referred to as 'uncut'. Finally, as in the previous experiment, an RD with no insert at all served as a control. All RDs were set up in triplicate. Aliquoting male pupae by weight had proved to be very successful in the previous experiment (aiming for aliquots of 300, mean was 295±13), so the same technique was used again. Again, one sample of the male pupae (size-separated from the females) was manually checked for female contamination (zero females in 1,000 males (0%)). The timeline of RD set up, draining and release was the same as previously described in Section 3.2.1.

Statistical Analysis

Analyses were again performed on the summed categories 'total survival' and 'total mortality'. Both were found not found to be normally distributed, due to one outlying replicate in the 'No insert, 600' treatment. Removal of this replicate was considered, but rejected, based on the impression from the previous two experiments that inserts improve *consistency* of survival (i.e. RDs with no inserts may have comparable survival with modified RDs in some instances, but in others their survival is markedly lower). It was felt that removal of a replicate displaying this undesirable variation would artificially improve the performance of the 'no insert' RD, and so the replicate was left in place. Again, neither log nor arcsine transformations conferred a normal distribution, so a Kruskal-Wallis test was used to compare 'total survival' and 'total mortality' across all treatments.

Results and Discussion

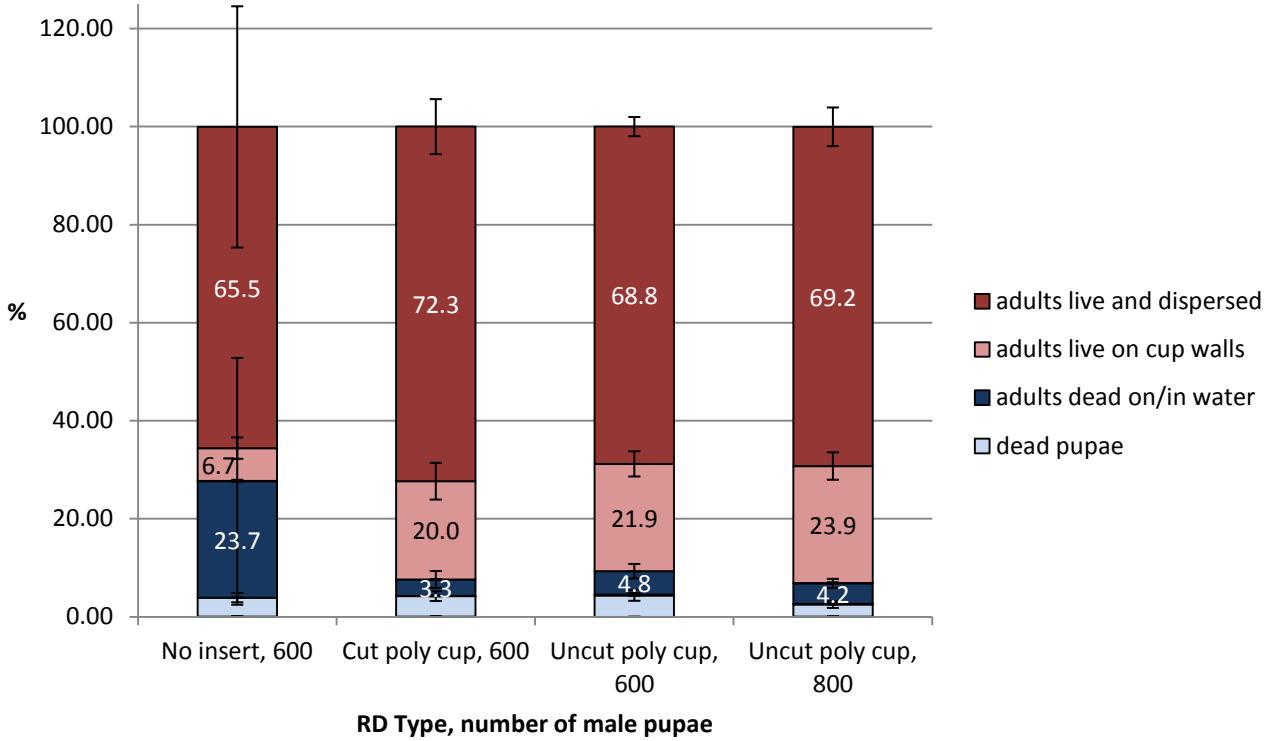


Figure 3-5. Fate of pupae across a range of RD types. Treatments were set up with the number of pupae indicated in the figure (600 or 800, means were 574±36 and 769±9 respectively). Error bars = sd, n=3. Categories shown in red are those which would successfully disperse and mate if released in the field, those in blue would not.

There were no statistical differences in total survival or total mortality between any treatments (χ^2 =7.67, p=0.053, Kruskal-Wallis test). All three RDs with inserts (three right-hand bars, Figure 3-5) had extremely high total survival (left-to-right: 92.3%, 90.7%, and 93.1%). As in the previous experiment, the control RD ('No insert, 600') had survival that was lower than - but not significantly different from - that of the RDs with inserts. Also like the previous experiment, this was due to one replicate of the control RD having a much higher proportion of 'adults dead on water' than the other two replicates (in fact over half of the 600 males in that outlying replicate died on the surface of the water).

In those RDs with 600 pupae, the number of pupae per cm^2 of water was 23.5. When *An. albimanus* pupae were eclosed at various densities from petri dishes into cages, approximately the same number of pupae per cm^2 resulted in 79.4% successful emergence (Bailey et al., 1979). In the present study, all treatments at that density with inserts far surpassed those values, despite the resultant adults eclosing into more constrained surroundings and an additional period spent in the RDs after emergence. In the treatment

with 800 pupae, there were 31.3 pupae per cm^2, which had previously resulted in 70.0% emergence (Bailey et al., 1979); again, this was far exceeded in the present study.

The results of this experiment were extremely positive. The lack of an insert was once again demonstrated to be responsible for unacceptable variation in the survival of males in the RD. By contrast the RDs with poly cup inserts showed consistently high survival, which even at 800 males per RD was comparable with the survival observed at only 300 males per RD. The brief for this set of experiments was that the RD needed to be able to hold 600 male pupae, allow them to eclose, and then provide a safe space for them prior to release. As the results of this experiment showed that this was possible with at least up to 800 males, this design of RD was deemed sufficiently developed for preliminary use in the field.

3.3 Release Device Prototype 2 - Cassette Cage

Introduction

The second prototype RD was based on a specific system used during the releases of sterilised adult male *An. albimanus* in El Salvador in the late 1970s (Dame et al., 1981). The best-performing adult RDs in that programme were 'flat' release cages (30.5 x 30.5 x 2 cm), split into two halves that opened about a hinge to 180°, like a book (Bailey et al., 1979; and Figure 3-6, panel A). The motivation for this design was (and remains) that while adult insects do not necessarily need to fly around whilst inside the RD (and indeed it is likely detrimental to their subsequent performance if they do; Bailey et al., 1979), they do need a surface on which to rest. Therefore surface area should be maximised and volume minimised as far as possible. Reduced volume maximises portability, which is important given the limited carrying capacity of programme vehicles and staff. The regular, flat shape also lends well to the RDs being stacked and stored together into a 'cassette', hence the name. Finally, the ability of the RD to open fully flat minimises the need for staff to agitate the RDs in order to compel the males to disperse.

As with the coffee cup RDs, each cassette cage RD needed to house 600 male mosquitoes during eclosion, sexual maturation, sugar-feeding (if required), transport to the field, and release. However, unlike the coffee cup RDs, the aim for the cassette cage RDs was for them to be reusable, not disposable. Rather than constructing cassette cage RDs from scratch, commercially available rigid plastic stationery folders (A4 'Tuff Boxes' by Tiger Stationery Ltd, UK) were used as a starting point, to which modifications were made (Figure 3-6).

In a preliminary (single repeat) experiment, 480 of 552 pupae (87.0%) successfully eclosed and dispersed from a cassette cage RD. This was sufficiently high to warrant a further experiment at a higher density, and so a density of 800 males per RD was chosen, in order to be an identical maximum test to that given to the coffee cup RDs. Again, as per the coffee cup RDs, the aim of the experiment was to test the suitability for purpose of the RD, by counting the number of males that survived to be able to successfully 'disperse' from the RD. Two different sizes of RD were investigated, along with two different release schedules: release after 3 days (representing the typical length of time males might spend in an RD in a

field release), and release after 10 days (to make any potential performance penalties that might be incurred by being kept at high densities more apparent).

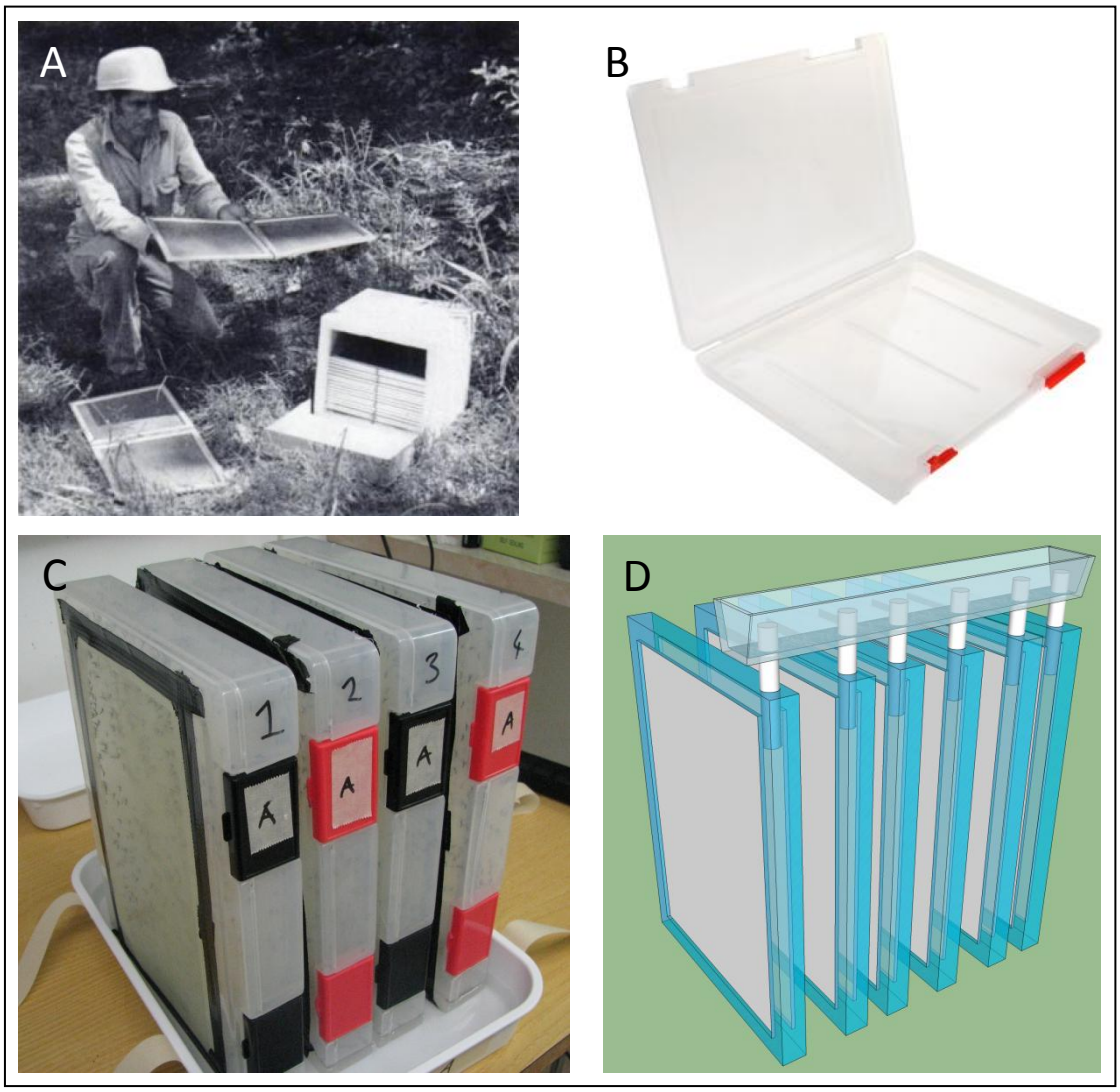


Figure 3-6. Cassette Cage RDs. (A) Flat release cages being opened to release sterile male *An. albimanus* in El Salvador in the late 1970s. When closed, the cages stacked inside the insulated container shown on the right. Figure modified from Lowe et al. (1980). **(B)** Stationery folder that formed the basis for the RDs used in the present experiment. Like those shown in panel A, each RD was hinged on one side, with clips on the other to snap shut. **(C)** Pupae were placed in the bottom of the RD, which was then stood in a few centimetres of water. Small holes in the bottom of the RD allowed the water to fill the lower few centimetres, providing a medium from which the pupae could emerge into adults. After eclosion was complete, the RD was simply lifted out of the water, which drained out through the holes. Adult males were then able to rest on all six internal sides of the RD, including the largest two sides which had been replaced with mesh. The mesh allowed provision of sugar solution (via foam blocks) without requiring access to the inside of the RD. The mesh also provided a resting substrate preferable to smooth plastic; the other four internal sides were roughened with sandpaper to produce a similar benefit. In the context of a real release, after draining the RDs would then be stacked, transported to the release site, and opened to release the adult males. **(D)** A future stage of development could include a parallel sugar-feeding mechanism via sugar-soaked cotton wicks.

Materials and Methods

Two sizes of RD were tested, one twice the depth of the other: 'slim' (31 x 23 x 2 cm), and 'deep' (31 x 23 x 4 cm) (Table 3-2). Pupae and larvae were separated by means of cold water separation, and male pupae were separated from the females by size (both as described in Section 3.2.1). Each RD received one aliquot of 800 males, before being closed and stood upright in water (≈2-3 cm depth). Sixteen RDs (eight of each size) were set up on Day 0. To act as controls, at the same time five 30 x 30 x 30 cm cages were set up, each with an aliquot of 800 male pupae. To ensure eclosion space was not limiting in the controls, the 800 males were spread across three weigh boats[12]. These controls allowed a daily assessment of survival in 'ideal' (non-space-limited) conditions, by daily removal of dead males. On Day 2, eclosion was complete and the RDs were lifted out of the water. On Day 3, four RDs of each size were separately opened inside large cages (BugDorm-2400 Insect Rearing Tent, 75 x 75 x 115 cm). Dispersed (flying) adults were aspirated and counted, as were those adults that were only capable of walking. Finally, dead adults (including semi-emerged adults) and dead pupae were counted from inside the opened RDs. The remaining four RDs of each size were kept for another 7 days prior to release, before releases and counts were performed the same way on Day 10. This cohort had access to 10% sucrose solution (via the mesh sides of the RDs) from Day 3 onwards, as did the controls. On Day 10, the remaining males in the controls were frozen and counted.

Statistical Analysis

The category of primary interest was the proportion of adult males that had successfully dispersed from the RDs. These data were found not to be normally distributed, so were arcsine transformed to make them so. The effects of treatment (slim RD, deep RD, or control) and release day (Day 3 or Day 10) on the number of males that successfully dispersed were tested by two-way ANOVA. As males in controls were never 'released', the numbers of flying males on Days 3 and 10 (calculated at the end of the experiment once all the males were counted) were taken to be equivalent to the numbers that would have successfully dispersed during a release on those days.

[12] Three 6 x 6 cm weigh boats = 108 cm^2 total eclosion space, therefore the number of pupae per cm^2 eclosion space (7.41) was less than, but still comparable with, that in the deep RDs (Table 3-2). However, unlike in the vertically-sided RDs, newly-emerged adult males in the controls had plenty of flat space around the eclosion medium onto which they could easily crawl. Previous experiments had shown this results in very low mortality (≈0-3%) during eclosion (Oxitec internal data).

Results

Cassette cage RDs, being larger, represented a significant reduction in the number of individual mosquitoes per unit space compared to coffee cup RDs (Table 3-2). This was true even when comparing 800 males per cassette cage RD to 600 males per coffee cup RD (the two densities actually tested for each of the two RD prototypes). Deep RDs had very similar 'adults per cm^2 resting space' value to slim RDs, but were twice as spacious in terms of pupal eclosion space and adult flying space. Part of the basis of the shape of the RD was the hypothesis that resting space was the most important factor; if slim RDs proved to have similar survival to deep RDs, this would provide strong evidence that this was the case. It would also justify the use of RDs of half the volume, a significant space-saver when releasing on a large scale.

Table 3-2. Number of individual mosquitoes per unit of space inside the two tested sizes of cassette cage RD, and controls. In the cassette cage RD experiment, only densities of 800 males per RD or control were actually tested (black text); other values (grey text) are given for comparison with the medium coffee cup RDs (italics).

	'Slim' cassette cage RD	**'Deep' cassette cage RD**	**Control cage**	***Medium coffee cup RD***
At 600 males per RD:				
pupae per cm^2 water	14.49	7.25	5.56	*23.51*
adults per cm^2 resting space	0.38	0.35	0.15	*1.69*
adults per cm^3 air volume	0.47	0.24	0.03	*1.10*
At 800 males per RD:				
pupae per cm^2 water	19.32	9.66	7.41	*31.35*
adults per cm^2 resting space	0.51	0.47	0.20	*2.26*
adults per cm^3 air volume	0.63	0.32	0.04	*1.46*

The average number of males able to disperse from the RDs was always reasonably high (>75%), regardless of day of release (Figure 3-7). However, Day 3 releases still resulted in significantly more successful dispersal than Day 10 releases ($F=26.3$, $p<0.001$, two-way ANOVA). There was no main effect of treatment, i.e. within a single release day males from slim RDs, deep RDs, and controls all dispersed equally well ($F=0.2$, $p=0.81$).

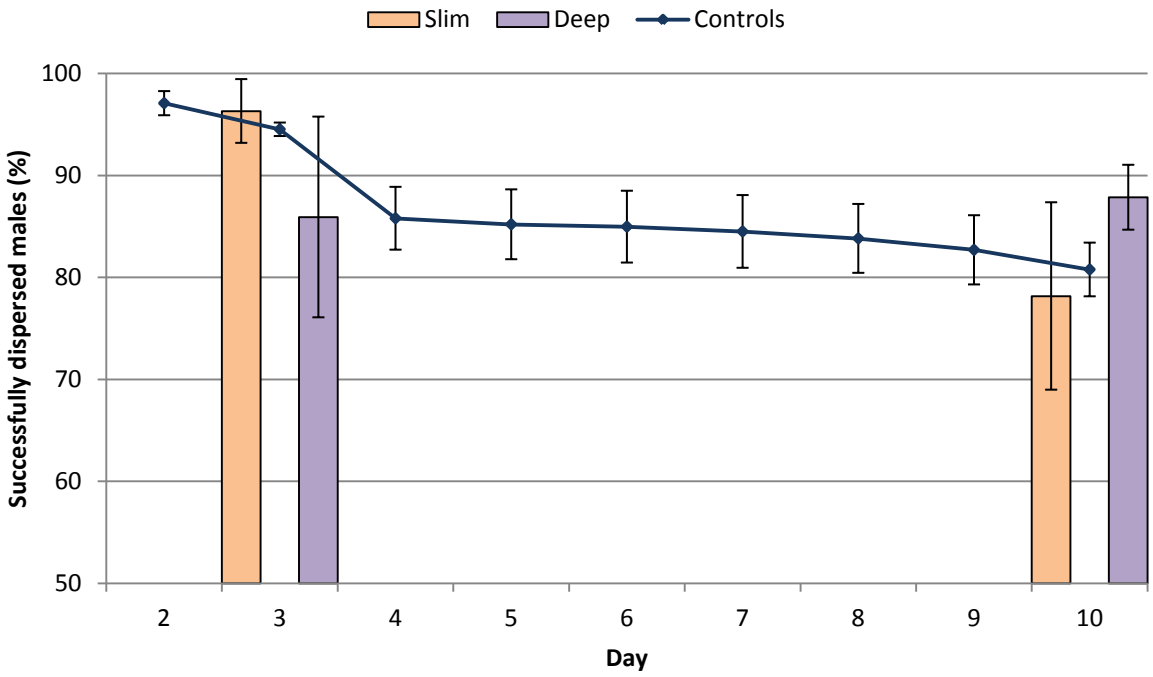


Figure 3-7. Dispersal success from cassette cage RDs, released after 3 or 10 days. Bars represent the 'slim' and 'deep' RDs, the line represents controls. Data are expressed as the percentage of total pupae introduced to each RD or control[13]. Error bars = sd, n=4 for RDs, n=5 for controls. N.B. For clarity the vertical axis only shows the range 50-100%.

Discussion

The most important result was that both 'slim' and 'deep' RD types showed relatively high (78.2 - 96.3%) levels of dispersal. Being kept in the RDs for an extra week did significantly reduce male dispersal; however this was also true of controls, which represented 'ideal' conditions. Within each release day (Day 3 or Day 10), the numbers of adults dispersed from the RDs were statistically indistinct from the numbers of live flying males in the controls. This suggests that - on a superficial level at least - the RDs were similar to the 'ideal' conditions found in the controls.

However, when anecdotally compared with males released on Day 3, the males released on Day 10 required more coercion to leave the opened RD, and then did not disperse as fully within the large 'release' cage. Although survival after 10 days was similar to that in the controls, these behavioural differences were likely a sign of sub-lethal damage occurring

[13] Total number of pupae in each treatment (±sd):
Day 3 releases from RDs: 771±71 and 766±50 for 'slim' and 'deep' RDs respectively.
Day 10 releases from RDs: 729±31 and 800±63 for 'slim' and 'deep' RDs respectively.
Control cages: 773±30.

when the males were kept for long periods of time in the RDs. Although the intention in the field would not be to keep the males for that long in the RDs prior to release, it is nonetheless evidence for the sub-lethal damage that could occur following a period of time spent in the RDs.

As in the coffee cup RDs, the most problematic issue was consistency. Of all the RDs released on Day 3 (4 slim and 4 deep), five out of eight repeats showed high dispersal (average 96.7%), similar to the controls (average 94.5%). However, the other three RDs released on Day 3 (3 out of the 4 'deep' RDs) had lower dispersal (average 81.8%). Similar inconsistency was seen in the 'slim' RDs released on Day 10, and in both cases is represented by the much larger error bars on those series in Figure 3-7. The fact that this higher variation was observed in both RD types and on both release days indicates that it is not a problem necessarily inherent to those conditions. Female contamination is a possibility. Males cohabiting with females have long been known to have shorter longevity than when caged in a single-sex environment (Liles and Delong, 1960). This is because the energy expenditure associated with courtship and mating is significant, especially as males appear to be unable to identify refractory females before making said energy investment (Bargielowski et al., 2011a). If some replicates had a very small number of females while others were female-free, this may explain the observed variation within a single treatment. However, variation in controls was always much lower. Perhaps by chance all control replicates were female-free, or more likely, that the greatly reduced amount of space per male in the RDs (Table 3-2) exacerbated the fitness cost of making mating attempts. The issue of female contamination in release cohorts is an important one. Their presence reduces the effectiveness of male releases by (i) depleting the energies of the males while being held together in RDs (Bargielowski et al., 2011a), and (ii) distracting the released males from mating with wild females at the time of release by presenting a more proximal potential mating opportunity (Rendon et al., 2004).

In both slim and deep RDs the number of adults per cm^2 resting space was approximately 0.5. At an equivalent density, the mortality in the hinged cages used by Bailey et al. (1979) was only 7-8%. In the present study, this low level of mortality was only achieved by one of the four treatments (slim RDs, 3 days). What therefore was the cause of the inferior adult survival in the present study? It is almost certainly a result of the length of time spent by adults in the RDs. In Bailey et al. (1979), males eclosed, matured, and sugar-fed in low

density holding cages for several days, after which they were chilled and aliquoted by volume into RDs. It is likely that damage occurred during this transfer process (Lowe et al., 1980), as the volumetric counting necessitated the packing together of the delicate adults. However, after this process males spent only four hours in the RDs prior to release. This short length of time may not have been long enough for any damage incurred during the transfer process to manifest as mortality. Nonetheless, it will still have affected subsequent performance in the field (Lowe et al., 1980), and so the low 7-8% adult mortality reported may be an underestimate of the overall fitness penalty borne by those males. By contrast, in the present study males both eclosed and matured at high density in the RDs, and then stayed there for much longer, either 3 or 10 days (only the former being representative of likely field release schedules). Therefore, although there was no transfer of adult males from one cage type to another during which damage could occur, this was traded-off against the necessity of a longer post-emergence holding period at high density.

3.4 Conclusions from Laboratory Release Device Experiments

The coffee cup RDs, after two rounds of optimisation, had demonstrated high and consistent male survival, at densities greater than that required by the upcoming suppression programme. The coffee cups themselves were cheap to purchase, easy to manufacture on-site, robust, and disposable. Moreover, similar alternatives were likely to be available in the event of supply issues.

The cassette cage RDs were highly modular and space efficient and showed potential for high survival and dispersal of male OX513A, also at densities greater than required. However, they still required further optimisation, including implementation of a more integrated sugar-delivery system. Furthermore, the prototypes used in the experiments were custom-built, a process which was time-consuming (although this could be alleviated by professional manufacture on a larger scale, at a cost).

At the point of conclusion of the cassette cage experiment, a decision was required on which RD solution to implement in the upcoming 2010 suppression trial in the Cayman Islands. The coffee cup RDs, being demonstrably fit for purpose in their current form, were therefore put into operation as the RDs for the first suppression trial using genetically engineered mosquitoes.

3.5 **Release Device Optimisation in the Field**

The suppression trial in the Cayman Islands in 2010 was a collaboration between Oxitec and the Cayman Islands' Mosquito Research and Control Unit (MRCU). The trial lasted several months and over the course of this period a number of staff and students from both Oxitec and MRCU were involved. The author was present for the majority of the trial, from May to September 2010. The results of the trial are reported by Harris et al. (2012). All three experiments in this Section 3.5 were collaborative efforts. Experiments 3.5.1, 3.5.2, and 3.5.3 were designed by Angela Harris (MRCU), Andrew McKemey, Zoe Curtis, Sian Morgan and the author (all Oxitec). The author conducted all practical work for 3.5.1 and 3.5.2, whilst practical work of 3.5.3 was shared. The author collated all data and performed all statistical analyses.



Figure 3-8. Coffee cup release devices ready to be taken to the field. Raisins proved to be problematic as a source of sugar, as they quickly developed mould and made the RDs attractive to ants. It was decided to replace them with florists' foam (Oasis® Floral Products, Smithers-Oasis Company, Ohio, USA) soaked in 10% sucrose solution. RDs were stored in flowerpot racks, all raised up on legs that sat in water, which protected them from ants.

Following the promising results from the laboratory, the coffee cup RD design (with poly cup insert) was implemented as part of the suppression trial on Grand Cayman, the largest of the Cayman Islands (Figure 3-8). However, after several releases using the RDs it became apparent that the transfer of technology from laboratory to field had not been at all successful. During the first release where full counts were performed, it was estimated that only ≈20% of the pupae that went into the RDs survived to be released as adults (compared to ≈90% in laboratory tests). This mortality seemed to be predominantly due to adults dying post-emergence (see Figure 3-14). Given this high rate of mortality, it seemed probable those that did survive were carrying severe levels of sub-lethal mortality, likely to impair their

competitiveness when compared to the WT males in the release area. The high level of mortality coupled with the poor condition of the survivors meant that these early releases were almost certainly ineffective in their purpose of achieving RIDL-WT matings.

In order to improve the effectiveness of the releases as soon as possible, a series of four key changes were immediately implemented. They were as follows:

(1) Reduction of time spent in RDs. The males in the first releases had spent 4-5 days in the RDs prior to release, in order to ensure all the males were sexually mature and supplied with sugar before they were released. In releases of sterilised adult males in El Salvador, a greater duration of time spent post-emergence in RDs or caged conditions was associated with higher mortality (Bailey et al., 1979; Dame et al., 1981). More recently, adult male *An. arabiensis* transported in drinking cups suffered higher mortality after just two extra hours spent in the cups (Helinski et al., 2008). In the present case, it seemed that, as might be expected, maintenance at high adult densities was similarly increasing mortality in the present case (although this mortality had not been apparent in the laboratory assessments of the RDs prior to the trial). As such, the time spent by males in the RDs prior to release was reduced to from 4-5 to 3 days. This was still deemed sufficient time for males to sexually mature (a process which takes 15-24 hours in *Ae. aegypti*; Roth, 1948; Klowden, 1990), and for sugar feeding to take place.

(2) Reduction of density from 600 males per RD to 300 males per RD. During releases of adult *An. albimanus* in El Salvador, high mortality in adult male release cohorts was attributed to high densities of adults in 'cup' RDs (Lowe et al., 1980). When considered in conjunction with previous experience of maintaining high-density adult colonies for egg production (Oxitec internal data), it was felt that halving the density in the RDs could be one way to quickly improve adult survival. This change resulted in the doubling of the number of RDs required for each release.

(3) Removal of the poly cup insert. The MRCU insectary on Grand Cayman provided a consistently warmer and more humid environment than that in Oxitec's UK laboratory. This meant that pupal eclosion happened over a shortened timescale, and so draining water from the RDs could be performed as early as Day 2 (in laboratory tests it was done on Day 4, to be sure of complete eclosion). This meant that protecting the adult males from the surface of the water was less of a priority than before. Furthermore the porous surface of the polystyrene insert provided an ideal substrate for mould. This had not been a problem

in laboratory tests, but it was hypothesised that the increased humidity and fungal load of the insectary on Grand Cayman was contributing to density-dependent mortality.

(4) Addition of nipagin to the sugar meals. It was also hypothesised that this issue of high fungal load in the environment might have been having a negative effect on the quality of the sugar meals provided to the adult males. This was delivered via absorbent foam blocks, which were especially vulnerable to fungal contamination (even more so than the polystyrene inserts). To combat this Nipagin® (methylparaben, or methyl 4-hydroxybenzoate) - an anti-fungal agent commonly used in the food media of *Drosophila* and commercially-reared butterflies - was added. Nipagin had also recently been shown to improve the longevity of *An. gambiae* in the laboratory (Benedict et al., 2009a), and so had been adopted as a standard diet additive by those authors.

Due to the necessity of producing males for release as part of the field suppression effort, there was not time to individually test the effects of these four changes before they were implemented in the rearing and release procedure. However, these four operational changes did significantly improve the numbers of males being released (quantified by sampling RDs from each release and counting mortality, data not shown). In this way the rearing and release schedule was stabilised, and the suppression trial could be said to have begun in full force (the results of the trial are reported by Harris et al., 2012). Alongside the suppression trial, a series of experiments was conducted that aimed to investigate exactly how these changes were improving survival. Changes (1 and 2), (3) and (4) are investigated in the following Sections 3.5.1, 3.5.2, and 3.5.3, respectively.

3.5.1 Daily Mortality in Release Devices

Introduction

This experiment was designed to quantify the effects of changes (1) and (2) mentioned above: reducing the length of time adults spent in the RD (from 4-5 days to 3 days), and reducing the density of males in the RDs (from 600 to 300). These changes had appeared to reduce the mortality occurring in the RDs being produced for the suppression trial. However, the extent and timing of this reduction in mortality needed to be quantified. Up to this point, the RDs had been stored entirely in the outdoor insectary prior to release, and it was hypothesised that such warm, humid conditions (with higher fungal load) might be contributing to the mortality in the RDs. An alternative location (an indoor air-conditioned laboratory) in which to store the RDs was available. This was much cooler and drier, and it was thought that perhaps keeping the adults in a cooler, less humid environment whilst they were in the RDs might improve survival, primarily by reducing fungal growth. Therefore, this experiment was designed to investigate the effects on RD mortality of:

1) length of time spent in the RDs;
2) density of males in the RDs; and
3) temperature and humidity of the RD environment during the pre-release period.

Materials and Methods

Two experiments were conducted, one at high density (600 males per RD) and one at low density (300 males per RD). Each experiment consisted of two environmental conditions: 'warm and humid' (outdoor insectary) and 'cool and dry' (indoor laboratory). Other conditions were kept as similar as possible to those experienced by the RDs used in the releases for the main suppression trial, which continued alongside. For example, no poly cup inserts were used in the RDs, and sugar solution (with nipagin) was provided daily. A 'spoon' of specific size was used to aliquot male pupae by volume; therefore three aliquots of each density were counted manually, and the mean of these counts was taken to be the number of pupae starting in every RD. On Day 1, pupae in the RDs eclosed into adults, and on Day 2 all the RDs were drained of water. For each of the four treatments, the number of dead males drained from five randomly-selected RDs was counted, and this number was applied to all RDs within that experiment. On each of Days 3-7 (Days 3-6 in Experiment 2),

five randomly-selected RDs were opened inside a cage to release the live adults, and the dead males inside the RD were counted. In this way, a daily 'snapshot' of the mortality in the RDs was obtained.

Table 3-3. Experimental treatments investigating daily mortality in RDs.

Experiment	Density (males per RD)	Location	Description	RD had dye?[3]
1	high (600)	outdoor insectary	warm, humid[1]	no
		indoor lab	cool, dry[2]	no
2	low (300)	outdoor insectary	warm, humid	yes
		indoor lab	cool, dry	yes

[1] Outdoor insectary was approximately 28±2°C and 80% RH.
[2] Indoor laboratory was 20±2°C. Exact RH unknown, but room was air-conditioned.
[3] The Experiment 2 RDs were extras from a release batch used for a mark-release-recapture (MRR) experiment (data not shown), which required their inside surfaces to be coated in fluorescent dye powder (Day Glo, Switzer Brothers, Cleveland, OH, USA). This powder is designed to mark insects for later identification, and does not affect survival of *Aedes* mosquitoes in a laboratory environment (Lacroix et al., 2009, 2012)[14].

Statistical Analysis

These experiments only counted mortality ('released' males were not counted). Three kinds of adult mortality were identified: 'stuck dead' (males that had died whilst in contact with the water); 'dry dead' (males that had died separate from the water); and 'live and walking' (males that were still alive when the RD was opened, but as they were unable to fly would not have been able to mate). These three adult mortality categories are all shown as the orange colours in the figures overleaf, and were summed to get 'total adult mortality' (TAM), the value used to make statistical comparisons. The mortality identified when draining the RDs on Day 2 (shown in the figures as blue and white bars) was counted in a sample of RDs (5 per treatment) and then applied to all RDs in that treatment; therefore these values were the same across all days of the experiment and no comparisons were made.

For Experiment 1 (high density RDs) and Experiment 2 (low density RDs), TAM was found to be normally distributed only after log transformation. Two-way ANOVA (with Tukey HSD post-hoc tests) was used to investigate the effects of location and day on TAM, separately

[14] Although more vigorous methods of application than those used here have been shown to reduce adult survival (Dickens and Brant, 2014).

within Experiments 1 and 2 (unfortunately Experiments 1 and 2 were not comparable with one another, as they were not run simultaneously, and furthermore only Experiment 2 was conducted with the use of fluorescent marking dye).

Results

Experiment 1 - High Density RDs (600 males)

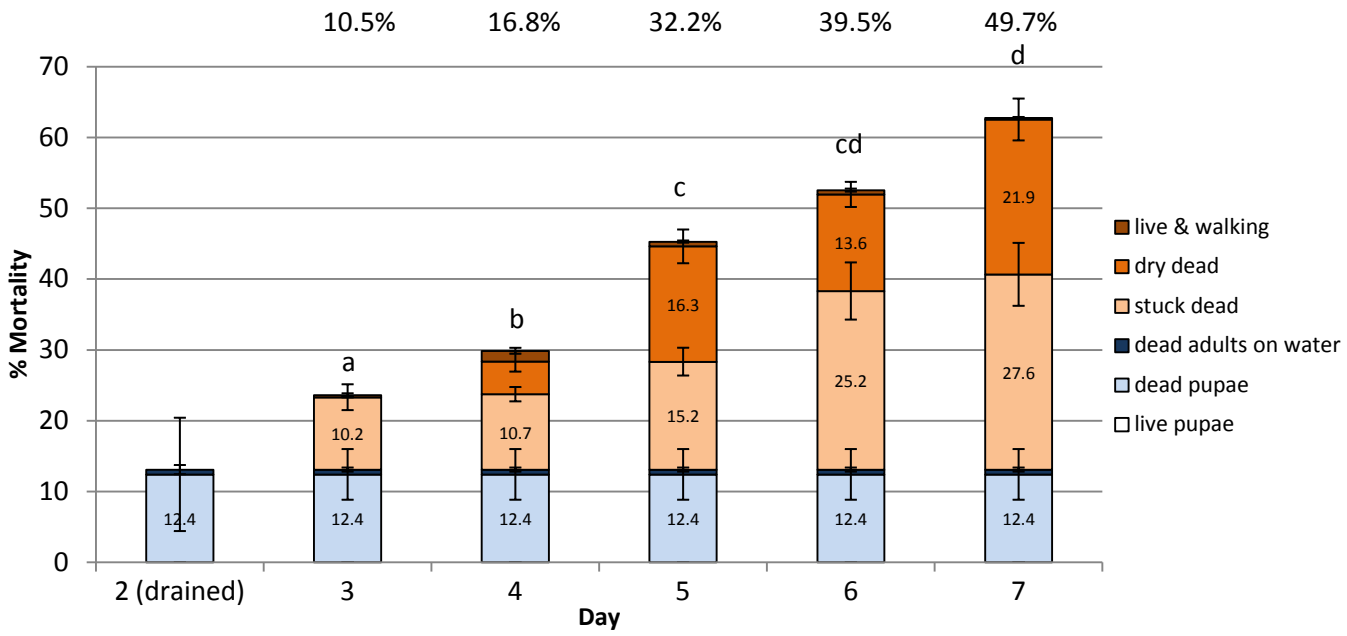


Figure 3-9. Mortality of males kept at high density (600 per RD), maintained in the *outdoor insectary*. n=5, error bars = sd. RDs were drained on Day 2. Days on which TAM (sum of all orange colours, values given above each bar) was statistically different are indicated by letters.

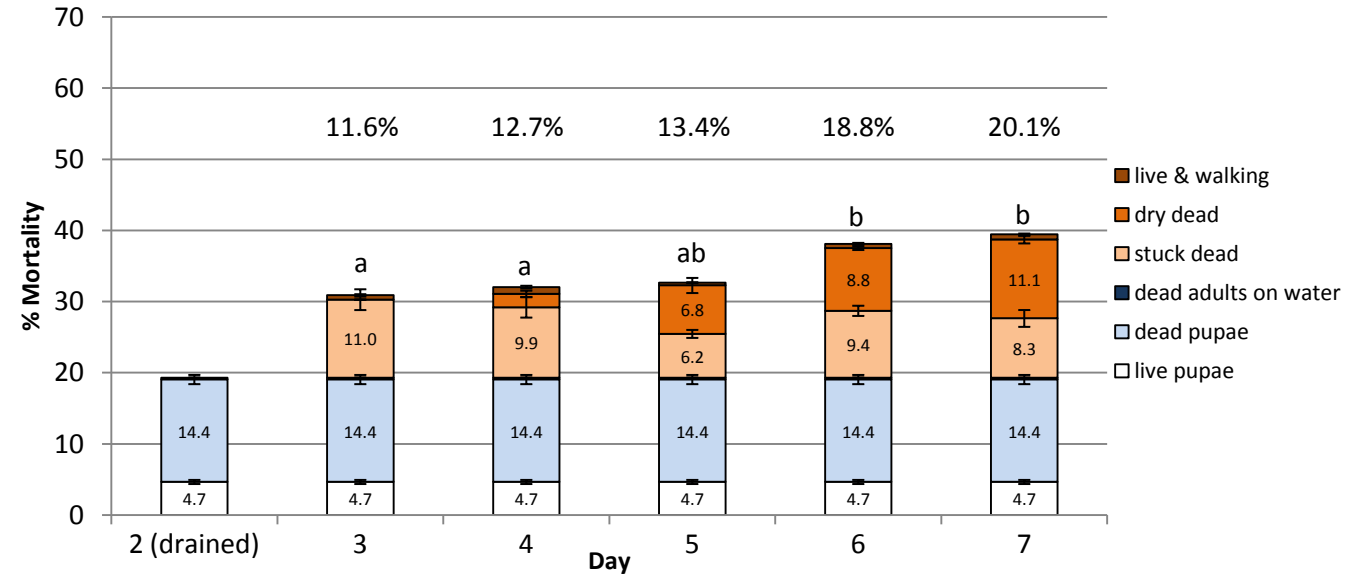


Figure 3-10. Mortality of males kept at high density (600 per RD), maintained in the *indoor laboratory*. n=5, error bars = sd. RDs were drained on Day 2. Days on which TAM (sum of all orange colours, values given above each bar) was statistically different are indicated by letters.

Experiment 2 - Low Density RDs (300 males)

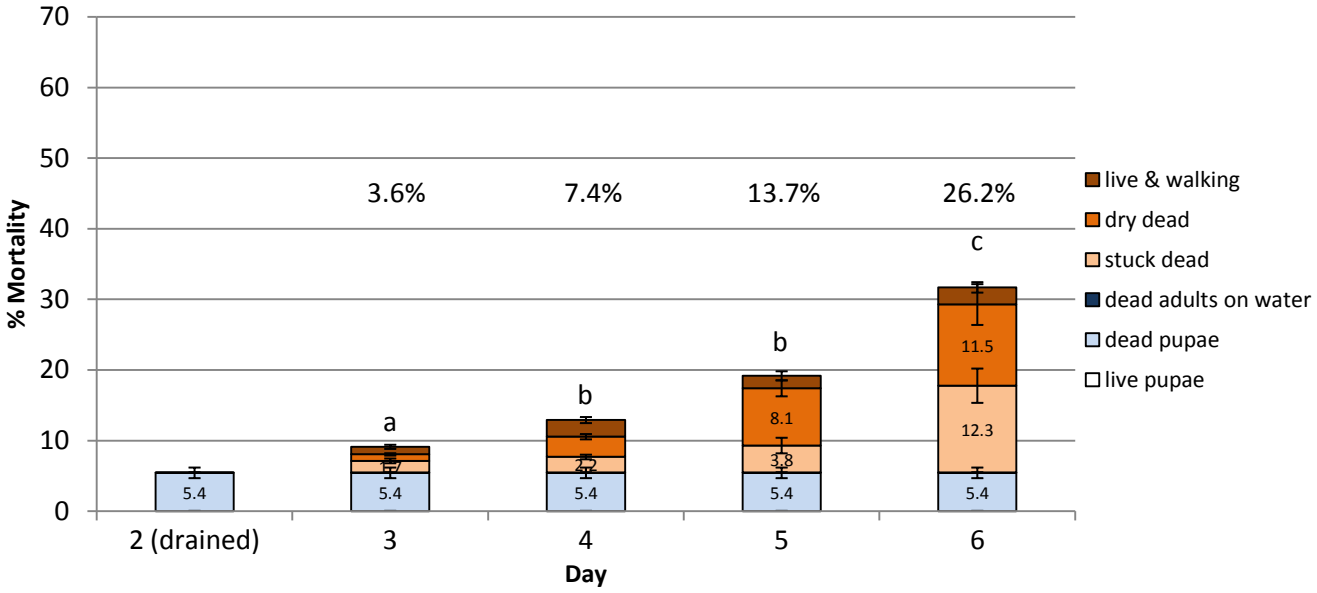


Figure 3-11. Mortality of males kept at low density (300 per RD), maintained in the *outdoor insectary*. Days 2-3 n=10, Days 4-6 n=5. Error bars = sd. RDs were drained on Day 2. Days on which TAM (sum of all orange colours, values given above each bar) was statistically different are indicated by letters.

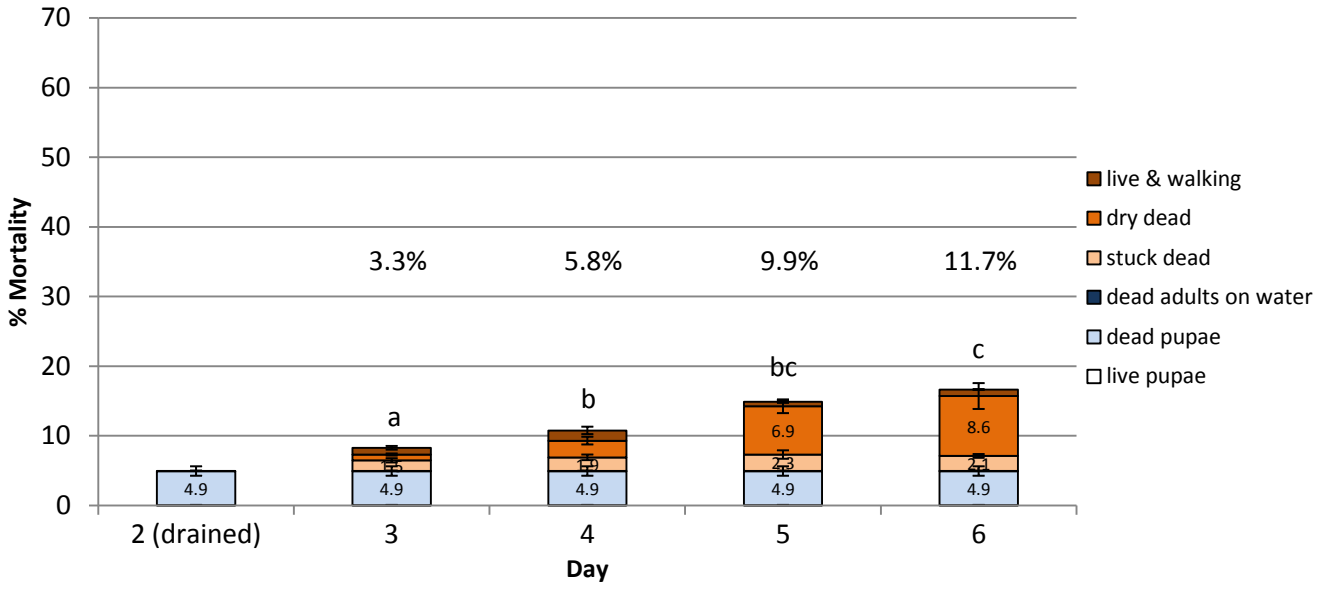


Figure 3-12. Mortality of males kept at low density (300 per RD), maintained in the *indoor laboratory*. Days 2-3 n=10, Days 4-6 n=5. Error bars = sd. RDs were drained on Day 2. Days on which TAM (sum of all orange colours, values given above each bar) was statistically different are indicated by letters.

Experiment 1 - High Density RDs

There were significant main effects of both location ($F=95.0$, $p<0.001$, two-way ANOVA) and day ($F=48.6$, $p<0.001$) on total adult mortality (TAM). TAM was higher in the outdoor insectary than in the indoor laboratory, and in both locations increased with day. In the outdoor insectary (Figure 3-9), the daily increase in TAM was almost always significant, with TAM reaching 49.7% by Day 7. However, in the indoor laboratory (Figure 3-10), TAM never significantly increased from one day to the next, and by day 7 had reached only 20.1%. Therefore at densities of 600 males per RD, keeping RDs in the outdoor insectary resulted in higher TAM overall, and increased the severity of the day-to-day increase.

Experiment 2 - Low Density RDs

As in the high density experiment, there were significant main effects of both location ($F=11.9$, $p=0.0013$) and day ($F=67.8$, $p<0.001$) on TAM. Again, TAM was higher in the outdoor insectary than in the indoor laboratory, and in both locations increased with day. In the outdoor insectary (Figure 3-11), TAM reached 26.2% by day 6, but this figure was only 11.7% on the same day in the indoor laboratory (Figure 3-12). However, while the effect of location was significant, it was much less so in this experiment than in Experiment 1.

Strictly speaking the results of Experiments 1 and 2 cannot be statistically compared. However, when looking at the day 6[15] TAM values in all four figures, the values in Experiment 2 (300 males per RD) are lower than those in Experiment 1 (600 males per RD), as was expected.

Discussion

The lower average TAM observed in Experiment 2 was an indication that the decision to halve the density in the RDs for release had been well founded, despite the logistical challenges it introduced by doubling the number of RDs that needed to be produced, transported, and released.

In both experiments, the TAM of RDs kept in the colder environment (the indoor lab) was reduced as compared to those kept in the outdoor insectary. Based on this result, RDs for release were stored in the indoor laboratory rather than the outdoor insectary. However,

[15] Experiment 2 only ran up to Day 6.

the colder temperature slowed pupal eclosion to the point where a considerable number of individuals were lost as live pupae during the draining process. This increase in the loss of live pupae was also observed in Experiment 1 (high density): 0% live pupal loss in the outdoor insectary (Figure 3-9), but 4.7% live pupal loss in the indoor laboratory (white bars, Figure 3-10). It is unclear why such an increase in live pupal loss was not also seen in Experiment 2: Experiments 1 and 2 were conducted with pupae from different larval rearing batches (spares from two batches reared 2 days apart for release as part of the suppression trial). Small variations in conditions (primarily lower temperatures) experienced during larval development occasionally resulted in lower numbers of pupae at the point of harvesting, and of those a higher proportion of male individuals that had only very recently pupated. By Day 2 these males in particular might have not yet eclosed (especially if the colder temperature in the indoor laboratory slowed down metamorphosis; Turnock et al., 1986), and so when the RD was drained they would be lost as live pupae. In addition to this there are possible density-dependent effects on the timing of pupation. The process of emergence requires many times more surface area per individual than that occupied by the pupa pre-emergence; it is therefore possible that when surface area is more limiting (i.e. at higher pupal densities), pupae stagger their emergence to prevent damage occurring as a result of crowding during the vulnerable period immediately following emergence. Those individuals that remain as pupae for longer would therefore be more likely to be lost as live pupae when the RDs were drained on Day 2.

As well as the concern of lost live pupae, there were other operational issues associated with storing the RDs in the indoor laboratory. Problems frequently encountered in the outdoor insectary (such as occasional adult escapees, and ants being attracted to the RDs), whilst tolerable in a self-contained outdoor insectary dedicated to the suppression trial, became more of a problem when in an indoor research environment shared with other researchers. At this point, TAM in the releases for the suppression trial had been sufficiently reduced by the 4 changes listed in Section 3.5; therefore the additional problems created by storing the RDs in the indoor laboratory could not be justified. As such, storage of RDs was returned to the outdoor insectary, and the TAM of RDs to be released continued to be monitored to ensure it stayed acceptably low (less than ≈15%).

3.5.2 Laboratory vs. Field Performance of Release Device Inserts

In the laboratory, using inserts in the coffee cup RDs was found to be an effective way of preventing adults from coming into contact with the surface of the water (Section 3.2.3). As already discussed, the initial male releases in the field had shown that the RDs with inserts, despite showing low mortality in the laboratory, showed the opposite in the field. Although the use of RDs with inserts had by this point been discontinued, it was necessary to quantify this discrepancy between laboratory and field performance. This experiment was designed to quantify the effects of change number (3): removal of the poly cup insert.

Materials and Methods

Routine releases of males as part of the suppression trial no longer used inserts in the RDs. However for one release, 10 experimental RDs were set up with inserts, and incorporated into the release. Ten randomly-selected RDs without inserts were used as controls. For all 20 of these RDs, care was taken to retain dead males inside the RD when opening the RD for release. These 20 RDs were brought back from the field, and an assessment made of the mortality inside each RD. Each RD had been set up with 300 male pupae (aliquoted volumetrically), drained on Day 2, and released on Day 3. Prior to release, RDs were stored in the outdoor insectary.

Statistical Analysis

As in the previous experiment, the three categories of dead adults were summed to give a value for total adult mortality (TAM). The TAM of RDs with and without inserts was compared with a 2-sample t-test.

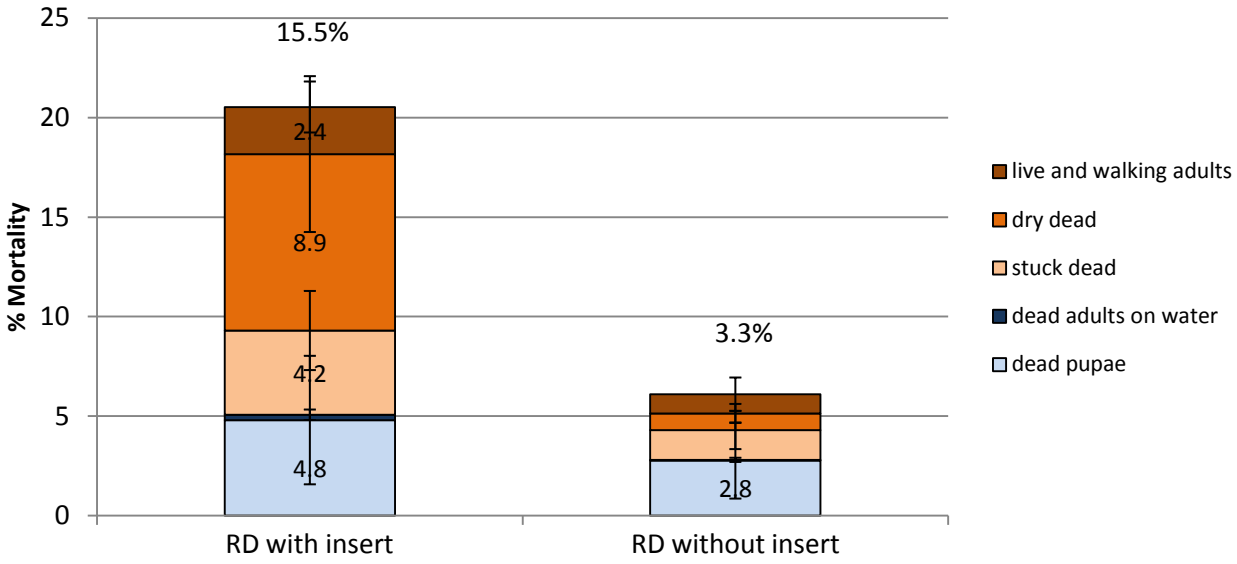


Figure 3-13. Mortality on day of release, RDs with inserts vs. those without. Error bars = sd, n=10. The values of TAM (sum of all orange colours) for each RD type are given above each bar.

Total adult mortality was significantly higher ($t=7.9$, $p<0.001$) in those RDs equipped with inserts than in those without (Figure 3-13). This experiment served its purpose of confirming and quantifying what was already suspected, that inserts - despite promising laboratory results - actually contributed to mortality in RDs when used in the field. It is worth highlighting that the 15.5% TAM seen in RDs with inserts had already occurred by Day 3 (release day) in this experiment; in equivalent RDs without inserts, that level of TAM did not occur until later, after Day 5 (Figure 3-11). Although this is an informal comparison between experiments, this indicates that the inserts accelerated mortality.

It is difficult to offer an explanation of the exact mechanism by which the inserts increased mortality without further laboratory testing of the interaction between insert, insect, and environmental factors. The initial hypothesis under which the inserts were removed from the RDs was that the intense humidity and fungal load of the atmosphere inside the insectary on Grand Cayman (which was not present in the UK laboratory in which they were tested prior) was causing mould or fungal spores to accumulate on the porous surface of the insert, increasing the microbial burden on the insects and reducing their longevity. Such an effect would likely be exacerbated by the high densities at which the males were being stored. This hypothesis is strengthened by the most severe mortality increase being observed in those adults classified as ‘dry dead’, i.e. those that successfully emerged and

avoided drowning, but died later of another cause (Figure 3-14). By this measure, in field releases the insert did at least continue to be successful in its initial aim of preventing adult males from drowning on the surface of the water.

Immediately after emergence, *Ae. aegypti* are delicate and require time to allow their exoskeleton to harden (Christophers, 1960), which takes place either while resting on the surface of the water, or on a vertical resting surface nearby (Oxitec internal data). At the high densities used in the RDs the surface of the water became crowded with emerging adults very quickly, and so emerging males would be stimulated to crawl from the water's surface and up onto the vertical walls of the RD, moving away from the congestion. However, in those RDs with inserts, the steep overhang and narrow exit of the insert (see Figure 3-3) would have made this climb much more difficult, creating a bottleneck and possibly weakening the already fragile newly-emerged adults.

A similar mechanism may have been at work in another recent study, of at-release sugar feeding of irradiated *Ae. albopictus* (Bellini et al., 2014). As part of testing a pupal RD which incorporated a sugar-feeding sponge element, an upturned conical funnel was added to the exit of the device to prevent successfully emerged and dispersed males coming back into the device to sugar-feed. The shape and intention of the funnel and the RD insert are similar. The authors of that study reported that the addition of the funnel increased average mortality in the devices by up to a multiple of twelve (although high variation meant the difference was not statistically significant). An explanation for this phenomenon was not offered.



Figure 3-14. Field release of adult males from RDs with inserts. The insert cup of this RD had accumulated a ring of 'dry dead' adults.

Alternatively, the mortality may have occurred at a later stage, after the adults had emerged and passed through the opening in the insert into the main chamber of the RD itself. The gap between the inside wall of the RD and the outside wall of the insert diminishes from top to bottom, and so it is possible that males which were in this gap, with more males following behind them, could not get back out. Certainly this correlates with the location and condition of the male corpses (Figure 3-14). This process would be greatly exacerbated by the agitation of RDs as they were transported to the field. This agitation did not occur to those RDs tested in the laboratory prior the programme start, and so this may explain why results differed between laboratory and field. Similar excessive and specifically transport-related mortality was also observed in certain types of RD used in El Salvador with *An. albimanus* adult males (Lowe et al., 1980).

3.5.3 Addition of Nipagin to the Sugar Diet

Introduction

The final change made to the released RDs was the addition of nipagin to the sugar solution provided to the males. Nipagin® (methylparaben, or methyl-4-hydroxybenzoate) is an anti-fungal agent that has been used as a preservative in the food and cosmetics industries for over 50 years. Within entomology, it is used in *Drosophila* food medium and commercial butterfly rearing nectars. It has been shown to increase the longevity of adult *An. gambiae* and *An. arabiensis* by preserving the quality of the sugar meal, by inhibiting bacterial and fungal growth. This in turn reduced the microbial load and immune challenge borne by the insects (Benedict et al., 2009a).

As the addition of nipagin to the sugar meal was introduced at the same time as other changes, its contribution to the observed improvement in survival was unknown. As such, this experiment aimed to quantify the effects of nipagin in the sugar diet on subsequent adult survival, and whether these effects were density-dependent.

Materials and Methods

Two male densities were tested: 600 and 300 males per RD, corresponding to the original and changed densities used for releases. Each density was tested with and without nipagin, giving treatments 1-4 (Table 3-4). Also set up were two control treatments (treatments 5-6), eclosed from weigh boats into cages (instead of eclosed within RDs) as an 'ideal' eclosion medium.

Table 3-4. Treatments used in nipagin-density experiment. All n=5.

Treatment	Eclosion Medium	Density	Nipagin
1	RD	300	+
2	RD	300	-
3	RD	600	+
4	RD	600	-
5	Weigh boat	300	+
6	Weigh boat	300	-

All six treatments (n=5 for each) were set up on Day 0, with male pupae aliquoted by volume. Treatments 1-4 were drained on Day 2, and the drained contents (mortality) counted. On Day 3 the males in the treatment 1-4 RDs were 'released' inside 15 x 15 x 15 cm cages, and any remaining dead individuals inside the RDs were counted as Day 3

mortality. Treatments 5-6 were eclosed directly into cages from weigh boats, which were removed on Day 2 and mortality within was assessed. For all treatments, on each of Days 3-9 any dead adults in the cages were counted and removed, and on Day 10 all remaining surviving adults were killed and counted. Throughout the experiment, sugar water - either with or without nipagin (0.2% weight/volume) as appropriate - was provided to the males via foam blocks. All treatments and controls were maintained in the outdoor insectary.

Statistical Analysis

The treatments were divided up to create two full-factorial experimental designs. The first analysis used the data for treatments 1-4 to investigate the effects of density and nipagin on those males that eclosed from RDs. The second analysis used the data for treatments 1-2 and 5-6 to investigate the effects of eclosion medium and nipagin on males eclosed at the lower density (300 males). Cox proportional hazards models were constructed as per the description of statistical analysis in Section 5.2. As the experiment was terminated on Day 10, the males that had survived to that point were censored.

Results

Effects of Density and Nipagin on Eclosion from RDs

Both density and nipagin affected hazard significantly, with (i) lower density and (ii) the presence of nipagin each resulting in improved survival. Males eclosed at 300 males per RD had 58% of the hazard of those eclosed at 600 males per RD ($p<0.0001$). Males provided with nipagin post-eclosion had 76% the hazard of those without ($p<0.0001$). In addition, there was a significant interaction between the two variables - for those males at the lower density, the addition of nipagin resulted in a further significant reduction in the hazard ($p=0.001$).

Effects of Eclosion Medium and Nipagin on Males Eclosed at 300 males per RD/weigh boat

Both eclosion medium and nipagin affected hazard significantly, with (i) weigh boats as the eclosion medium and (ii) the presence of nipagin each resulting in improved survival. Males eclosed from weigh boats had 32% of the hazard of those eclosed from RDs ($p<0.0001$). Males provided with nipagin post-eclosion had 59% the hazard of those without ($p<0.0001$). There were no interaction effects between eclosion medium and nipagin.

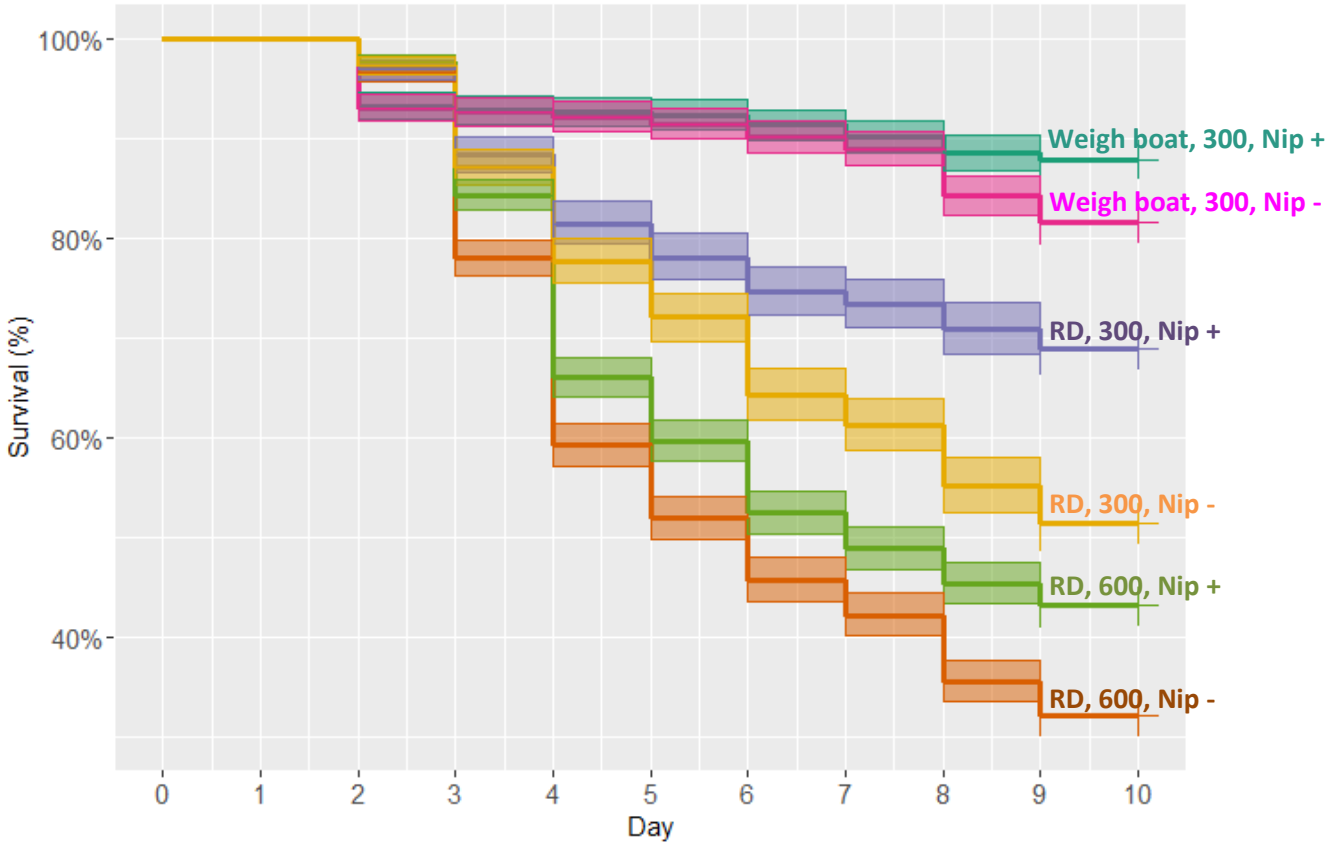


Figure 3-15. Survival of adult males as dependent on eclosion medium, eclosion density, and presence of nipagin. Shaded areas indicate 95% confidence intervals, n = 5 (apart from 'RD, 600, Nip - ' where n =4). RDs were set up on Day 0, drained on Day 2, and 'released' on Day 3. Weigh boats were set up on Day 0, allowed to eclose directly into cages, and removed from the cages on Day 2. Daily mortality counts were made for all treatments on Days 3-9. Treatments are as listed in Table 3-4. By the conclusion of the experiment, those males supplied with nipagin always exhibited higher survival than those without (other factors being equal).

Discussion

The primary outcome of this experiment was extremely positive: the treatment that was equivalent to the current conditions experienced by released males resulted in substantially higher survival than the treatment equivalent to conditions in the early releases. 'RD, 600, Nip -' (equivalent to early releases) males were reduced to just ≈35% survival after only 9 days, even with access to sugar, and despite the fact experimental densities were likely not

as high as they were in the first releases[16]. By contrast, 'RD, 300, Nip +' (equivalent to current releases) males were at ≈70% survival at Day 9. Therefore, this experiment showed that the changes made as an immediate reaction to the high mortality of early releases had directly contributed to an improvement in the overall male mosquito output of the trial.

The greatest difference in survival that was attributable solely to the presence of nipagin was seen in the 'RD, 300' males (≈20% difference between 'Nip +' and 'Nip - ' in survival by Day 9). This was also a positive outcome for the experiment, as it showed that in conditions currently experienced by the released males, nipagin resulted in a demonstrable improvement of subsequent survival. It is suspected that the 'RD, 600' treatments were already under considerable density-dependent stress, which masked any great mortality reduction caused by nipagin. Similarly, it is likely the controls ('weigh boat' treatments) were under near-optimum conditions, and so nipagin was not sufficient to significantly improve their (already very high) survival.

The two 'RD, 300' treatments looked to have similar rates of decline up until Day 6, at which point their respective survival curves began to diverge, with those not being provided with nipagin faring more poorly. This was likely because in the early stages of the experiment, other factors had a greater influence on mortality (e.g. mortality associated with eclosion). After this point, the fungal deterioration of the sugar diet started to impact survival, and so the benefit of nipagin as an anti-fungal became more apparent. It is suspected that further divergence between nipagin and non-nipagin treatments would have been observed had the post-release (adult survival) portion of the experiment been run for a greater length of time. The curtailing of the experiment on Day 10 was a deliberate result of expending the minimum amount of (limited) manpower in order to get the answers to our questions - by that point in time the relative benefits of nipagin and density had become apparent, and so the experiment was stopped.

Similar practical constraints meant that the experimental design was not fully multiplicative; there were no treatments where 600 males were eclosed from a weigh boat. This is unfortunate as the inclusion of those treatments to make a full factorial experimental design

[16] Mean number of male pupae (±sd) starting in '300' RDs was 253±4, in '300' weigh boats was 235±6, and in '600' RDs was 461±11. By this point in the suppression trial the feeding regimen had been altered to provide more food per larva; the males used in this experiment were spares from this production, and so the higher food per larva may have increased the average size of the male pupae. Since they were aliquoted into the RDs volumetrically, this would have resulted in fewer males per aliquot and so lower-than-intended densities in the experimental RDs.

would have allowed a more complete analysis, without the need to split the analysis into two. In addition, a 'weigh boat, 600' treatment would also have shown the 'crossover point' of the benefits of the better eclosion medium (weigh boats) versus the benefits of the better density (300); i.e. would 600 males eclosing from a weigh boat have higher or lower subsequent survival than 300 males eclosing from an RD, and how would this be affected by nipagin?

However, despite these two shortcomings, the aim of the experiment was to quickly show that reducing the density of males in the RD, and providing nipagin as part of the sugar diet, improved subsequent survival. Based on these aims, the experiment was a success.

Of course, the observed improvement in survival as a result of these changes would not be applicable to field survival, at least not in absolute terms. The survival in the field of males that have been mass reared and then released - approximately two days (Lacroix et al., 2012; Neira et al., 2014; Carvalho et al., 2015; Winskill et al., 2015) - is far lower than those subsequently kept in laboratory conditions. A crude calculation would therefore equate an observed extension of laboratory life of many days to one of only a few hours in the field. Furthermore, released males would only have access to nipagin until the day of release (Day 3). Therefore it could be argued that whilst nipagin appeared in this experiment to be beneficial following nine days of access, this is less likely to be the case following only three days of access. A more field-representative experiment would have been to provide sugar with nipagin prior to 'release', and sugar alone afterwards. However, given that this approach would likely have reduced the magnitude (and therefore detectability) of any beneficial effects, such an experiment would have required a prohibitively high number of replicates in order for the data to have been informative for the purposes of on-going field releases.

Regardless of the actual field longevity or performance of male OX513A following release, in this experiment nipagin provided a benefit to OX513A males, even if slight. Experiments on *An. gambiae* and *An. arabiensis* with a longer duration of nipagin provision found a more significant increase in longevity (Benedict et al., 2009a), although the authors of that study pointed out that the same effect was achieved by refreshing sugar solutions daily, and therefore the primary benefit of nipagin was simply maintaining the quality of the sugar diet. Mosquitoes have a bacteriolytic factor that is released during sugar feeding, and which is present in the crop after a sugar meal (Rossignol and Lueders, 1986; Pimentel and

Rossignol, 1990), providing protection against any pathogens ingested with the sugar meal. Of course, many species of insect, including mosquitoes, also depend on symbiotic relationships with microorganisms for nutritional purposes (Douglas, 1998; Alverson and Cohen, 2002; Baumann et al., 2006; Siozios et al., 2008). The effect on these gut flora of nipagin ingestion is unknown, and would be complicated by the presence of the antibiotic tetracycline in the larval rearing medium (used to repress the lethal RIDL construct). Further work is needed to better understand the ways in which altering the makeup of microorganisms within the insect alters its potential fitness. In this case, addition of nipagin to the diet seemed to be of benefit to the insect, if slight. Therefore from this point in the suppression trial onwards, nipagin was incorporated as a routine addition to the sugar diet of released males.

Conclusions

This was the first longevity assay OX513A males reared during the suppression trial had been subject to. It was found to be an excellent way of discriminating potential fitness differences that arose as a result of sub-lethal effects of the production, release, and storage stages of mass production; for example the clearly reduced fitness of males kept at higher densities prior to release. Furthermore, longevity studies need not take weeks to perform; in this case differences in performance became apparent after less than 10 days. It is recommended that during the lead-in time to a suppression trial (after the start of operations, but prior to the first releases), several rearing and release protocols should be tested and the resulting males monitored for longevity. By starting a trial with several options in terms of these protocols - even if they are variations on a theme - the variant that gives the best results in-situ can be implemented, while the others provide some redundancy in case of unforeseen problems.

3.6 Conclusions and Further Work

Summary of Results

During preparatory tests for the 2010 suppression trial of OX513A in the Cayman Islands, the coffee cup RDs had allowed excellent survival of the adult males in the laboratory. However it quickly became apparent that the same could not be said of the first field releases. The high mortality in these first few releases of adult males was a significant problem; the numbers being released were below target values, and those males which did survive to be released were almost certainly non-competitive with wild males. Therefore, a suite of changes was made in an effort to improve the quality of releases. The effects of these changes were then examined in laboratory experiments on-site.

The first experiment examined the effects on mortality of (i) reducing the density in the RDs from 600 per RD to 300 per RD, and (ii) reducing the time spent by males in the RDs prior to release from 4-5 days to 3 days. This experiment established that mortality was significantly reduced by halving the density of males in each RD, and that mortality on day 3 was significantly less than on day 5. The operational implications of these changes were significant. Halving the adult density doubled the numbers of RDs that were required, which significantly increased the work required to prepare, manage, and distribute them. Reducing the numbers of days spent in the RDs had knock-on effects on the weekly rearing and harvesting schedule. Therefore, although the results of this experiment were expected (based on similar findings from previous studies where high density adult confinement was detrimental to survival; Bailey et al., 1979; Dame et al., 1981; Helinski et al., 2008), it was important to quantify the level of benefit to the release cohort in order to justify the changes made.

The removal of the poly cup insert did not have such a large operational impact; in fact, discontinuing its use reduced the time taken to construct and dispose of each RD. Nonetheless, it was important to quantify the contribution to mortality that the insert had been making. This experiment demonstrated that the contribution had been substantial, most likely as a result of inhibiting males from moving freely around the inside of the RD, and possibly exacerbated by agitation during transport.

Finally, the addition of nipagin to the pre-release sugar diet of the adult males was found to improve subsequent longevity in males, as had previously been demonstrated in *An.*

gambiae (Benedict et al., 2009a) . This change only required a small degree of extra labour to implement, and was probably the least impactful change in terms of improving subsequent performance of released males. However, given that the expense and effort of including nipagin the sugar diet was minimal, and it was shown to increase male vigour (even slightly), it seemed worthwhile to continue its use.

Difficulty of Transfer of Technology to the Field

It would be extremely naive to assume that a field release programme will always go exactly as planned; until release operations actually begin, some questions will always remain unanswered and some problems unforeseen (Dame et al., 2009). The discrepancy in performance of the coffee cup RDs between laboratory and field was the most significant example of this principle. Ultimately, this issue was overcome by quickly implementing the series of changes to release methods described above. These changes were confirmed by both visual observation and post-hoc experimentation to improve the quantity and vigour of the released males. Similarly in the SIT programmes against *An. albimanus* in El Salvador in the 1970s, methods of transporting and releasing adults evolved over the course of the trials (Bailey et al., 1979; Lowe et al., 1980). Indeed, those programmes encountered several problems that required in-situ changes to be made. For example, the plywood boxes used as RDs in preliminary releases got very hot during transport to the field, resulting in mortality exceeding 40% (Lowe et al., 1980). Similar levels of mortality were observed using Styrofoam cups, also specifically incurred during transport to the field. Both of these examples highlight the need for a period of establishment and testing of rearing and release procedures well in advance of the scaling-up of production to maximum levels. This is because some conditions - in this case those experienced by adults in RDs during transport - cannot be easily or accurately simulated prior to operations at the field site itself. In El Salvador several other practices had to be changed as a result of their observed negative effects on male survival and vigour, such as the vacuuming of cold-immobilised males from holding cages into RDs, and the use of certain materials to screen RDs from one another (Lowe et al., 1980).

Further Work

By the approximate mid-point of the trial , the area in which releases were conducted had been reduced twice in order to increase the ratio of OX513A males: wild males (Harris et al., 2012). Also at this time, the effort required to manufacture, populate, manage, transport,

and release hundreds of coffee cup RDs was becoming prohibitive. The reduction in release area (and corresponding reduction in the number of release points) meant that fewer, larger releases were required. Therefore, rather than releasing many coffee cup RDs at each release point, laboratory cages (capable of holding many times more adults) were used as RDs. Although these presented new challenges of their own (they were difficult to open, for example), the resulting economies of scale more than compensated for these difficulties.

The reduction in release area and the above changes to adult release both contributed to the 80% reduction of the wild population that was observed by the end of the trial, following the release of 3.3 million OX513A males over a period of 23 weeks (Harris et al., 2012). Thus, the trial having been a successful demonstration of the ability of the technology, releases stopped. Subsequent successful trials in Brazil (Carvalho et al., 2014, 2015) and Panama (Gorman et al., 2016) used the same release principle demonstrated in this first trial: that of a single hand-held RD into which males emerged directly, were sugar-fed, and which was then transported to the field by ground vehicle and opened by hand. These systems have the advantages of being low-tech, robust, and easily-modifiable to suit local conditions (as indeed was required in the Cayman Islands trial). However, as suppression programmes using transgenic or sterile males become more ambitious and aim to reduce mosquito populations over increasingly large areas, ground-based adult releases may become less practical (Dame et al., 2009). The challenges associated with such scaling-up are discussed in Section 8.2.2.

4 General Introduction to Chapters 5 - 7

The problems encountered with releases of OX513A males during the 2010 Cayman Islands suppression trial highlighted a lack of knowledge of the competitiveness of released males. During the first releases, high mortality (≈80%) was observed in the release cohorts. Not only did this severely reduce the number of males being released, it almost certainly meant that those males that did survive to be released were carrying severe sub-lethal damage. This competitiveness penalty would have certainly impaired their ability to compete against wild males for mating opportunities. Certainly, no fluorescent larvae were collected from the field during the period of these early, low-quality releases. By taking the steps outlined at the beginning of Section 3.5 (such as reducing the time males spent in the RD, and reducing their density during this time), mortality was much reduced. However, low observed mortality by no means implied maximal competitiveness, and questions still remained over how the competitiveness of the released males could be further improved, and how such improvements might be quantified.

This is an extremely important subject, as the competitive ability of the released males is often a major factor in the success or failure of a trial that has the aim of population reduction or elimination (reviewed in Benedict and Robinson, 2003). Despite being reared under intensive conditions, the produced release cohort must still be of maximum possible vigour in order to ensure the success of the programme. In the case of releases of mosquitoes, optimal husbandry of adult males, particularly in the period between pupal eclosion and release as adults, can significantly affect their field performance by ensuring they are in the optimum physiological state before release. To that end, the following three chapters will investigate how treatment of the males in the period leading up to their release can affect their subsequent performance in the field. The three most important behaviours of released males are longevity, dispersal ability, and mating competitiveness (Lacroix et al., 2012); these are investigated in the upcoming Chapters 5, 6, and 7.

Knowledge of which male traits are most important for mating success, and how treatment of males prior to release can alter their subsequent performance, is distinctly lacking. Indeed, male mosquito biology in general has been fairly neglected over the last 30 years (Ferguson et al., 2005, 2010), due to a justifiable concentration of effort on understanding the role of female mosquito biology in disease transmission. However, the emergence in

recent years of genetically engineered mosquito strains as potentially viable solution to vector control has resulted in a resurgence of interest in understanding the aspects of male mosquito biology that impact on their performance. The experiments in the following chapters will go some way to answering pertinent questions about male mosquito performance. While the results will be of most relevance to the strain being used experimentally (*Ae. aegypti* RIDL strain OX513A), the general principles will likely also be applicable to releases of other strains or species.

Specifically, this thesis will examine the effects of three variables on male performance (discussed in more detail below):

a) male size;

b) concentration of sugar diet provided to the adults prior to release; and

c) duration of this pre-release period (i.e. how many days males are provided with sugar prior to release).

Male Size

The size of the pupa and resultant adult is primarily a result of larval rearing conditions and is an important aspect of mosquito life history. The final body size of *Ae. aegypti* is largely determined by the dietary conditions present during the larval phases, with more nutritionally challenging larval environments resulting in smaller individuals (Briegel, 1990; Timmermann and Briegel, 1993, 1999). The fourth and final instar is of particular importance; it is at this stage that 80-90% of a larva's growth and biosynthesis takes place (Briegel, 2003), and if food is scarce during this time they will increase their active foraging in an effort to find more (Reiskind and Janairo, 2015). Larger individuals possess greater stores of energetic reserves (Briegel et al., 2001a, 2001b), and so in the laboratory size is well known to be positively correlated with longevity (Steinwascher, 1982; Briegel et al., 2001a, 2001b; Gary et al., 2009; Reiskind and Lounibos, 2009; Bargielowski et al., 2011b). In the field, the picture is more complicated. Larger females (Hawley, 1985) and males have been found to live longer than smaller males, but the same effect was not found for females (Maciel-de-Freitas et al., 2007). Size can also affect flight and dispersal ability. Similarly to longevity, when flight is measured in the laboratory larger individuals tend to exhibit greater flight performance (Briegel et al., 2001a; Kaufmann et al., 2013b). However, in full-field studies using mark-release-recapture methods this trend can be more difficult to detect

(Valerio et al., 2012), non-existent, or even reversed (Maciel-de-Freitas et al., 2007). Finally, the role of male body size in mating success, while possibly important, is generally poorly understood. In *An. gambiae*, for example, intermediate-sized males tend to have a higher mating success than smaller ones (Ng'habi et al., 2005, 2008, Maïga et al., 2012, 2014), although this picture may be complicated by variations in sugar availability and temperature (Gary et al., 2009). In *Ae. aegypti*, larger males have been shown to produce and transfer more sperm than smaller males (Ponlawat and Harrington, 2007, 2009), and also to father more eggs across more females (Helinski and Harrington, 2011). However, a more recent study found that this mating advantage possessed by larger males was present in *Ae. albopictus* but not in *Ae. aegypti* (De Jesus and Reiskind, 2016).

Sugar Diet

In the wild, mosquitoes rely on wide range floral nectars and other sources to subsist (Foster, 1995). In a mass production environment, the provision of sugar can be included operationally. Of the variety of sugars present in naturally-occurring sugar sources, sucrose is particularly prevalent (Van Handel, 1972). While other aedines such as *Ae. taeniorhynchus* are content to feed on a range of sugars (Nayar and Sauerman, 1971a), *Ae. aegypti* will actively select sucrose, being of greatest nutritional value (Ignell et al., 2010). Furthermore, the saliva of *Ae. aegypti* contains a sugar-digesting α-glucosidase that functions optimally on sucrose (James et al., 1989; Marinotti and James, 1990; Marinotti et al., 1990). Sucrose concentrations below 0.5% are of no nutritive effect to *Ae. aegypti,* whereas all higher concentrations (tested up to 50%) extend survivorship and allow synthesis of reserves (Briegel et al., 2001a). Nayar and Pierce (1980) found that during the first five days of the adult stage, although female *Culex nigripalpus* imbibed higher volumes of lower-concentration (5%) solutions, greatest caloric intake was achieved by provision of higher-concentration solutions (25%), even if the volumes consumed were significantly less. This trend was also seen in *Ae. taeniorhynchus* (Nayar and Sauerman, 1974). However, Kingsolver and Daniel (1979) have suggested that some species may struggle to imbibe sugar solutions of high concentration, and Andersson (1992) found that lower volumes of the highest sugar concentrations (50%) were imbibed, their hypertonicity possibly being toxic. The high viscosity of more concentrated sugar solutions can also present a physical hazard to feeding adults, which can become fatally entangled (Briegel et al., 2001a).

Length of Sugar Provision

The extent to which sugar diet can be synthesised into glycogen and lipid reserves depends on the length of time adults have access to it, but also on body size and concentration of diet. Briegel et al. (2001a) investigated the process of reserve synthesis in female *Ae. aegypti*. For both small and large females fed a range of sucrose concentrations, they calculated the time taken to synthesise maximal reserves of both glycogen and lipids. Those authors found that even within a single treatment group this time ranged considerably, although in general smaller individuals built up reserves more quickly.

Both in the laboratory and in the field, *Aedes* species of both sexes start foraging for sugar almost immediately after emergence, often precluding other behaviours such as host- or mate-seeking (Haeger, 1960; Nayar and Sauerman, 1975; Nayar, 1981; Magnarelli, 1983; Haramis and Foster, 1990; Smith and Gadawski, 1994; Foster, 1995). Certainly then, the first few days post-eclosion are crucial for determining the subsequent performance of mosquitoes. Andersson (1992) found that *Ae. communis* females provided with a sugar meal prior to blood or water meals survived longer than those that had a delay in sugar provision, highlighting the importance of the initial sugar meal. Nayar and Pierce (1980) quantified volumes of sugar solutions imbibed over the lifetime of female *Culex nigripalpus*. They found that most sugar feeding occurred in the first five days after eclosion, after which feeding occurred at a lower level to maintain reserves. This initial five-day period was vital for building on the insects' teneral reserves.

The three variables of (i) male size, (ii) sugar concentration, and (iii) duration of sugar provision are all tightly linked to the nutritional status of the males. At the point of emergence, larger mosquitoes have greater quantities of stored energy than smaller ones (Briegel, 1990; Takken et al., 1998). A higher concentration of sugar meal provided to the adults will increase their ability to synthesise reserves post-emergence, as will increasing the length of time they are granted access to it (Briegel et al., 2001a). However, each of these variables is also limited by considerations other than the nutritional status of the males. Rearing larger males requires lower larval densities, and therefore significantly more laboratory or factory space. This consequently reduces the number of males that can be produced per unit area of a mass production facility. Therefore, either the total number of males able to be released is reduced, or greater rearing capacity (laboratory space, equipment, personnel) must be acquired, increasing the financial cost of the programme.

Provision of high-concentration sugar meals is limited by both practical and physiological constraints. Finally, increasing the length of time males have access to sugar also increases the time spent at high density prior to release, which was seen in Section 3.5.1 to be a major contributing factor to high mortality. A similar trend has also been observed in previous field studies (Bailey et al., 1979; Dame et al., 1981; Helinski et al., 2008).

As well as being thematically linked, these variables were chosen because they are all under direct control of project personnel during a release programme. The type and duration of sugar feeding can be altered to suit specific requirements, whilst male size is a function of larval rearing conditions. All three of these variables have the potential to significantly affect the subsequent performance of released males. However, it is not expected that any observed improvements to insect vigour observed in the experiments described here will confer an equal advantage on males released into the wild. The difficulty of translating insect performance from the laboratory to the field has been a perennial problem from the earliest SIT trials right up to the 2010 Cayman Islands suppression trial using OX513A. Rather, the aim of the laboratory experiments described in the following three chapters is to draw out general principles which will improve the overall efficacy of male releases. These can then be further tested in semi-field or full-field experiments, before being incorporated into the design of future large-scale suppression programmes.

5 Longevity of Male OX513A

5.1 Introduction

The longevity of female mosquitoes, both in the laboratory and the field, has been well documented. Larger females, possessing greater teneral reserves, are better equipped to survive in environments where sources of adult nutrition are scarce (Andersson, 1992). From a disease transmission perspective, longer-lived females have a higher probability of becoming competent to transmit a pathogen; this principle was recently demonstrated in wild-caught female *Ae. aegypti* during a dengue outbreak in Brazil (Juliano et al., 2014). From the point of view of a genetic control programme such as RIDL, greater field longevity of released males is a positive trait; longer-lived males have a greater likelihood of mating success and therefore an improved chance of passing on the genetic control element into the wild population. Although the emergence of RIDL and other genetic control strategies has spurred interest in field longevity of wild and released males, there are still gaps in our knowledge, notably about how longevity may be effectively augmented by improving the nutritional status of the released males.

An adult male mosquito's capacity for survival is primarily dependent on two types of energy resource: those accumulated during the larval stage (teneral reserves) (Timmermann and Briegel, 1993, 1999), and additional reserves synthesised as an adult from ingested carbohydrates, typically in the form of plant nectars (Foster, 1995). Teneral reserves general comprise a combination of glycogen and triglycerides, quantities of which are dependent on the availability of food at the larval stages (Nayar, 1968, 1969; Nayar and Sauerman, 1975; Yuval, 1992; Foster, 1995). Larger individuals possess greater teneral reserves than smaller males (Briegel et al., 2001a), and in general will survive longer as a result (Maciel-de-Freitas et al., 2007; Xue et al., 2010). As males do not blood feed, sugar acquisition is the only way in which teneral reserves can be added to, and is crucial for survival beyond a few days (Nayar and Sauerman, 1971b; Briegel and Kaiser, 1973; Briegel et al., 2001a). As sugar is imbibed in liquid form, this can ensure that the insect also takes on enough water to prevent dehydration (although it can also be taken on separately if necessary).

In the laboratory, longevity is perhaps the simplest measure of adult insect vigour, and is commonly used as a proxy for fitness in many insect species. As the behaviour being

measured - essentially just staying alive - is a reasonably simple one, longevity studies can provide a sensitive way of gauging the effects of even subtle imposed physiological changes (Briegel et al., 2001a). Such changes are likely to be more difficult to detect experimentally as part of assays that test a more complex behaviour, such as mating. This sensitivity is further enhanced by the ability to perform longevity analyses on large numbers of insects, as the labour per insect is extremely low (daily removal of dead individuals). This makes longevity studies ideal for gaining an overview of the effects of multiple variables, before selection of those that warrant more in-depth and labour intensive investigation. However, there are disadvantages associated with longevity studies. The relationship between longevity and other components of fitness is uncertain, and elucidation of background effects can be problematic (a notable example being that laboratory insect colonies may have been inadvertently selected for shorter generation time). Of course, like any fitness assay, it can be difficult to relate results obtained in a laboratory to those from the field, with the expectation being that survival estimates obtained from laboratory data will significantly overestimate field longevity (Hawley, 1988).

The aim of the experiments in this chapter was to assess how the nutritional status of OX513A males affects their longevity. Nutritional status was modulated by altering the males' physical size (itself a result of larval rearing conditions), and the type and duration of sugar feeding provided immediately following eclosion. By assessing longevity after exposure to different feeding regimens, it was possible to assess which modifications to nutritional status were the most significant determinants of subsequent longevity. The results of these experiments also informed which factors warranted further investigation of their effects on more complex behaviours in subsequent chapters.

5.1.1 Experimental Design

This chapter set out to investigate the effects of three variables - male size, concentration of sugar diet, and duration of sugar provision - on the longevity of OX513A adult males. As previously mentioned, although adult mosquitoes emerge with teneral reserves accumulated during the larval stages, an initial sugar feed is important for the fuelling of subsequent adult behaviours. As such, newly-emerged wild adults of both sexes will often undertake to obtain a sugar feed immediately, before beginning mate- or host-seeking behaviours (Haeger, 1960; Nayar and Sauerman, 1975; Nayar, 1981; Magnarelli, 1983;

Haramis and Foster, 1990; Smith and Gadawski, 1994; Foster, 1995)[17]. Accordingly, during the time that mass reared males are maintained prior to release, a source of sugar and/or water may be provided to them (they also reach sexual maturity during this time). It therefore seems reasonable to assume that upon release, a sugar-fed male's first priority will be mate-seeking, and not sugar-feeding. The expected lifespan of released OX513A males is short, having been variously estimated at 1.4 days (Carvalho et al., 2015); 1.6 days (Winskill et al., 2015); 2.0 days (Lacroix et al., 2012); 2.2 days (Winskill et al., 2015); and 2.3 days (Neira et al., 2014). As such, it is unlikely that any will survive long enough to exhaust their on-board energy reserves and be driven to seek energy replenishment from wild sources of sugar. Therefore, neither of the two experimental designs contemplated any post-release sugar feeding. Experiment 1 was a 'worst-case scenario', where males were provided with sugar for a short period of time (equivalent to the duration of time spent in release devices between eclosion and release), and thereafter being completely deprived of sugar and water. This examined how long males could maintain themselves with only the energetic and water resources that were provided to them prior to 'release'. Experiment 2 was identical, except once sugar access was removed water was still provided continuously. This contemplated a second possible nutritive scenario, in which released males still do not expend effort in seeking a wild source of sugar, but are able to take on water. This would be the case if the males had access to a water source as a by-product of seeking females, for example if the females were in the process of eclosing from standing water. The experimental provision of water after 'release' also served to extend longevity compared to Experiment 1, with the intention of revealing any more subtle differences between treatments that may not be evident under the more compressed survival times of Experiment 1. Furthermore, in female *Ae. aegypti*, the ability of an individual to actually utilise its reserves was partly dependent on the concurrent availability of water (Briegel, 1990).

The treatments used in each experiment were the same. Three variables were used: male size class ('small' and 'medium'); sugar concentration (water, 1%, and 10%); and length of sugar provision (2, 3, and 4 days). Each combination of variable levels was tested, giving a total of eighteen treatments (Table 5-1).

[17] Although mating prior to sugar feeding has occasionally been observed (Haeger, 1955).

The two male size classes used ('medium' and 'small') were products of two rearing densities (3 and 5 larvae ml^{-1}) within the likely range of densities used to mass rear males for release in the field. Whilst these two densities are quite similar, and there is often overlap in the sizes of males that are produced (see Figure 9-1, Appendix), longevity studies are often able to reveal subtle changes in survival that occur as a result of small physiological differences. Choosing more extreme rearing densities would likely have produced a more significant result; however the aim was to investigate treatments that are actually field-applicable.

Table 5-1. List of treatments used in longevity experiments.

Treatment	Male	Sugar	Days of provision
1	small	water	2
2	small	water	3
3	small	water	4
4	medium	water	2
5	medium	water	3
6	medium	water	4
7	small	1	2
8	small	1	3
9	small	1	4
10	small	10	2
11	small	10	3
12	small	10	4
13	medium	1	2
14	medium	1	3
15	medium	1	4
16	medium	10	2
17	medium	10	3
18	medium	10	4

The three sugar concentrations were chosen to range on a log scale from being of no nutritive value (water), through slight nutritive value (1%), to a commonly used standard (10%). Very similar sucrose concentrations have been used in other studies of *Ae. aegypti* energetics in females (Kaufmann et al., 2013b), notably by Briegel et al. (2001a) who found that female *Ae. aegypti* of a range of sizes were able to proportionally increase their stored reserves to the greatest extent when maintained on 10% sucrose, compared to

concentrations below or above this (tested up to 50%). A preliminary longevity experiment (data not shown) was conducted with 40% sucrose solution as a sugar treatment. In those treatments the viscosity of the sugar diet caused feeding adults to fatally stick to the sugar feeder. Furthermore the sugar rapidly crystallised on the sugar feeders, making it unavailable to the surviving adults.

The numbers of days of provision (2, 3, or 4) were chosen based on experiences from the Cayman Islands suppression trial in 2010. Keeping males for less than 2 days prior to release risked losing a substantial portion of the release cohort as uneclosed pupae. Keeping for 5 days prior to release increases mortality inside RDs (see Section 3.5.1), and depending on the number of releases per week can cause scheduling and storage problems. For these reasons the tested numbers of days of sugar provision were 2, 3, and 4.

5.2 Materials and Methods

Larvae were reared according to the regimen in General Methods (Section 2.2.2). Male pupae were harvested and allowed to eclose in groups of 50 into 15 x 15 x 15 cm cages. Each of the 18 treatments consisted of four cages of 50 males. Pupae that failed to eclose were not counted toward the survival analysis, therefore the sample size for each treatment was slightly below 200 males (range = 175-195). Sugar was provided for the first 2, 3, or 4 days of adult life (Table 5-2) then removed. In Experiment 2, water was also provided throughout the experiment, in addition to the initial sugar provision. Mortality was assessed and dead males removed daily. Insectary conditions during longevity experiments were 25.6(±1.1)°C and 70.1(±6.3)% RH.

Table 5-2. Procedure for the set-up of longevity assays.

Day	Action
0	Pupae placed into cages, sugar provision started
1	First eclosion of adult males
2	Eclosion complete, eclosion medium removed, non-eclosed males counted
3	First daily mortality count, sugar removed from '2 days of provision' cages
4	Second daily mortality count, sugar removed from '3 days of provision' cages
5	Third daily mortality count, sugar removed from '4 days of provision' cages
≥6	Daily mortality counts

Statistical Analysis

Cox proportional hazards regression[18] (Cox, 1972) was used to assess the effects of the three variables - male size, sugar concentration, and number of days of sugar provision - on adult male longevity. The Cox proportional hazards model is a semi-parametric model (in that it that does not specify a baseline hazard function) and allows estimation of the effect of multiple covariates on an individual's hazard, i.e. their instantaneous risk of death over the course of the study. For each variable, those levels perceived by the author to be the most favourable (medium males, 10% sugar, and four days of provision) were designated as reference levels, and so individuals in those levels were assigned a hazard ratio of 1. Hazard ratios for other factor levels were then expressed as multiples of 1. For example, in Table 5-3, those males provided with 1% sugar had a hazard ratio of 1.78, which can be read as having a 78% higher chance of death (over the course of the study) when compared to those on 10%. Each hazard ratio is presented with 95% confidence intervals, and a p-value as a measure of statistical significance. As both experiments were run until every individual died, no data were censored.

Each of the variables in the Cox proportional hazards model was tested to see if it violated the assumption of proportionality of hazards, i.e. that the survival functions for each factor change proportionally over time. In each case, variables were tested for proportional hazards by means of:

- the function `cox.zph`; and
- visual inspection of:
    - survival functions for each variable (Kaplan-Meier plots);
    - Schoenfeld residuals; and
    - log-minus-log plots.

In each case, none of the variables were found to grossly violate the assumption of proportional hazards, upon which the validity of the model depended.

Kaplan-Meier plots are also presented as a graphical representation of survival.

[18] R function `coxph` within package *survival*.

5.3 Results

5.3.1 Results - Experiment 1

In Experiment 1, males were provided with sugar of the concentration and for the duration relevant to their treatment (see treatment list in Table 5-1). Once the sugar was removed after two, three, or four days, males were provided with no other form of sustenance.

Main Effects

Across the whole experiment, mean age at death was 6.4 days, and maximum age at death was 10 days. For each of sugar concentration and days of provision, hazard ratios between the reference levels (10%, and 4 days) and the remaining levels all differed significantly (all $p<0.0001$, Table 5-3). **Of the two factors, reducing the days of provision increased the hazard ratio to a much greater extent than reducing the sugar concentration**, especially when reducing from three days of provision to two.

Table 5-3. Main effects of male size, sugar concentration, and days of provision on survival of males in Experiment 1 (n=3,350). For each factor, the hazard ratio of the first level is set to 1, with the other levels expressed as multiples of 1.

	Hazard Ratio	95% CIs	p
Male Size:			
medium	1	-	-
small	0.94	0.88 - 1.01	0.068
Sugar concentration:			
10%	1	-	-
1%	1.78	1.64 - 1.94	<0.0001
water	6.16	5.59 - 6.80	<0.0001
Days of provision:			
4	1	-	-
3	7.26	6.56 - 8.03	<0.0001
2	35.09	31.03 - 39.69	<0.0001

Surprisingly, small males did not have a significantly different hazard than medium males. A sample of wing length measurements taken after the conclusion of the experiment showed that 'medium' males were in fact only marginally larger than 'small' males (see Figure 9-1, panel A, Appendix). This lack of size distinction was certainly the reason for Experiment 1's lack of significant effect of male size. The significant main effects of sugar concentration and days of provision can usefully be visualised using Kaplan-Meier plots (Figure 5-1). In

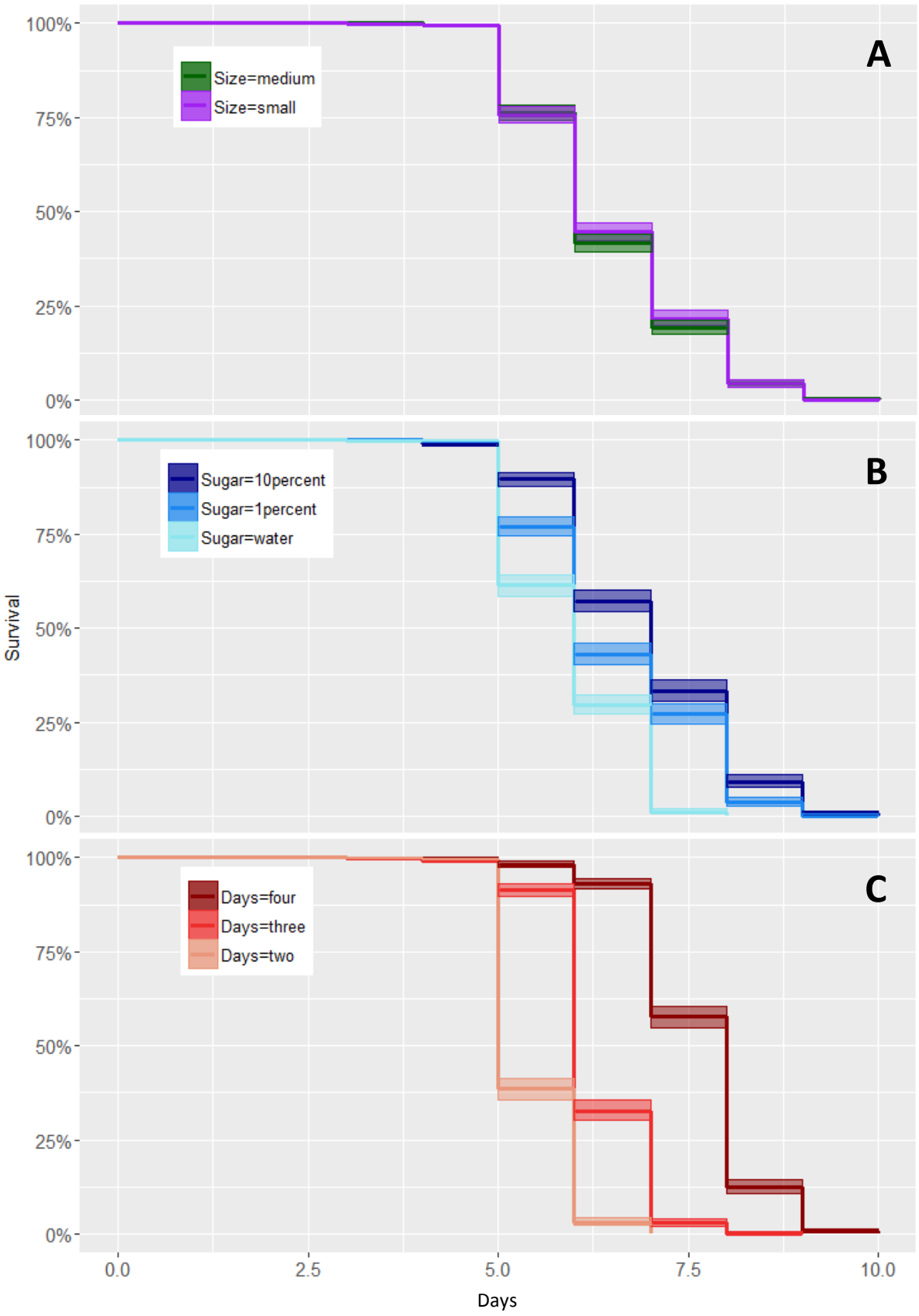


Figure 5-1. Survival of Experiment 1 males as dependent upon (A) size class, (B) sugar concentration, and (C) days of provision. Shaded areas indicate 95% confidence intervals. While the effects of both sugar concentration and days of provision were significant ($p<0.0001$ for both, log-rank test), the effect of days of provision was the stronger of the two. Male size did not significantly affect survival ($p=0.217$, log-rank test).

each case, the survival curves of the three factor levels diverge at Day 5, and remain distinct until each is at a near-zero population.

Interaction Effects

In small males, there was a significant interaction between sugar concentration and days of provision. For small males provided with **1% sugar**, a reduction in the number of days of provision resulted in additional significant increases in hazard ratio versus those provided with 10% sugar ($p=0.004$ between 4 days and 3 days, and $p<0.001$ between 4 days and 2 days). In other words, a reduction in the number of days of provision had a greater adverse effect on small males provided with 1% sugar than those provided with 10% sugar, and the greater the reduction in days of provision, the greater the severity of the effect. Interestingly, this effect did not extend to those small males provided with **water**.

Similarly, in medium males there was also a significant interaction between sugar concentration and days of provision. For medium males provided with **water**, a reduction in the number of days of provision resulted in additional significant increases in hazard ratio versus those provided with 10% sugar ($p<0.0001$ between 4 days and 3 days, and $p=0.033$ between 4 days and 2 days). In other words, a reduction in the number of days of provision had a greater adverse effect on medium males provided with water than those provided with 10% sugar. This effect was not observed for those medium males provided with **1% sugar**, and so is the converse of the effect found in the small males.

5.3.2 Results - Experiment 2

In Experiment 2, males were provided with sugar of the concentration and for the duration relevant to their treatment (see treatment list in Table 5-1). Once the sugar was removed after two, three, or four days, males were provided with water for the remainder of the experiment.

Main Effects

Across the whole experiment, mean age at death was 11.4 days (significantly older than in Experiment 1, $p<0.0001$, two-sample t-test), and maximum age at death was 19 days. For all three variables, hazard ratios between the reference levels (medium, 10%, and 4 days) and the remaining levels all differed significantly (all $p<0.0001$, Table 5-4). **Overall, and in the reverse of Experiment 1, reducing the sugar concentration increased the hazard ratio to a greater extent than reducing the days of provision**. However, the difference in effect size between the two factors was not as stark as was the case in Experiment 1.

Table 5-4. Main effects of male size, sugar concentration, and days of provision on survival of males in Experiment 2 (n=3,322). For each factor, the hazard ratio of the first level is set to 1, with the other levels expressed as multiples of 1.

	Hazard Ratio	95% CIs	p
Male Size:			
medium	1	-	-
small	3.11	2.89 - 3.35	<0.0001
Sugar concentration:			
10%	1	-	-
1%	2.59	2.37 - 2.84	<0.0001
water	5.05	4.59 - 5.56	<0.0001
Days of provision:			
4	1	-	-
3	1.39	1.27 - 1.51	<0.0001
2	2.40	2.20 - 2.63	<0.0001

Also in contrast to Experiment 1, small males had an as-expected significantly higher hazard than medium males ($p<0.0001$, Table 5-4). This was a result of the increased distinction between the size classes, which had not been present in Experiment 1 (see Figure 9-1, panel B, Appendix). Kaplan-Meier plots for Experiment 2 are shown in Figure 5-2. The factor

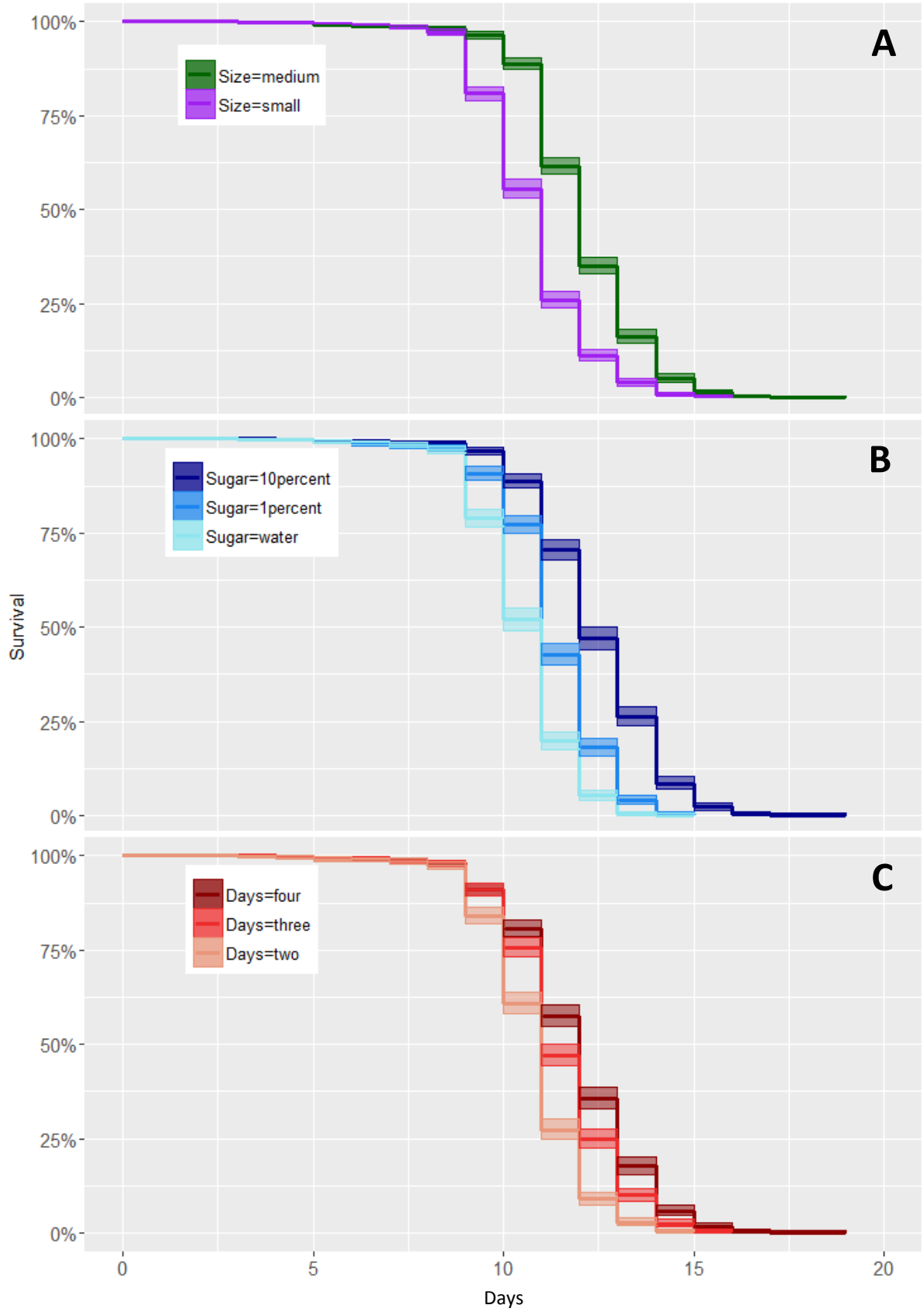


Figure 5-2. Survival of Experiment 2 males as dependent upon (A) size class, (B) sugar concentration, and (C) days of provision. Shaded areas indicate 95% confidence intervals. While the effects of both sugar concentration and days of provision were significant ($p<0.0001$ for both, log-rank test), in contrast to Experiment 1 the effect of sugar concentration was the stronger of the two. Also in contrast to Experiment 1, male size did significantly affect survival ($p<0.0001$, log-rank test).

levels of each variable diverge at Day 9 (in contrast to Day 5 in Experiment 1), and again remain distinct until each is at a near-zero population. The significance of male size in Experiment 2 (versus its non-significance in Experiment 1) can clearly be seen by comparing panel A in each of Figure 5-1 and Figure 5-2. The variable which most impacted survival in Experiment 2 was sugar concentration (Table 5-4); this is reflected in Figure 5-2 where panel B has the most distinct survival curves of the three variables. By providing water throughout the study, Experiment 2 reduced the effects of dehydration that were limiting in Experiment 1. In Experiment 1, males provided with water only (which was then removed after the initial 'feeding period') lived an average of 6 days. In Experiment 2, males provided with water only (provision of which was continued through their lifetime) lived an average of 10.5 days.

Interaction Effects

In small males, there was a significant interaction between sugar concentration and days of provision. For small males provided with **water**, a reduction in the number of days of provision resulted in additional significant increases in hazard ratio versus those provided with 10% sugar ($p=0.004$ between 4 days and 3 days, and $p<0.0001$ between 4 days and 2 days). In other words, a reduction in the number of days of provision had a greater adverse effect on small males provided with water than those provided with 10% sugar, and the greater the reduction in days of provision, the greater the severity of the effect. This effect was also observed for those small males provided with **1% sugar**, but only when comparing 4 days and 2 days ($p<0.0001$).

Similarly, in medium males there was also a significant interaction between sugar concentration and days of provision. For medium males provided with **water**, a reduction in the number of days of provision resulted in additional significant increases in hazard ratio versus those provided with 10% sugar ($p<0.0001$ between 4 days and 2 days; p was marginally non-significant between 4 days and 3 days). In the same way, for medium males provided with **1% sugar**, a reduction in the number of days of provision resulted in additional significant increases in hazard ratio versus those provided with 10% sugar ($p=0.022$ between 4 days and 3 days, and $p=0.006$ between 4 days and 2 days). In other words, a reduction in the number of days of provision had a greater adverse effect on medium males provided with either water or 1%, than those provided with 10% sugar, and the greater the reduction in days of provision, the greater the severity of the effect.

5.3.3 Results - Wing Length as a Covariate

In Experiments 1 and 2, males were either of 'small' or 'medium' size. This distinction was made based on prior knowledge of how rearing regimen affected the size of the individuals produced (with higher-density rearing generally producing smaller individuals; see Figure 2-7). However, even with the desired separation between the size classes seen in Experiment 2, in both experiments there was still considerable overlap between the two size classes (see Figure 9-1, Appendix). In order to allow a more accurate assessment of the effect of male size on longevity, a subset of males from each of Experiments 1 and 2 had their wing lengths measured after death (description of method in Section 2.2.3). Wing length is a commonly-used proxy for adult size (Nasci, 1990), and so these direct size measurements allowed an analysis of the effects of absolute male size (in combination with the other two variables: sugar concentration, and days of provision) on longevity.

For each treatment on each day, up to three dead males (when available) had wing length measurements taken. In Experiment 1, 197 males had their wings measured, from 3,350 total males (5.9%). In Experiment 2, 352 males had their wings measured, from 3,322 total males (10.6%). Experiment 1 had fewer total days on which mortality occurred, so fewer measurements were made than in Experiment 2. Cox proportional hazards models were constructed in the same way as detailed for Experiments 1 and 2, except with size class being replaced by wing length as a continuous covariate.

Main Effects

In both Experiment 1 and Experiment 2, wing length had a significant main effect on the hazard ratio. An increase of 0.01mm in wing length was associated with a 7% and 8% reduction in the hazard[19] for Experiments 1 and 2 respectively (both $p<0.0001$, Table 5-5). Neither Experiment 1 nor Experiment 2 showed significant interaction effects between wing length and either of sugar concentration or days of provision.

[19] With continuous covariates, the hazard ratio can be interpreted to read "for every 1 unit increase in the covariate, the hazard increases/decreases by 'x'%". Wing lengths were measured in mm, meaning 1 unit = 1 mm. As the range of wing lengths obtained spanned <0.5 mm, this would have resulted in extremely small hazard ratios. As such, wing length values were multiplied by 100 prior to analysis, to enable hazard ratios to be read as pertaining to increases of 0.01 mm, and not 1 mm.

Table 5-5. Main effects of wing length[20] on survival of males in Experiment 1 (n=197) and Experiment 2 (n=352). The size and significance of the effect of increasing wing length on reducing the hazard ratio were very similar in each experiment. The confidence intervals in Experiment 2 were slightly smaller than in Experiment 1, likely a result of greater sample size increasing statistical power.

	Hazard Ratio	95% CIs	p
Experiment 1	0.93	0.89 - 0.96	<0.0001
Experiment 2	0.92	0.90 - 0.95	<0.0001

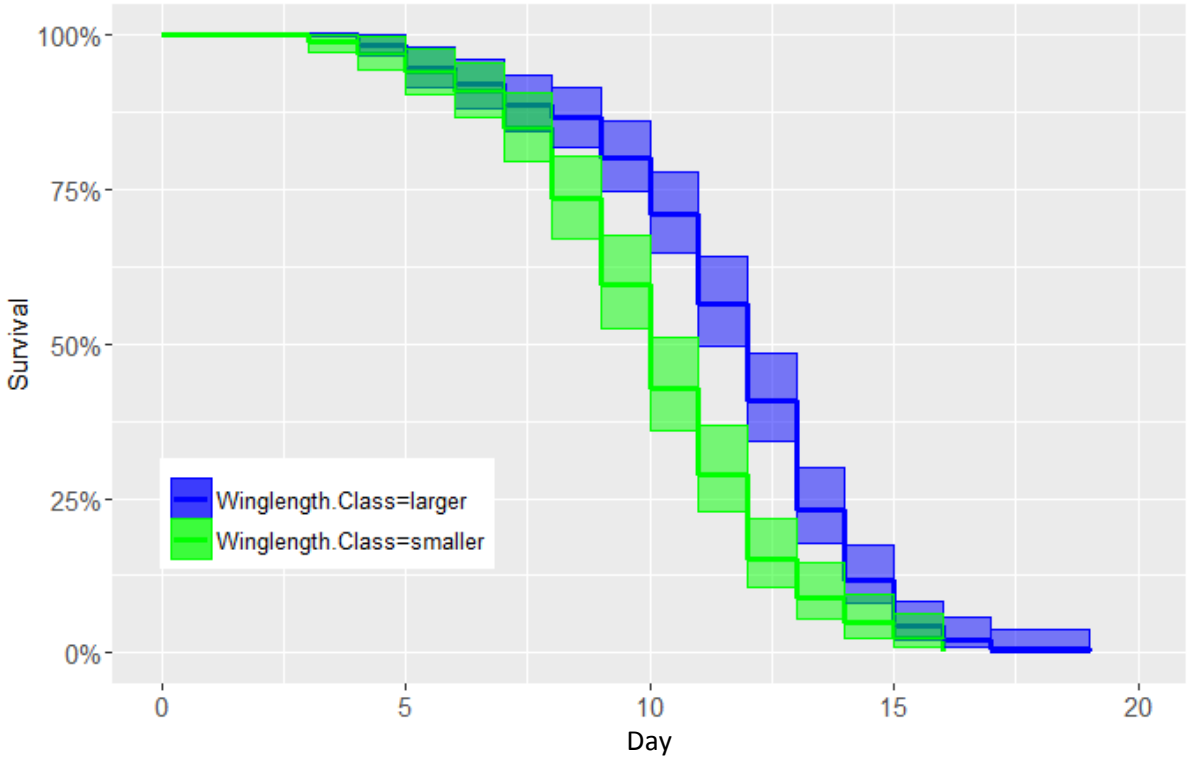


Figure 5-3. Survival of those males in Experiment 2 for which direct wing length measurements were obtained. In this figure, males are split into two size classes dependent on whether they are larger or smaller than the average wing length. Shaded areas indicate 95% confidence intervals. Larger males lived significantly longer than smaller males (p<0.0001, log-rank test).

Panel A of Figure 5-2 shows survival in Experiment 2 with Kaplan-Meier curves for each size class. Males were assigned as 'small' or 'medium' by virtue of their rearing conditions and not their actual measured size; as such there is some overlap in wing length between the two size classes (see Figure 9-1, panel B, Appendix). Figure 5-3 also shows survival in Experiment 2 based on size, but disregards the potentially ambiguous distinction between

[20] The models constructed for this analysis also produced hazard ratios (and corresponding 95% confidence intervals and p-values) for the variables sugar concentration and days of provision. However, given that those results were obtained from a subset of the individuals used in the primary analyses and therefore are less informative, they are not included in Table 5-5.

'small' and 'medium' and replaces it with an absolute distinction - males were directly measured and were either 'smaller' or 'larger' than the average size (and so by definition there is no overlap in wing lengths between these two new classes). Both methods of size differentiation show that all things being equal, larger males lived longer than smaller males.

For further analysis, it is illuminating to use 'age at death' (measured in days) as the measure of survival. There was a strong positive linear correlation between wing length and age at death ($F=38.26$, $p<0.001$), analogous to the significant reduction of the hazard ratio by increasing wing length ($p<0.0001$, Table 5-5). However, graphical examination of the wing length data yields trends of further interest. Panel A of Figure 5-4 below shows age at death versus wing length, plotted by the concentration of sugar with which the males were provided. The males provided with 10% sugar (blue circles) died later than those on 1% and water (yellow and open circles) (significant main effect of sugar concentration on hazard ratio, $p<0.0001$, Table 5-4). Of those males on 1% and water (yellow and open circles), only larger than average males (above the dashed red line) lived for 14 days or longer, i.e. to survive for a long time, males on poor-quality diets needed to be larger. Conversely, those males on the highest-quality sugar meal (blue circles) were capable of living for a long time even if they were much smaller than average (lower right-hand area of Chart A).

Overall, the mean wing length of males dying each day (solid black line) plots a remarkably straight course through the data, i.e. generally the mean wing length of males dying on a particular day was very slightly greater than the preceding day. However, this near-perfect linear trend was interrupted during Days 4-6, where wing length sharply increased to around the grand mean (dotted red line), before decreasing again.

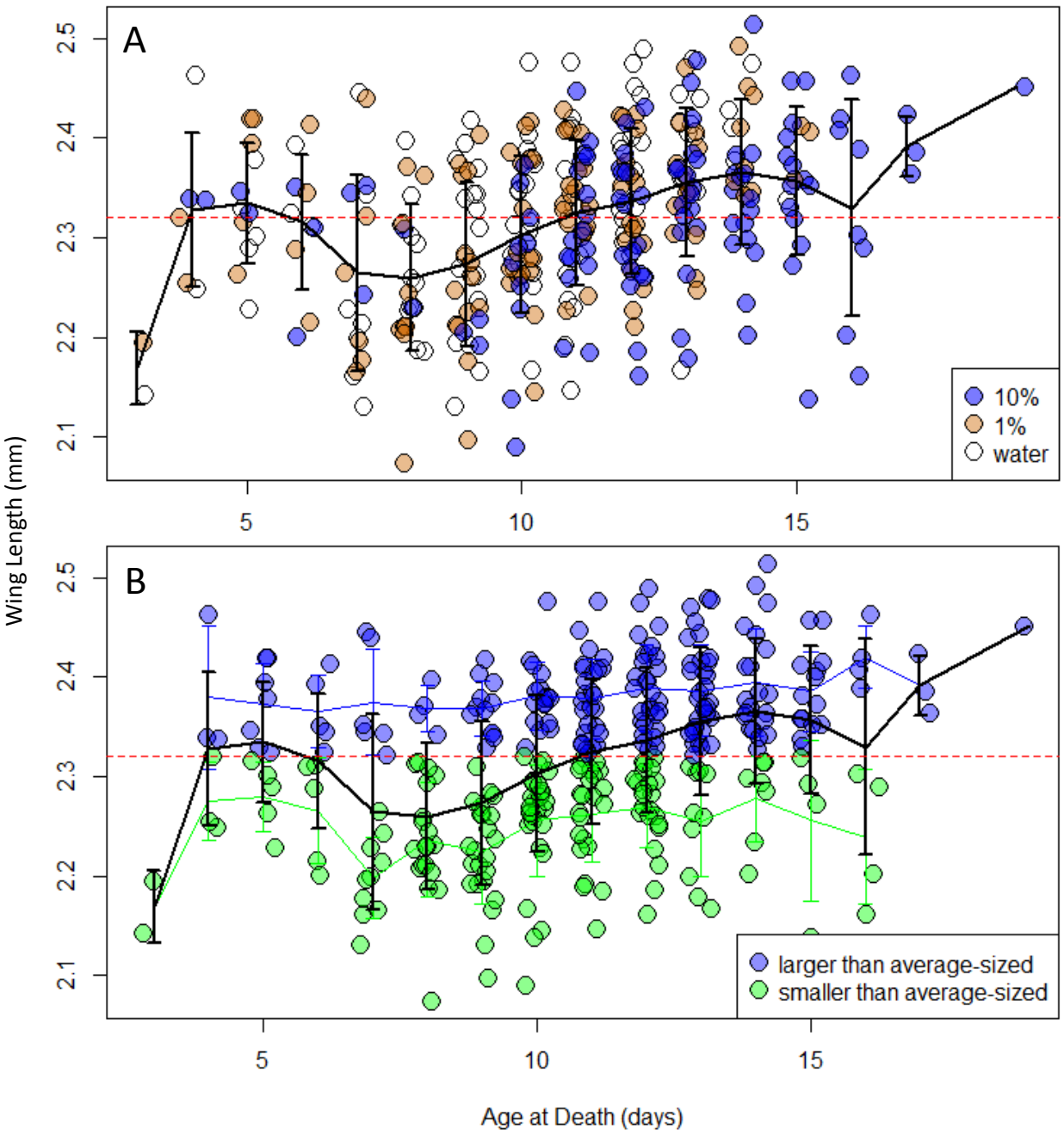


Figure 5-4. Age at death versus wing length for Experiment 2 males. Each data point is a single male[21]. The same data are plotted in both panels, which are coloured according to (A) sugar concentration, and (B) male size (larger or smaller than the average). Data plotted by number of days of provision are not shown. In both panels, the mean wing length of all males that died on a particular day is shown as a solid black line, ±sd, and the dashed red line is the mean wing length of all measured males (2.320 mm). Chart B also includes lines showing the mean wing lengths (±sd) of males in both size subgroups that that died on a particular day. For each day of death, points are artificially horizontally off-set to prevent points from completely obscuring each other, resulting in the slightly different location of each point between graphs. This also symbolically represents the fact males died throughout each day, not only at the exact time of measurement.

[21] N.B. As the number of males measured per day of death per treatment was capped at three, the number of data points per day in Figure 5-4 is not proportionally representative of the number of males that died on each day of Experiment 2 overall.

5.4 Discussion

The success of genetic control mechanisms such as RIDL depends on the ability of the released males to survive, disperse, and mate with wild females. Currently, knowledge is lacking about how the effectiveness of these releases may be maximised by ensuring the released males are in a nutritionally optimal state. The experiments in this chapter represent a full assessment of how the nutritional status of male *Ae. aegypti* OX513A affects their survival, subsequent to a simulated release in a laboratory setting.

Experiments 1 and 2

In Experiment 1, nutritionally speaking, conditions were harsh; no other sustenance (not even water) was provided after the initial few days of provision. As a result, the number of days of initial sugar provision was the most important factor in determining longevity; those males provided with their respective sugar for only 2 days had more than 35 times the hazard of those males who had access for 4 days. By means of comparison, the effect of providing even a non-nutritive diet (water) as compared to 10% sugar was to multiply the hazard ratio by only six times. Although dehydration was clearly a limiting factor for longevity, there was also an indication of reserve synthesis. Whilst providing water for an extra day merely extended lifespan by one day, providing 10% sugar for an extra day extended lifespan by slightly more than one day. This is broadly consistent with prior experiments on females, where reserve synthesis was shown to be possible within the first few days of adult life (Briegel et al., 2001a).

In Experiment 2, the continued provision of water after the initial sugar feed lifted dehydration as a limiting factor, and significantly extended lifespan overall; from a mean of 6.4 days in Experiment 1 to 11.4 days in Experiment 2. The elimination of dehydration as an impediment to survival, coupled with the addition of truly distinct male size classes, meant that 'days of provision' went from being the most important factor in Experiment 1 to the least in Experiment 2, where sugar concentration had the greatest effect. All things being equal, medium-sized males lived longer in absolute terms than their smaller counterparts. The experiment also demonstrated a remarkable ability of males provided with water to survive on their teneral reserves alone; the average day of death for all males provided with only water was 10.5 days. This was likely a result of being held in a single-sex environment;

water-fed males with access to females typically live for only 4 days (Chadee et al., 2014). This discrepancy is a result of the high energetic costs of courting females and making mating attempts (Bargielowski et al., 2011a), which highlights the importance of reducing female contamination of male release cohorts to the absolute minimum.

Experiment 2 had a shared aim with a recent similar experiment performed with *Ae. albopictus*, which examined the impact on longevity of sugar feeding immediately prior to release (Bellini et al., 2014). In that experiment, male pupae were allowed to eclose from pupal release devices equipped with a sponge sugar-feeding element, over which the adults passed as they exited. As in Experiment 2, water was provided to the males post 'release'. Those male cohorts offered sugar immediately after emergence had significantly higher subsequent survival than those offered water only. This significant effect of sugar concentration on longevity was analogous to the results of Experiment 2, and reinforces the importance of an early nutritive sugar meal for subsequent male vigour. There is also a good deal of field evidence for male aedines sugar feeding early in adult life. Wild male *Ae. vexans* have been observed feeding on flowers in the very first few hours after emergence, even before their adult bodies had completely hardened or the terminalia part of their genitalia had rotated (Breeland and Pickard, 1961, 1967). Similar observations have been recorded of males of *Ae. impiger* and *Ae. nigripes* (Corbet and Danks, 1973), *Ae. taeniorhynchus* (Haeger, 1960), *Ae. albopictus* and *Ae. togoi* (Harada et al., 1975), and *Ae. canadensis* and *Ae. stimulans* (Magnarelli, 1983).

One of the aims of the longevity experiments in this chapter was to examine more subtle effects of size and sugar feeding, that may not be visible in experiments that examine more complex behaviours and/or have smaller sample sizes. In particular, the interaction effects observed in Experiment 2 reveal interesting trends. Within each size class of male, a reduction in the number of days of sugar provision prior to 'release' had a greater adverse effect on those males provided with the inferior energy sources (1% sugar and water) as compared to those on the most nutritive energy source (10% sugar). Universally within that adverse effect, the greater the reduction in feeding time, the more severe the effect. This indicated that those males on the highest-quality sugar diet not only lived the longest (all else being equal), but were the most well-equipped to cope with a feeding window of reduced duration. In respect of this trend both male size classes responded the same way. This question - of higher-quality sugar meals mitigating the subsequent loss of fitness

potential as a result of reduced pre-release feeding duration - is directly applicable to field release strategies. If it is expected that released males will not have access to any sustenance (even water) after release (analogous to Experiment 1, perhaps applicable to early-season releases during a dry season[22]), then providing sugar for an extra day prior to release might be the best strategy to maximise field longevity. However, if males are being released into a nutritionally less harsh environment (analogous to Experiment 2, with plenty of water and sugar sources available), then the optimal strategy might be to provide a higher-quality sugar diet for a shorter period of time, and mitigate possible damage and mortality from being kept at high density for too long prior to release. This is a good example of the need for male-release suppression programmes to be anticipatory of, and responsive to, the wider environmental context into which the males are released.

A similar question arises over the ability of adult sugar meals to compensate for reduced absolute levels of teneral reserves possessed by smaller males (having experienced more nutritionally challenging larval rearing conditions). In a comparison of artificial *Ae. albopictus* larval diets (Puggioli et al., 2013), a specialist mass rearing diet reduced the time to pupation and adult emergence, whereas a standard laboratory diet was found to significantly improve the longevity of the adults. Both of these outcomes are potentially advantageous from a mass production perspective. Reducing the time to pupation and emergence would increase the numerical output of a production facility over a set period, whereas improved adult longevity indicates an improvement in insect quality. The authors of that study hypothesised that because of the reduced duration spent feeding as larvae, those individuals on the specialist mass rearing diet (which was found to be carbohydrate-poor) were able to accumulate less reserves, which reduced their longevity (see also Van Handel, 1988; Briegel and Timmermann, 2001). However, this longevity reduction was only present if the adults emerged into an environment devoid of energy sources; provision of 10% sugar post-emergence allowed the adults reared on the specialist mass rearing diet to synthesise enough new reserves as adults to attain equal longevity with those adults which had been reared on the standard laboratory diet. Nonetheless, this compensatory reserve synthesis as adults was demonstrated only when the males were provided with 10% sugar

[22] For example, a recent assessment of mosquito sugar-feeding behaviour in the wild was carried out during the dry season in Ecuador. This study used coloured dyes to track sugar feeding on local vegetation; not one of the 172 collected male *Ae. aegypti* had sugar-fed, compared to one-third of the collected females (Qualls et al., 2016).

from emergence until their death, some 30 days later. This duration of sugar provision is an unrealistic scenario for released males, which would usually be released after only a few days. A more realistic assessment can be made by comparing treatments within Experiment 2. In a separate sub-analysis (results not shown), the 'small' males (being reared in a poorer nutritional environment and possessing less reserves) were found to have a similar compensatory ability when given sugar post-emergence, broadly dose-dependent on sugar concentration and days of provision. However, given that their sugar-feeding window in which compensatory reserves could be taken on was only 2-4 days (compared to the males' entire lifetime in Puggioli et al.'s experiment), this was not enough to improve their longevity to be commensurate with that of the medium-sized males. Given that 2-4 days between eclosion and release is a realistic timeframe for a field suppression programme, it can be concluded that sugar feeding prior to release, although necessary and advisable in order to maximise the energetic resources of the released males, is insufficient to compensate for poorer larval rearing conditions.

In Experiments 1 and 2 there was potential for variation in longevity within treatments based on variable sugar uptake, as it could not be guaranteed that males would regularly imbibe the solutions provided to them. Any effect of this nature would likely be exacerbated in those treatments provided with sugar for the shortest duration. In tests on individually housed *Ae. albopictus* with easy access to a sugar meal, 50% of newly-emerged males fed from 10% sugar solution within six hours, and 90% fed within 19 hours (Bellini et al., 2014). If one assumes similar rates of uptake in Experiments 1 and 2, the vast majority of males offered a 10% sugar meal would have taken the opportunity to do so at least once before the sugar was removed. Nonetheless, it is conceivable that the same may not apply to those males on 1% sugar or water, being less attractive energy sources (Lindh et al., 2006). From examination of the survival curves of those individual treatments provided with water only (data not shown), it was apparent that almost all of the males died exactly two days after the water was removed. The synchronicity of this mortality indicates that the males were likely utilising the water source right up until its removal. Indeed, it is quite possible that in actual fact the lower quality sugar meals incited a greater rate of consumption, as was observed in the first week of life of female *Ae. taeniorhynchus* (Nayar and Sauerman, 1974).

This issue of adult males' propensity to sugar feed was also investigated during the tests mentioned above of a pupal release device with sugar-feeding element (Bellini et al., 2014).

As mentioned, the authors of that study found that when eclosed and housed individually (i.e. under 'low stress' conditions), 50% of irradiated *Ae. albopictus* males had sugar-fed within six hours of emergence. Interestingly, when offered sugar as they exited a crowded pupal release device (i.e. 'high stress' conditions), around the same proportion of males took the opportunity to sugar feed. Given that the males' compunction to disperse and leave the crowded conditions of the pupal release device may cause them to forgo an initial sugar feeding opportunity, achieving parity with 'low stress' conditions represents a positive result in terms of imparting energetic resources to a release cohort. Adult release - which has been the primary release method for all OX513A field trials to date - presents a longer time window during which sugar meals can be offered to the adults. Therefore, one would expect that the proportion of adults sugar-fed at the point of release to be at least as great as that in Bellini's pupal releases. Indeed, part of the rationale for adult release is the desire to hold back adult males until they are sexually mature (a process which takes 15-24 hours in *Ae. aegypti*; Roth, 1948; Klowden, 1990). Therefore, it makes sense to use this time period to load them with a sugar meal. A modified version of the adult release device described in Section 3.2 - which includes a mechanism for sugar feeding - is currently being used for field releases of OX513A in Brazil (Carvalho et al., 2015). It would be very interesting to examine the proportion of males in these cohorts that take the opportunity to consume a sugar meal prior to release, in order that this proportion may be maximised.

Wing Length as a Covariate

In the primary analysis of Experiments 1 and 2, 'male size' was a categorical variable, with males being either of 'small' or 'medium' size, as dependent on their rearing conditions. The two rearing densities used (3 and 5 larvae ml^{-1}), while being potentially field applicable, produce individuals of overlapping size ranges (see Figure 9-1, Appendix). The direct measurement of wing lengths of males that died on each day throughout the experiments allowed a quantitative analysis of the link between absolute male size, sugar feeding, and longevity.

Overall, larger males lived significantly longer than smaller males; each incremental increase of 0.01 mm in wing length was associated with a 7-8% reduction in the hazard. Similarly, when males were split into two groups based on their measured wing lengths (being either 'smaller' or 'larger' than average, Figure 5-3), 'larger' males lived significantly longer. This

was an expected result; the reduced density-dependent competition resulting from lower larval rearing densities results in greater food availability per individual, allowing a higher degree of physical growth and reserve synthesis (Timmermann and Briegel, 1993, 1999). These larvae then develop into larger adults, which - being nutritionally advantaged - are able to survive for longer than adults that were subject to more intense density-dependent competition at the larval stages. These results echo similar findings in *Ae. albopictus* males, where being of larger size extends lifespan, whether provided with only water or provided with sugar (Xue et al., 2010). This advantage of size has also been show to be present in the wild; in a mark-release-recapture experiment in Brazil, larger *Ae. aegypti* males tended to be recaptured later, implying a survival benefit (Maciel-de-Freitas et al., 2007).

Although the interactions between wing length and each of sugar concentration and days of provision were not significant, informal observations still yielded information of value. A poorer sugar diet (1% or water) precluded males from living longer than two weeks, unless they were in the upper half of the size range. This is similar to results in females provided with only water, where larger females lived more than twice as long as their smaller sisters (Briegel et al., 2001a). Similarly, a higher-quality sugar meal (10%) allowed even some smaller-than-average males to live until the very latter stages of the experiment. Both observations indicate the potential for body size and sugar diet to compensate for each other in order to extend lifespan.

Generally speaking, the relationship between body size and longevity in *Ae. aegypti* is represented in the mosquito literature as linear, with larger individuals living for longer (Steinwascher, 1982; Hawley, 1985; Briegel et al., 2001a, 2001b; Reiskind and Lounibos, 2009). However, some field studies have failed to find this relationship (Scott et al., 2000), or found it only in males and not females (Maciel-de-Freitas et al., 2007). An example of a very clearly non-linear relationship was found in a recent laboratory study of *Ae. aegypti*; where females of intermediate size had the greatest longevity, followed by the very smallest females, with the very largest females having the shortest longevity (Juliano et al., 2014). The authors of that study did not suggest an explanation for such a relationship. A deviation from linearity (albeit one less extreme) was also found in Experiment 2. For the most part, the relationship between wing length and longevity was linear, with the average wing length of males dying on a particular day generally being slightly larger than those dying on the preceding day (Figure 5-4) (this was also reflected in the highly significant reduction of the

hazard ratio with increasing wing length). However, there was an observed 'bump' in this otherwise near-perfect relationship, occurring during days 4-6 of Experiment 2; males dying on those days appeared to be larger than those dying on the days immediately preceding or following. This feature of the data manifested equally in Experiment 1 (with allowance for variation in experiment length), and so is unlikely to be an artefact of random variation and/or the reduced sample sizes near the beginning and end of each experiment. Perhaps the males dying on days 4-6 were a subset of males that, for whatever reason, were more prone to either (i) mistakenly trying to mate with other males (as is occasionally observed in male-only cages), or (ii) were more likely to be subject to repeated erroneous mating attempts by other males, which required fending off. Each of these activities is separately known to be a significant drain on available energy (Bargielowski et al., 2011a; Helinski and Harrington, 2012), and so could have caused earlier death of these males than their size alone would predict. Another possible explanation is that the 'bump' was caused by males that either (i) did not fully utilise (imbibe) the water or sugar resource during the initial 2, 3, or 4 day period when it was available to them, and/or (ii) were less able to synthesise reserves from any meals taken during this time. This could have resulted in earlier-than-expected death based on their size. A reduction of feeding propensity as described would likely more severely affect those males on the less nutritious sugars; indeed, the majority of males that died on days 4-6 were provided with the 1% or water diets. If such a lack of feeding propensity does exist in certain subgroups, then it did not occur in any males that were in the bottom third or so of the size range; no males of that size died on those days at all. Leaving aside this phenomenon, the near-perfect linearity (that existed with only minor perturbance during Days 7-19) was remarkable given the range of variables other than wing length that would likely have been exerting an effect on age at death.

5.5 Conclusions and Further Work

When males were not provided with water after the initial period of sugar provision they died fairly quickly, and the most important determinant of longevity was the number of days of sugar provision. When water was provided after the initial sugar feed males lived much longer overall, and the most important determinant of longevity was concentration of the pre-'release' sugar meal. In the second of these scenarios, medium-sized males lived longer than their smaller counterparts. In small males, the initial sugar feed was not enough to compensate for their lack of teneral reserves relative to their larger brothers. Similarly, there was a significant correlation between wing length and longevity. To survive for more than 14 days, males on the poor-quality sugar diets (0% or 1%) needed to be larger; conversely, those males on 10% sugar were capable of surviving for this length of time even if they were much smaller than average size. In general, as day of death increased so did the average wing length of the males dying on that day; however, males dying on days 4-6 were unexpectedly large.

As well as being an indicator of improved vigour in general, males that survive for longer in the field after release have a greater chance of encountering and mating with a wild female. The longevity experiments presented in this chapter showed how sugar concentration and duration of sugar provision each affect the subsequent longevity of males of different size. The relative importance of these variables was also demonstrated to change dependent on external conditions, specifically the availability of water. Using this information, these factors will be taken forward in subsequent chapters for investigation using more complex male behaviours.

The experiments presented in this chapter did not attempt to understand to what extent any change in adult male longevity observed in the laboratory would translate into change in a field environment. The first efforts towards establishing this relationship in males have been made in *Ae. albopictus* (Bellini et al., 2014), but no such study has yet taken place using *Ae. aegypti.* Establishing this relationship would certainly be challenging, not least because it would likely vary heavily by study area. A possible method would be to use a series of mark-release-recapture experiments. For example, releasing two cohorts of males, one nutritionally advantaged (larger individuals given sugar access for several days), and one nutritionally disadvantaged (smaller individuals given little or no sugar access). Both cohorts

would need to be released simultaneously (and so be differently-marked) in order that weather conditions were identical for each cohort. In addition, males of each treatment should be co-released from multiple release points, which should be geographically spread as if for a suppression programme. This will maximise applicability of the results, as well as ensuring that each cohort is exposed to a range of potential habitat types and microclimates. In this way, for each broad type of release environment (hot and dry with little vegetation; temperate with more extensive plant cover; etc) it may be possible to infer a set of principles that dictate how to maximise the nutritional advantage possessed by released males.

Although the sugar-feeding behaviour of wild *Ae. aegypti* remains surprisingly cryptic, the generally accepted most common sugar sources are floral and extrafloral nectaries, and sugar concentration (although subject to variation with humidity) can be in the range 20-50% (Foster, 1995). Although 10% sucrose is a commonly-used laboratory standard, higher concentrations have shown to be of survival benefit to females (Andersson, 1992; Briegel et al., 2001a). However, in a preliminary experiment using 40% sucrose, the high viscosity of the solution resulted in indirect death via the adults becoming stuck to the sugar feeder. Moreover, the solution crystallised very rapidly after which it was unavailable for consumption by the insects. Therefore a sucrose concentration of 25% would be a good target for further investigation as a 'high' concentration sugar diet with the potential to further increase reserve synthesis in newly-emerged males.

Another further avenue of investigation is the propensity of male OX513A to feed prior to release, as mentioned in the discussion above. This could be examined by assessing the proportion of males that actually imbibe a sugar meal during the pre-release period (during which they are held in release devices). This data could be obtained relatively easily either by simple visual examination of the crop, or more quantitatively by the use of the cold anthrone reaction (Van Handel, 1972, 1985). Such tests should be performed after two, three, or four days provision as per the experiments presented in this chapter.

The results presented in this chapter represent the first formal assessment of the effects of body size and external sources of adult nutrition on longevity of male OX513A *Ae. aegypti*. They provide a baseline to which further changes to rearing and sugar-feeding procedures

can be compared, and furthermore demonstrate the importance of these factors to subsequent overall male performance.

6 Flight Capacity of Male OX513A

6.1 Introduction

The flight capacity of OX513A males is an aspect of their performance that warrants in-depth investigation, as it plays a crucial role in the mating biology of the insect. Mating often occurs near the host (Hartberg, 1971), so males are required to travel from their eclosion site (or in the case of RIDL males, their release point) to seek out females. An accurate assessment of the ability of males to disperse informs the number and spacing of release points, as well as the numbers released at each point, in order to ensure complete coverage of the target area for control.

In the case of mosquitoes, flight capacity is primarily estimated either in the field using mark-release-recapture (MRR) experiments, or in the laboratory using flight mills. These are two fundamentally different methods. MRR experiments date back to studies on populations of plaice by Petersen in 1896 (Southwood and Henderson, 2000) and waterfowl (Lincoln, 1930), and are used to infer information about the size, dispersal, and longevity of a wide variety of animal populations. In the case of dispersal of mosquitoes, large numbers of adults are marked (e.g. with fluorescent powder) and the distances at which they are recovered via trapping allow estimates to be made of field dispersal distances. These distances vary by location and environmental conditions (among other factors), but for male *Ae. aegypti* average distances travelled are generally less than 100 m and often less than 60 m (Reuben et al., 1975; Trpis and Hausermann, 1986; Muir and Kay, 1998; Tsuda et al., 2001; Harrington et al., 2005; Maciel-de-Freitas et al., 2007; Lacroix et al., 2012; Valerio et al., 2012). Similarly, the estimated mean dispersal distance of released OX513A males is 50 - 60 m, although models indicate that the coverage of released males rapidly decreases at distances greater than 33 m from the release point (Winskill et al., 2015). The total distance a mosquito travels from release to recapture is likely to be the net effect of a series of flights in multiple directions (Hawkes, 2009), and in the presence of suitable topography (such as a tower block), such flight can be vertical as well as horizontal (Liew and Curtis, 2003; Chadee, 2004; Lau et al., 2013).

By comparison, flight mill studies aim to measure the equivalent of maximal straight-line flight. Once mounted on a flight mill, a mosquito is compelled to fly by the tarsal reflex

(Rowley et al., 1968; Dudley, 2002), where loss of ground contact with the feet stimulates wing movement. In this way a mosquito is flown to exhaustion (which is unlikely to ever happen in nature), and a measure of a mosquito's maximal flight capacity can be obtained in a controlled and uniform environment. Females of several mosquito species have had their flight quantified on flight mills, including: *Ae. aegypti* (Briegel et al., 2001a); *Ae. albopictus* (Kaufmann et al., 2013a); *Ae. vexans* (Briegel et al., 2001b); *Ae. triseriatus* (Clarke and Rowley 1987); *Ae. sollicitans* and *Ae. taeniorhynchus* (Nayar and Van Handel, 1971); and *An. gambiae* and *An. atroparvus* (Kaufmann and Briegel, 2004). Distances achieved by female *Ae. aegypti* on flight mills are many times greater than those recorded in the field, typically in the range 1-4 km (Rowley and Graham, 1968a, 1968b; Nayar and Sauerman, 1973; Briegel et al., 2001a; Kaufmann et al., 2013b). Only one flight mill study of *Ae. aegypti* males has been performed; it found that on average males will fly around 2 km (Bargielowski et al., 2012).

A study by Briegel et al. (2001a) gives an excellent insight into the relationship between size, nutritional status, and flight capacity of female *Ae. aegypti*. As might be expected, the provision of sugar prior to flight greatly increased the flight potential of females when compared to those provided with water only. However, being of large size was possibly a more important factor in determining flight ability, with starved larger females flying as well as sugar-fed smaller females. Interestingly, the reverse relationship has been found in wild female dispersal, with smaller individuals travelling further (Maciel-de-Freitas et al., 2007). In those females with access to sugar, there was no *overall* correlation between age and flight performance, with strong flight performance possible throughout the experimental lifespan of the females (Briegel et al., 2001a), as has also more recently been found in *Ae. albopictus* (Kaufmann et al., 2013a). However in those same *Ae. aegypti* females, there was a consistent and significant drop in flight performance observed between days 3 and 4 of adult life (Briegel et al., 2001a). Such a trend - if true in males - might have implications for releases of OX513A mosquitoes, which tend to be released at about this age.

As demonstrated in the previous chapter, size and nutritional status were strong determinants of longevity in male OX513A *Ae. aegypti.* Those same factors, as described above, have been shown by others to similarly influence flight capacity in *Ae. aegypti* females. As such, the experiments in this chapter undertook to investigate - for the first time - the influence of body size and nutritional status on the flight capacity of male *Ae.*

aegypti. Any positive relationship between flight capacity and body size and/or improved nutritional status would be taken as an indicator of overall improved male vigour, as well as potentially increased dispersal ability in the field.

That sugar-fed mosquitoes demonstrate flight capability far in excess of starved individuals has been thoroughly demonstrated (Nayar and Sauerman, 1971a, 1971b, 1973; Nayar and Van Handel, 1971; Briegel et al., 2001a, 2001b; Kaufmann and Briegel, 2004; Kaufmann et al., 2013a, 2013b; Boyer et al., 2013). Furthermore, given the clear longevity benefit of sugar feeding demonstrated in the previous chapter, it seems likely that OX513A males will be sugar-fed wherever possible prior to release. Therefore, and in an effort to maximise the quantity of data produced from limited study time and equipment, a treatment comprising starved males was not included in the plans to use a flight mill system to investigate the effects of (i) male size, and (ii) days of sugar provision, on the flight of male OX513A mosquitoes. The flights of 'small' and 'medium' males, fed 10% sucrose solution for either 3 or 4 days, were compared. This was achieved using three measures of flight performance: distance flown (km), time spent in flight (h), and average speed (m/s).

6.2 Materials and Methods

Flight capacity experiments were performed on OX513A males reared at two densities, 3 and 5 larvae ml^{-1}. These are two rearing densities which are likely to be relevant to field production. By feeding all trays equally, the amount of food per larva was lower in the higher-density trays and higher in the low-density trays. This produced males of two different size classes, respectively termed 'small' and 'medium' (General Methods, Section 2.2.2). On each of days 9-13, 100 male pupae were picked and placed into a 15 x 15 x 15 cm cage to eclose.

The flight mills were constructed according to Figure 6-1, and were built specifically for the purposes of this experiment[23] by Jason Lim at Rothamsted Research (Harpenden, UK) where the experiments were performed. Eight flight mills were arranged in an array, which was entirely contained within a sealed tank during flights, keeping temperature and humidity extremely constant (26 °C (±0.3 °C) and 37% RH (±2% RH)). The tank also served to protect the mills from outside disturbance such as researchers moving inside the laboratory, and the turbulence generated by the insectary air conditioning. Individual flight mills were further enclosed in cubicles, preventing males from being disturbed by directional biases or the activity of surrounding males.

Of the daily cohorts of 100 males, those to be flown on the mills were selected according to certain attributes. Each was fully sugar-fed (observed as having an abdomen swollen with sucrose solution), and free from physical damage such as missing limbs or damaged wing tips. Furthermore, males were selected based on their propensity to fly; those most reluctant to fly when their collection tube was agitated were excluded. After either 3 or 4 days of being provided with sucrose solution, males were selected according to these criteria and individually attached to one of the eight flight mills. This was achieved by first anaesthetising each male with cold (90 s at -20°C), after which it was attached to the end of

[23] Much useful advice on the use of mills for the assessment of mosquito flight was provided by Irka Bargielowski and Christian Kaufmann, the latter especially having a great deal of experience from his research at the University of Zurich (Kaufmann and Briegel, 2004; Kaufmann and Brown, 2008; Bargielowski et al., 2012; Kaufmann et al., 2013a, 2013b). However, as the flight mills used to generate the results herein were newly-built, they differ from the equipment used by Kaufmann in a number of ways. Notably, a shorter rotor of novel construction reduces the burden of weight and inertia on the insect, allowing for greater freedom of flight. As such, direct comparisons between the data presented here and previous literature should not be made. At the time of thesis submission, this new flight mill design was subject to a patent application (publication number WO2015022522 A1).

the rotor with a single human hair 1-2 cm long, glued to its scutum with a tiny spot of superglue (Loctite® Super Glue Liquid, Henkel Corp., USA). In preliminary flights the angle of attachment was found to be an important determinant of flight ability, therefore care was taken to ensure all males were attached in a uniform manner (Figure 6-2). Flights were started between 4 pm and 6 pm each evening, and ran for ≈20 hours (Figure 6-3) to give the males ample opportunity to fly to exhaustion. The insectary lights were switched off between 6 pm and 2 am (as they were during the rearing period), resulting in complete darkness during this time. After the flights, all males were individually frozen to allow for later assessment of wing length.

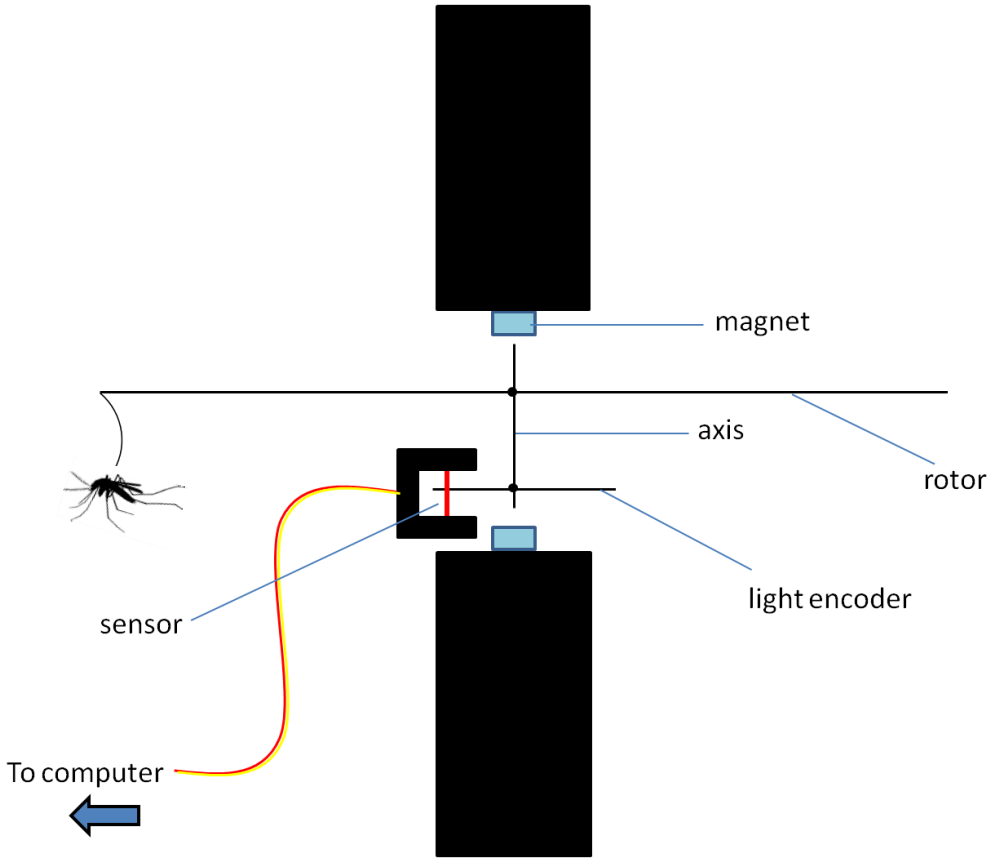


Figure 6-1. Side-view schematic of the mosquito flight mill. Each male mosquito was attached via a single human hair to the end of a rotor, which was affixed perpendicular to an axis. This axis consisted of a metal pin, held vertically by two magnets. The flight of the mosquito caused the rotor to rotate about the axis, describing a circle of circumference 0.25 m. A light encoder (in this instance a flat card circle with light and dark sections) was also attached to the axis. The light encoder rotated at the same rate as the rotor, and the passage of the light and dark sections was 'read' by a sensor. From this information the number of rotations (and hence distance flown by the mosquito) was calculated per unit of time. Distances were recorded by the computer every five seconds.

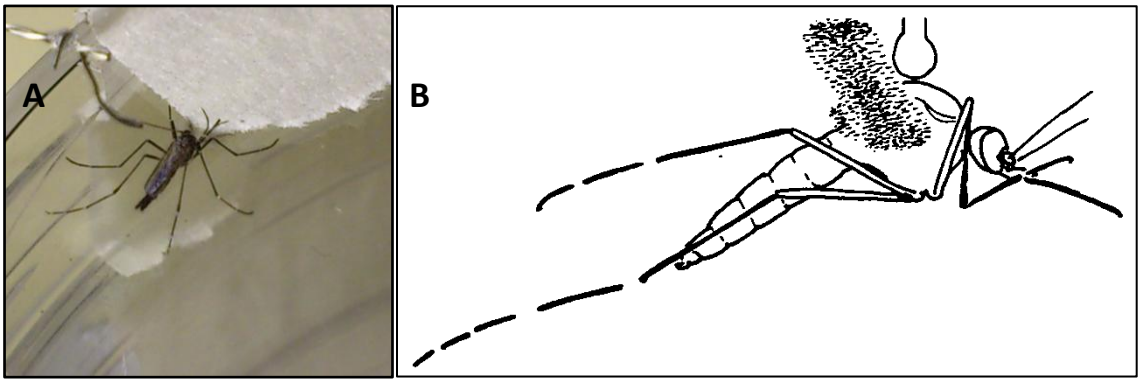


Figure 6-2. Mounting adult male *Ae. aegypti* onto the flight mills. (A) Cold-anaesthetised males were put into a natural resting position on all six legs, before the human hair (with tiny spot of glue) was lowered vertically, such that the point of attachment was on the uppermost point on the scutum. (B) This meant that once the glue had dried and the male had recovered, the body position of the male in flight was as shown. All males were flown in an anticlockwise direction. Panel B modified from Christophers (1960).

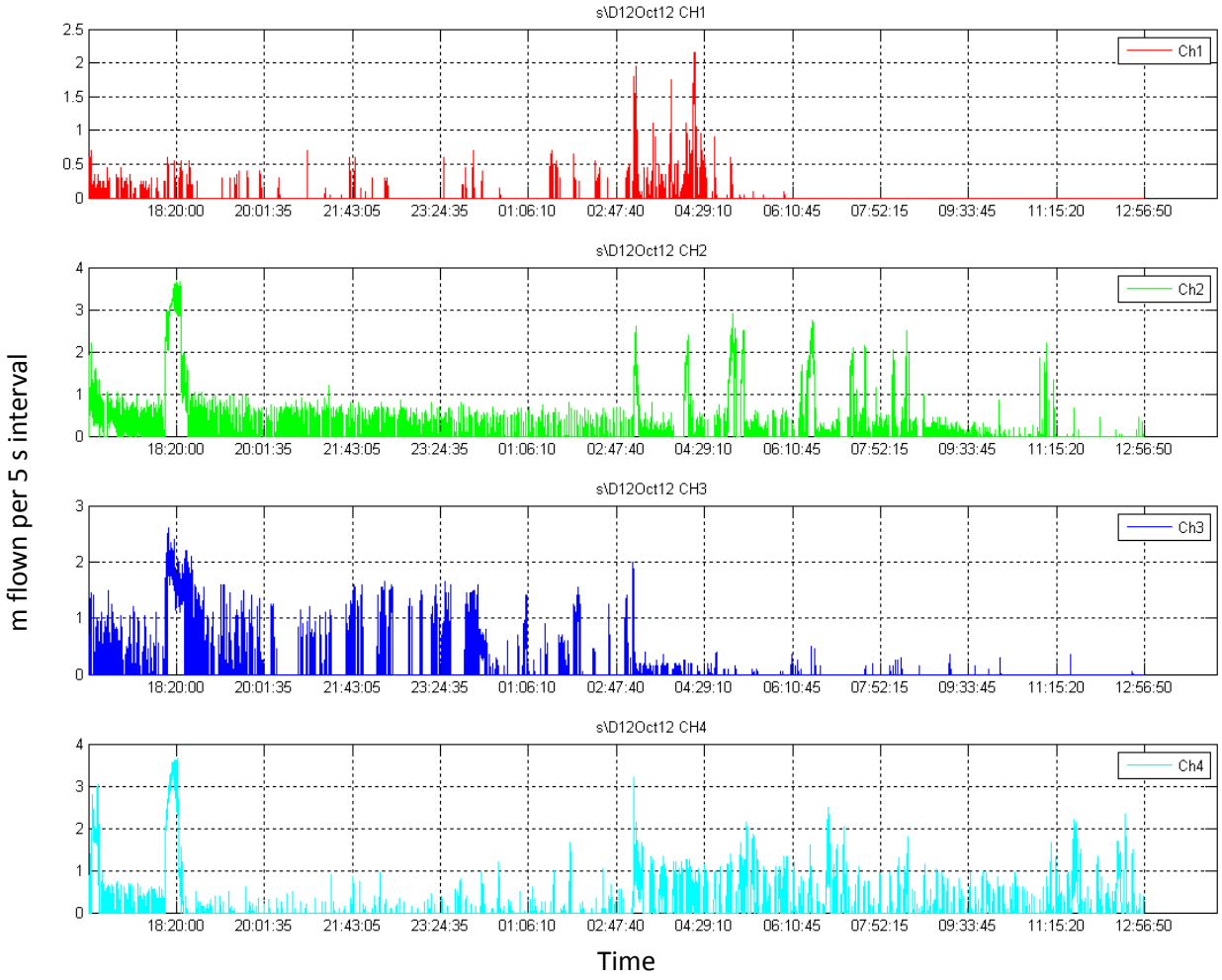


Figure 6-3. Example graphical outputs from the flight mills. Each chart represents the flight of a single male OX513A male over ≈20 hours. The y-axis represents the speed of the insect, measured every five seconds (displayed as metres per recorded five-second interval).

Statistical Analysis

Prior to statistical analysis, values for distance flown and average speed were log transformed, and values for time spent in flight were square-root transformed, in order to have normal distributions. For each of these three measures of flight performance, a maximally complex model was constructed, incorporating the following variables: flight mill number, male size class, days of sugar provision, and wing length (plus interactions thereof). Using backwards stepwise analysis, variables and interactions that did not contribute to the predictive ability of the model were dropped in turn until the model was simplified as far as possible. Then the significance (if any) of the effects of the remaining variable(s) on each measure of flight performance were tested using ANOVA.

6.3 Results

6.3.1 Data Processing

In total, 200 individual males were flown on the flight mills. Very short bursts of flight (<0.2m) were treated as noise and removed from the data. These were equivalent to short 'hops', rather than sustained flight. Although treatments were distributed randomly amongst the eight mills, initial plotting of the data by flight mill number revealed significant between-mill variation in both 'mean distance flown' and 'mean time spent in flight' (Figure 6-4).

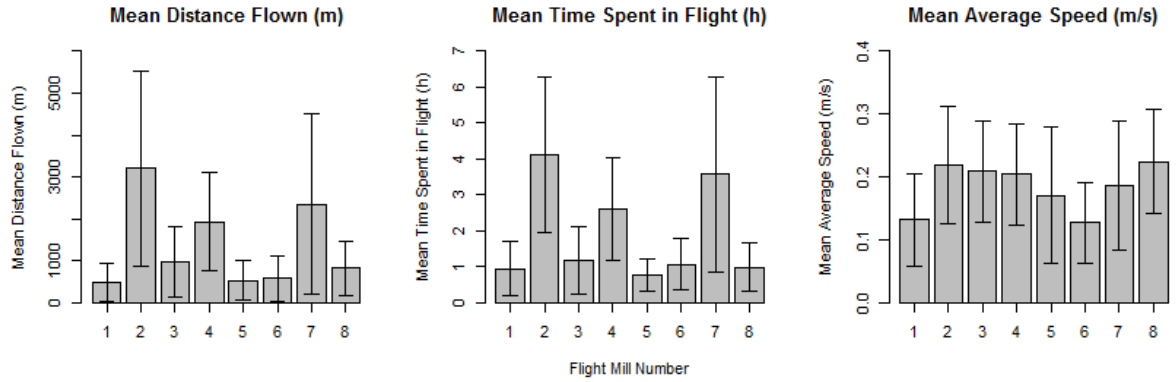


Figure 6-4. Means of distance flown, time spent in flight, and average speed, for all males, plotted by flight mill number. Error bars = sd, n=25. Mean distance flown and mean time spent flying displayed similar patterns of between-mill variation, with mill numbers 2, 4, and 7 (termed 'low-friction' mills) seemingly allowing males to fly farther and for longer duration than the other mills. Mean average speed was not affected by such variation.

It is interesting that distance flown and time spent in flight were affected by differences between mills, but average speed was not. One explanation is that the 'low-friction' mills (mills 2, 4, and 7; i.e. those with higher 'mean distance flown' and 'mean time spent in flight' values) simply allowed flight more freely, so males on those mills were able to fly farther and for longer duration; the converse being true for 'high-friction' mills. Average speeds were approximately the same across both 'types' of mill, as average speed is simply distance flown divided by time spent in flight. An alternative is that males were modulating their flight behaviour dependent on conditions, i.e. those males on 'high-friction' mills expended more energy per unit of time in order to maintain a 'preferred' average speed (of ≈0.2 m/s), which was observed across males on both 'types' of mill. Consequently they exhausted themselves within a shorter amount of time, and so given less time to fly were able to cover less

distance. Both explanations are plausible, and are difficult to disentangle since distance, speed, and time are all dependent on one another. Certainly, and as expected, there was a clear association between time spent in flight and distance flown; the longer a mosquito spent in flight, the farther it travelled (Figure 6-5).

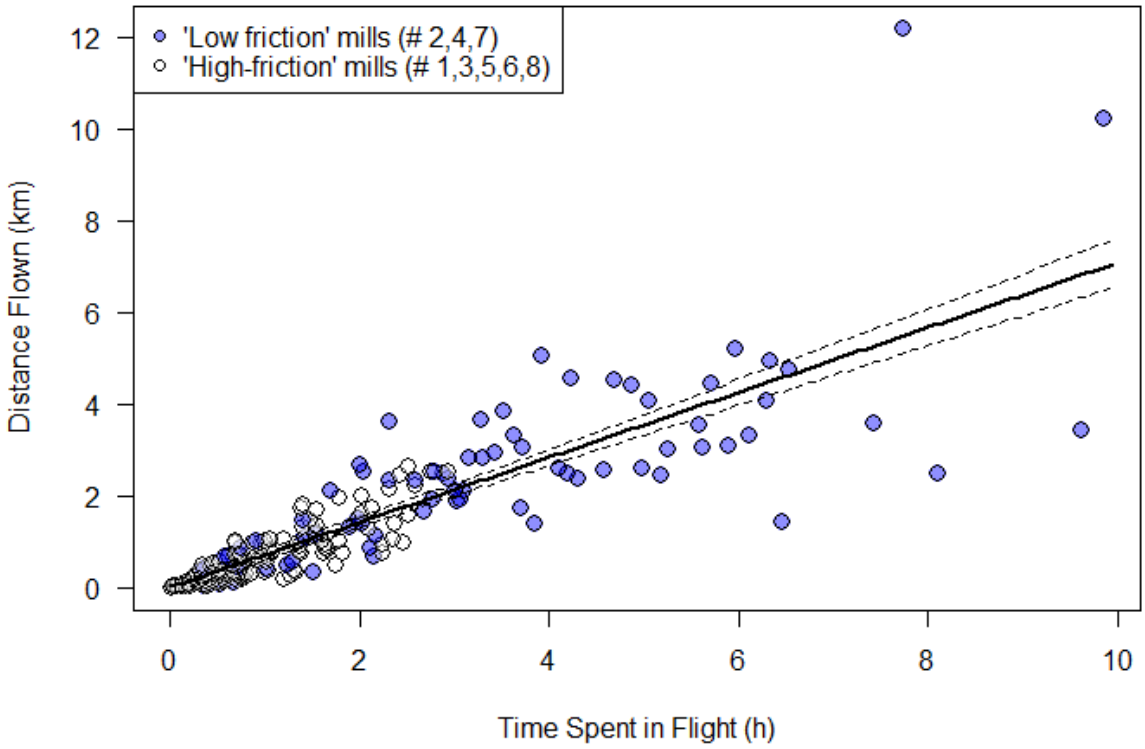


Figure 6-5. Linear relationship between time spent in flight and distance flown. The solid line represents the significant ($F=496.8$, $p<0.0001$) linear relationship between time spent in flight and distance flown; dashed lines are 95% confidence intervals. Males that spent longer in flight flew for greater distances, although only 'low-friction' mills (blue filled circles) permitted flights of longer than ≈3 h or ≈3 km. Both of the extremely far-flying males (the two outliers in the figure) were on 'low-friction' mills.

Treatments were randomly but not evenly distributed amongst the mills, and so without post-hoc intervention the variation between mills would affect treatments disproportionately (conversely, it was also possible that a higher proportion of a certain treatment on one mill could disproportionately affect that mill's 'performance'). Therefore, within each mill individual flights were randomly selected for exclusion, such that each treatment was represented the same number of times on each mill. This reduced the total number of flights from 200 to 136.

To then further account for between-mill variation, the median value of each mill was calculated, for each of the three measures of flight performance (median was used rather than mean due to the presence of two extreme outliers). These medians were plotted in ascending order (Figure 6-6). Then, the mean of these eight mill medians was taken as the performance of an 'average' mill for that particular measure of flight performance. This mean is represented by the red line in each plot of Figure 6-6, and was termed the 'baseline mill performance' (BMP).

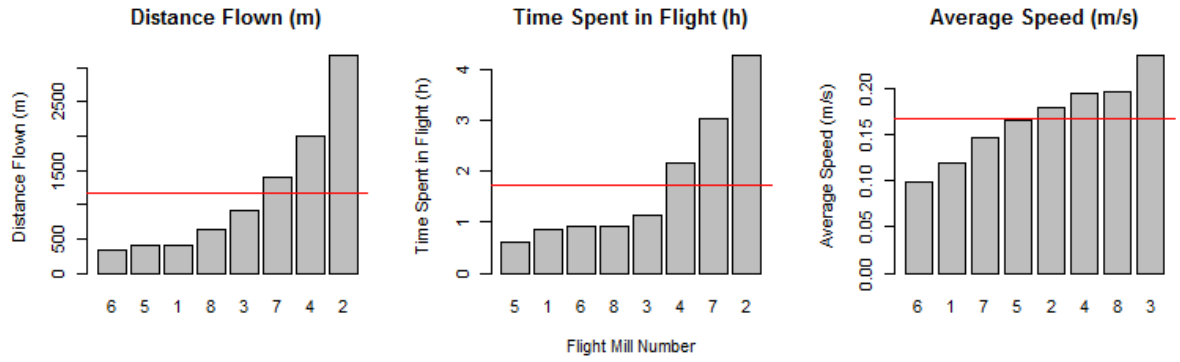


Figure 6-6. Median measures of flight performance achieved by all males, plotted by mill number. Mill medians are plotted in ascending order. Red lines represent the mean of all eight medians, the baseline mill performance (BMP). Note that for distance flown and time spent in flight, the 'low-friction' mills (numbers 2, 4, and 7) are the mills with the highest median values in each case.

Each flight, for each measure of flight performance, was then corrected using the following formula (distance flown used here by example):

$$corrected\ distance\ flown\ = orginal\ distance\ flown\ \times \left(\frac{BMP}{\frac{median\ distance}{flown\ for\ that\ mill}} \right)$$

For example, if a mill tended to produce shorter than average distances flown, then the multiplier was > 1, and so all distances flown were proportionally increased. Conversely a mill that produced farther than average distances flown had a multiplier < 1, and so all distances flown were proportionally decreased. The corrected data are shown in Figure 6-7,

and demonstrate that between-mill variation was significantly reduced by this method. All analyses that followed were performed on the corrected data.

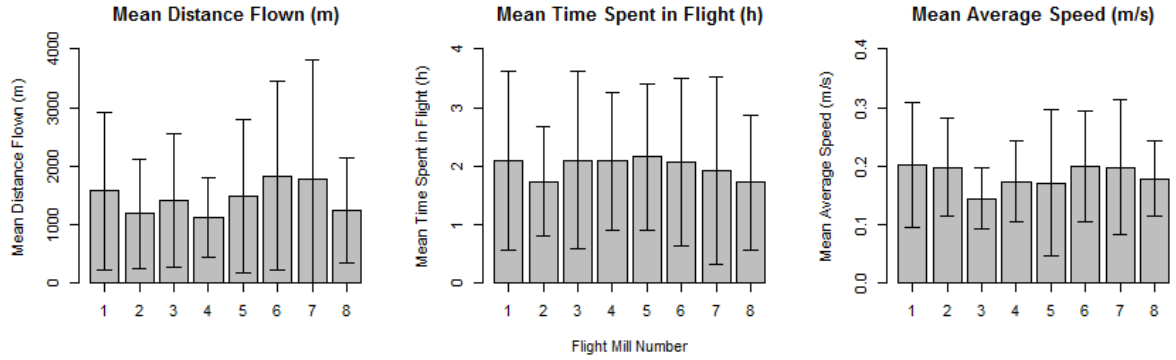


Figure 6-7. Corrected data for mean distance flown, time spent in flight, and average speed for all males, plotted by flight mill number. Error bars = sd, n=12-24. Following correction, between-mill variation was greatly reduced (compare to Figure 6-4, the same data before correction).

Other corrections were considered. For example, it could be argued that all males - regardless of which mill they were flown on - were impeded in their 'true' maximum flight capacity by the (very low) weight and inertia of the flight mill rotor. In which case, the 'least impeded' males would have been those flown on mill 2 (as mill 2 had the highest mean values for distance flown and time spent in flight). Therefore an alternative method could have been to use the mean value for the males flown on mill 2 as the BMP, with the values for males on the other seven mills proportionally multiplied up. However, this method yielded a large number of values that were above the maximum values recorded over the entire study, and therefore potentially above the maximum physiological capability of the insects. This was not the case in the chosen correction method; although the poorer-flying males did have their values increased beyond what was actually observed, this was balanced by the best-flying males having their values reduced. Furthermore, in the chosen method all corrected values remained within the bounds of actually observed values.

Flights shorter than 500 m were most likely caused by incorrect attachment, causing injury or inhibition of the wings, and so following the correction for between-mill variation described above, flights of <500 m in total were excluded from the analysis (as per Kaufmann and Briegel, 2004; Bargielowski et al., 2012; Kaufmann et al., 2013a). Out of 136 flights available for analysis, 31 were excluded (22.8%). After applying the cut-off, the data

were checked to ensure that no male size class, days of sugar provision, or mill number was under- or over-represented in the excluded data[24].

6.3.2 Flight Capacity of OX513A Males

The statistical model showed there was no significant effect of any of the variables (mill number, male size class, days of sugar provision, wing length) on any of the three measures of flight performance (distance flown, time spent in flight, average speed). The mean values of each measure of flight performance are given in Table 6-1.

Table 6-1. Mean values of each measure of flight performance for each of the four treatments. Means (±sd) are calculated from corrected data, from males that flew >500 m (n=105). The full spread of these values can be seen as box plots in Figure 9-2, Appendix. For each treatment the number of flights analysed is given, followed by the total number of flights performed (in brackets).

Measure of Flight Performance	***Treatment 1*** **medium males, 3 days of sugar n=21 (60)**	***Treatment 2*** **medium males, 4 days of sugar n=29 (44)**	***Treatment 3*** **small males, 3 days of sugar n=32 (48)**	***Treatment 4*** **small males, 4 days of sugar n=23 (48)**
Distance flown (km)	1.60 (±1.05)	2.23 (±1.71)	1.77 (±0.98)	1.44 (±0.79)
Time spent in flight (h)	2.36 (±1.34)	2.70 (±1.15)	2.35 (±1.12)	2.10 (±1.13)
Average speed (m/s)	0.19 (±0.07)	0.22 (±0.10)	0.21 (±0.07)	0.20 (±0.08)

All males had their wings measured after being flown; the 'small' and 'medium' size classes were found to not be significantly different in size (one-way ANOVA, F=2.62, p=0.109; see Figure 9-3, Appendix). As such, treatments 1 & 3 and treatments 2 & 4 were combined to test for any effects on the three measures of flight performance of days of provision on its own; no significant effects were found. Therefore, there was no difference in flight performance between males that had sugar fed for 3 days versus those that had fed for 4 days.

[24] The 31 excluded flights comprised:
- 18 flights by medium-sized males, and 13 flights by small males;
- 15 flights by males on 3 days of sugar, and 16 flights by males on 4 days of sugar; and
- Between three and five flights from each of the eight mills.

In keeping with previous studies of mosquito flight using data from both flight mills (Briegel et al., 2001a) and field releases (Winskill et al., 2015), prior to transformation the data for distance flown and time spent in flight were heavily right-skewed (i.e. the majority of values were relatively low; see Figure 9-2, top-left panel, Appendix), and were also comparable to results from the other flight mill study of male *Ae. aegypti* (Bargielowski et al., 2012). Although the range of corrected distances flown was large (510 - 8,445 m), 68% of males flew 2 km or less. However, in this study two individuals in particular displayed exceptional flight ability. 'Individual A' flew 12,178 m and spent 7.7 hours in flight (uncorrected data, post-correction this was reduced to 4,464 m and 3.2 hours). 'Individual B' flew 10,223 m and spent 9.8 hours in flight (uncorrected data, post-correction this was reduced to 8,445m and 5.6 hours).

6.3.3 Effect of Light Intensity

It was noticed throughout the flight experiments that when the lights in the insectary were turned off, flight was often stimulated; males already in flight increased their flight speed, and stationary males began flying. A small experiment was performed to investigate this further. Males attached to the flight mills were exposed to two additional 30-minute periods of dark, prior to the lights switching off for 'night' as usual at 6 pm. In total 16 males were flown, of which seven flew >500 m and so could be included in the analysis[25].

Prior to this experiment it was suspected that it was simply a sudden *change* in light intensity (in either direction) that stimulated the males to fly, perhaps as a startle response. However, from the results above there does seem to be a correlation between darkness and increased flight activity. The transition specifically from light to dark seemed to encourage flight, both increasing the speed of those males which were already flying, and stimulating those stationary males to begin flying. In contrast, the transition from dark to light generally had the opposite effect, although some males maintained their increased flight speed for a short while after the lights were switched back on.

[25] This experiment was concerned with directly measuring flight speed moment-to-moment. Therefore data correction for the effects of between-mill variation was not performed, and nor were flights <0.2 m removed as noise, each as had been the case in the primary experiment.

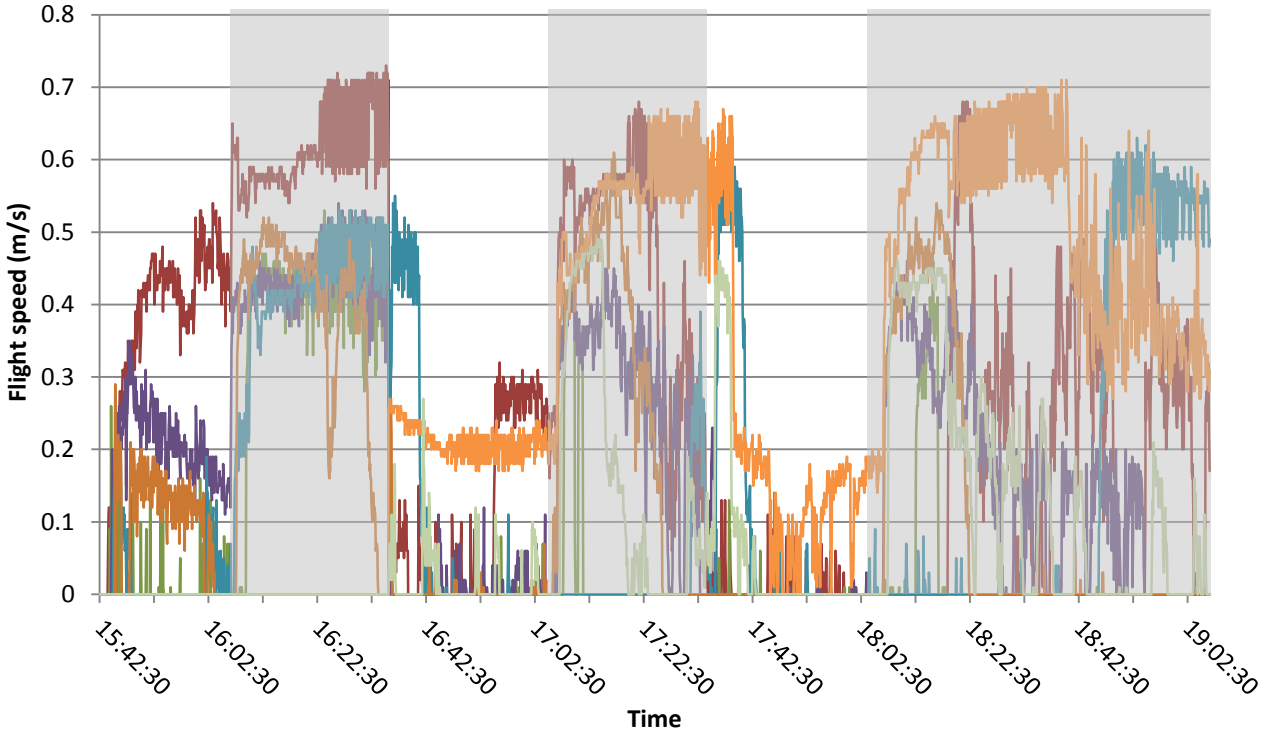


Figure 6-8. Flights of seven individual male mosquitoes subjected to periods of varying light intensity. Periods of darkness are indicated by the shaded areas. The first two dark periods lasted 30 mins each and were additional to the routine 'night' period starting at 6 pm. Only the first few hours of the flights are shown here, (i) to allow better examination of the initial periods subject to variable light, and (ii) because most flight activity occurred early on.

Also of note is that for the vast majority of the first dark period, not a single male was inactive, and flight speeds were consistently high for the entire 30-minute dark period. By the second and third dark periods, although flight was still stimulated by darkness, flight speeds began to decrease before the lights came back on, with several males slowing down to complete inactivity at points. It may be that the depletion of energy reserves resulted in this decreasing ability to maintain the high flight speeds stimulated by the dark conditions.

6.4 Discussion

The flight capacity of male mosquitoes, as investigated by performing tethered flights on flight mills, is a likely indicator of the males' ability and propensity to disperse in the field, a possible indicator of mating success, and a proxy for overall male vigour. However, such is the sex bias in mosquito ecology research (Ferguson et al., 2005, 2010), that to the author's knowledge there are only three published flight mill studies of male mosquitoes, across four species[26]: *Ae. sollicitans* and *Ae. taeniorhynchus* (Nayar and Van Handel, 1971); *Ae. sierrensis* (Yee and Anderson, 1995); and *Ae. aegypti* (Bargielowski et al., 2012). The present study was the first to investigate the effects of body size and nutritional status on flight performance of male *Ae. aegypti*.

Of the few flight mill studies of males performed to date, the most relevant comparator for the results presented here is the study conducted by Bargielowski et al. (2012), which was a comparison of the flight ability of males of two transgenic lines (including OX513A) and a wild-type line. Discounting the exceptional flight performances of Individuals A and B, the maximum and average recorded values of all three flight measures of flight performance were broadly consistent across both studies (I. Bargielowski, personal communication). However, it did appear that the males in the present study generally flew slightly faster but for shorter duration. This difference was likely a result of the reduced size and weight of the flight mills used in this study, which allowed the males to fly faster and so consequently exhaust their reserves more quickly.

There was a significant range of values recorded for 'distance flown' and 'time spent in flight', and so variation around each treatment mean was substantial (on average ±68.6% for distance flown and ±49.3% for time spent in flight[27]). This was exacerbated by the loss of replicates as a result of (i) the <500 m cut-off and (ii) balancing the numbers of treatments flown on each mill, which in combination reduced the total number of analysed flights to 105 from a performed total of 200. Although this represented a substantial loss of 47.5% of the performed replicates (giving a mean number of replicates per treatment of 26), this was

[26] In addition, a study by Briegel et al. (2001b) makes brief mention of flights performed with male *Ae. vexans*, although the primary focus of the study was females.

[27] Standard deviation as a percentage of overall means.

still an improvement on the 63.2% loss (mean 20 replicates per treatment) reported by Bargielowski et al. (2012).

The presence of this sufficient body of data gave confidence to the primary finding that there was no significant effect of days of provision (either 3 days or 4 days) on flight performance. Similarly, one the earliest flight mill studies of (female) *Ae. aegypti* suggested that there is little day-to-day difference in flight ability within the entire first two weeks of adult life (Rowley and Graham, 1968b). However, a more recent study of *Ae. aegypti* found marked differences in the day-to-day flight capacity over the first week of an adult female's life (Briegel et al., 2001a), which is discussed in more detail below. Similar daily changes were also found in female *Ae. vexans* (Briegel et al., 2001b), although the authors of that study go on to note that a similar trend was not observed in the males.

Of the three measures of flight performance, distance flown is of most direct relevance to suppression programmes based on male release. As well as influencing the spacing of release points, knowledge of the flight capacity of wild males (and females) will inform the size of designated barrier zones around the test site (Lee et al., 2013a). Low-level releases into barrier zones are used to minimise the potentially severe detrimental effect of immigration on the efficacy of the programme (Asman et al., 1981), and so need to be large enough to prevent wild individuals from traversing them. For example, a suppression programme using OX513A in Brazil used barrier zones (in which lower-level releases were performed) of 150 m (Carvalho et al., 2015), based on previously reported estimates of male *Ae. aegypti* field dispersal of 100 m or less.

In this study, of particular note were two extremely far-flying males, Individuals A and B. Although they were both flown on 'low-friction' mills (and so their measures of flight performance were 'corrected down' for the purposes of analysis of the entire data set), their actual observed flight performance may still be considered individually. Individual B flew 10.2 km, and spent a total of 9.9 h in flight (the longest total time spent in flight of any male). The majority of its flight total comprised a single continuous flight of 9.5 km / 8.4 h (the longest single flight by duration or distance). Individual A flew even further in total, 12.2 km, and had the highest average (0.44 m/s) and maximum (0.84 m/s) speeds recorded during the study. Both of these males were from the 'medium' size class, and were in fact the largest and third-largest males in the entire study. They had both been given 4 days of

sugar provision, and were flown (on the same day) on 'low-friction' mills. It seems that an alignment of these variables conspired to produce exceptional flight performance. Indeed, the total distances flown by Individuals A and B were more than double the absolute maximum flight distance observed previously in male *Ae. aegypti* (I. Bargielowski, personal communication), and were comparable to the maximum distances achieved by female *Ae. aegypti* (Rowley and Graham, 1968a, 1968b; Briegel et al., 2001a; Kaufmann et al., 2013b), which are generally longer-flying than males due to their inherent larger size and greater reserve stores (Kaufmann et al., 2013b). Although caution should be exercised when extolling the flight performance of just two individuals from an analysed group of 105, they nonetheless represent an unprecedented potential maximum outlay of energy by males of this species.

On measuring the wing lengths of flown males, the 'small' and 'medium' males were found to not be significantly different in size (Figure 9-3). These two size classes has previously been demonstrated to have overlapping but statistically distinct wing length ranges (Figure 2-7) when reared at Oxitec's laboratories (where the rearing protocol was developed). Although every effort was made to replicate rearing procedures at the laboratories of Rothamsted Research (where the flight capacity experiments took place), some equipment was not available. Most notably, the battery-powered pipette controller (used for aliquoting first instar larvae into rearing trays) had to be replaced by a manual equivalent with reduced suction power and consistency. It is suspected that, similar to the manner previously discussed (see Section 2.5.3, page 44, final paragraph), the reduced suction power of the manual pipette resulted in under-stocking of the rearing trays. As such, the wing lengths of both size classes used in the flight capacity experiments (Figure 9-3) were notably larger than those produced when establishing the rearing technique (Figure 2-7). In addition, the reduced consistency and accuracy of the pipette may have blurred the distinction between the two intended larval densities of 3 and 5 larvae ml^{-1}, resulting in non-distinct male size classes. It is suspected that had the male size classes been sufficiently different, this would have manifested as a significant difference in performance between size treatments. Certainly in female mosquitoes, larger individuals have been shown to have increased flight potential, to the point that large females deprived of all sugar access fly similar distances to smaller females that had constant access to sugar (Briegel et al., 2001a). This implies that being of small size is of sufficient disadvantage to flight potential that it cannot be

compensated for by being given a nutritional advantage in the form of sugar post-eclosion. It should also be noted that in that same study, the small and large size classes of female were highly distinct (<2.8 mm and >3.1 mm in wing length respectively); the authors deliberately selected individuals at the very extremes of the size range in order to illustrate hypothesised trends based on body size. In the case of the experiments presented here, the aim was to assess potential performance differences in males reared according to more subtly different regimens, both of which would be relevant and practical in a large scale field suppression programme. Indeed, in a separate linear model of absolute wing length (in the place of size class) and distance flown, there was in this study a marginally non-significant ($F=3.655$, $p=0.0587$) positive correlation between the two variables.

An additional finding of Briegel et al. (2001a) that was deemed to be of particular relevance prior to these experiments was the significant drop in female flight capacity between days 3 and 4 of adult life[28]. The authors of that study found that with constant access to 10% sucrose, flight ability increased over the first 3 days, up to average flights of 6 km and 7 h for 3-day old females. However, at 4 days old this trend reversed, with females flying on average for only 1 km and 3 h (nearly as poorly as 1 day-old females). An explanation for this sudden drop in flight ability between days 3 and 4 was not offered by the authors, but may have been an indication of the failure of some females to fully imbibe or utilise the sugar made available to them. This would only have manifested as poorer flight performance once those females were old enough to have significantly depleted their teneral reserves. If so, this would be analogous to the suggested cause of the 'bump' in the size of males that died on days 4-6 of longevity Experiment 2 in the previous chapter. Alternatively, it has been demonstrated that in female *Ae. aegypti* sugar feeding triggers a temporal (but marked) decrease in time spent in spontaneous flight, inversely proportional to the concentration of imbibed sugar (Jones and Madhukar, 1976). This suppression of flight behaviour is likely to be associated with abdominal inflation, and so would also apply to males. Whatever the reason, a similar drop in male flight potential over that period would present some cause for concern, particularly as OX513A males tend to be released 3 to 4 days post-eclosion (Harris et al., 2012; Neira et al., 2014; Carvalho et al., 2015). This study was designed partly to investigate this issue, and no such difference in flight

[28] In all cases individual females were flown only once; the number of days refers to the age of a previously unflown female.

performance was found between days 3 and 4 of adult male life (see right-most column of Figure 9-2, Appendix).

Following the 'drop' in flight ability of *Ae. aegypti* females in the study described above (Briegel et al., 2001a), from day 5 in that study flight capacity recovered and subsequently further increased to its maximum. When provided with constant access to sugar, this maximal flight ability was then possible throughout the rest of the insects' experimental lifetime (six weeks). This persistence of maximal or near-maximal flight performance several weeks into mosquito lifetimes has also been observed in *Ae. albopictus* (Kaufmann et al., 2013a) and in *An. gambiae* and *An. atroparvus* (Kaufmann and Briegel, 2004). These observations may indicate that after the first few days with access to a sugar source, the females had synthesised the maximum quantity of reserves capable for their body size. Accordingly, flights to exhaustion made at any point after this threshold had been reached yielded the same flight capabilities, regardless of female age. Although such full-lifetime assessments of flight ability were beyond the practical constraints of this study, it would be interesting to investigate whether maximal flight capabilities of males were similarly ever-present after an initial period of reserve synthesis. If so, it would provide a compelling reason to extend (if necessary) the pre-release sugar feeding period to fully encompass this crucial first few days.

In this study all flown males were visibly recently sugar-fed. As such, there was a question over whether sugar feeding would actually impair flight performance. In female *Ae. aegypti*, a sugar meal inhibits subsequent flight behaviour (Jones and Madhukar, 1976), although this may be a by-product of inhibiting blood avidity, which does not apply to males. Furthermore, a recent study has found that female *Ae. aegypti* can disperse at least 60 m once they have taken a sugar meal (Qualls et al., 2016). Another possible implication of a pre-flight sugar meal is that flight ability may be inhibited by the substantial increase in weight and drag that occurs when a mosquito takes on a liquid meal (Christophers, 1960; Foster, 1995; Roitberg, 2003). Carrying extra weight is known to reduce the flight speed of *Ae. albopictus* males (Boyer et al., 2013), although this was in the context of free, untethered flight. The authors of that study suggested that the slower flight speed as a result of sugar feeding would increase the predation risk of an individual - as was found in blood fed females (Roitberg, 2003) - but that in the context of a sterile release this may be balanced by the increased fitness and dispersal ability of a sugar-fed release population. In this study, all

the males were selected for inclusion based on the same criteria: that they were visibly sugar-fed but also displayed a propensity to fly within their cage. Therefore, any physical impediment caused by the recent sugar meal was similar across all replicates.

Finally, the storage of males at high densities prior to field release can result in physical damage occurring, and occasionally the loss of a limb (author's anecdotal observation). In the four instances where males were flown with limbs missing (usually accidentally removed during attachment to the mill), this did not preclude excellent flight performance, with one five-legged male flying 4.5 km (uncorrected value).

Effect of Light Intensity on Flight Activity

The flights of the seven males shown in Figure 6-8 demonstrate a striking effect of light intensity on flight activity, with flight activity being initiated and/or increased by the specific transition from light to dark conditions. This is in direct contrast to the results of a comparable study by Taylor and Jones (1969), who studied the circadian rhythms of free-flying *Ae. aegypti* females by manipulating the photoperiod. Their results demonstrated that over a period of many days female flight activity was strongly associated with periods of light, with periods of 'night' likewise strongly inhibiting flight activity. In particular, they noted that the 'early' onset of a dark period resulted in an according rapid reduction in flight activity; this was also found to be the case by Kawada et al. (2005). In the wild *Ae. aegypti* is similarly associated with daylight activity, with two diel peaks occurring at dawn and dusk (Chadee and Gilles, 2014); this has given rise to the moniker 'day-flying' mosquito. In rural areas flight and biting activity will all but cease after sunset, although artificial lighting in urban areas can override this rhythm and allow biting to continue (Chadee and Martinez, 2000). These activity peaks also exist in laboratory experiments: the greater of the two peaks occurs at 'dusk' and starts before the daylight begins to fade (Chadee et al., 2014), although as in the wild once darkness falls this will inhibit flight activity (Yee and Foster, 1992).

It would seem therefore that the results presented from this experiment - of darkness stimulating rather than inhibiting flight behaviour - run contrary to the previous literature from both laboratory and field studies. However, the previous literature examined activity over periods of many days or even weeks, and often with natural or near-natural periods of intermediate dawn or dusk light. By contrast, the light intensity experiment presented here

was carried out under constant forced flight conditions, with sudden and unnatural changes between full-light and full-dark conditions occurring multiple times over the space of only a few hours. It is suggested therefore that the findings presented here are indications of short-term responses to abrupt external stimuli, rather than being symptomatic of an endogenous rhythm contrary to that already established by other researchers. The exact reason for such a response is unclear. Given the demonstrated aversion of this species to fly in dark conditions, it could be that the sudden onset of complete darkness causes the mosquito to rapidly increase flight speed in order to find a suitable resting site, in which it would prefer to safely spend the night. Under free-flying conditions (either caged or in the wild), rapid flight in a single direction would usually be the best strategy for happening upon a resting site as soon as possible. Of course, under the tethered-flight conditions in this experiment no resting site was reached and so rapid flight continued until 'daylight' suddenly resumed some 30 minutes later.

6.5 Conclusions and Further Work

In terms of distance flown, time spent in flight, and average speed, the results from these experiments were comparable to those of the other flight mill study of male *Ae. aegypti* to date (Bargielowski et al., 2012). This showed that the novel flight mills used were fit for purpose, although the between-mill data correction that was required indicated that some additional machine calibration may have been prudent. There was no detectable effect of male size or sugar-feeding regimen on any of the three measures of flight performance. In the case of the latter, it may well be that an extra day of sugar provision does not provide sufficient nutritional advantage to manifest as greater flight performance, possibly as 3 days is enough time for males to synthesise the maximal amount of reserves possible for their body size. In the case of male size, the high variation inherent in flight mill data leads most researchers to group flown individuals into distinct size classes, which due to rearing issues was not possible in this case. The elimination of these rearing issues, combined with the use of more highly-differentiated sizes - for example, the inclusion of 'large' males reared at 1 larva ml^{-1} along with medium (3 larvae ml^{-1}) and small males (5 larvae ml^{-1}) - would have allowed a more informative investigation to be made into the effects of body size on flight performance. It is thought highly likely that, given the literature on females of other mosquito species, such an investigation would reveal that larger male OX513A have a greater flight capability than smaller males.

Two unusual results were obtained. The first was the extraordinary flight capacity of two males, which flew more than twice the distance of the previous recorded maximum for this species. Both males were part of the treatment group with the expected highest flight capacity, and - possibly more significantly - were the largest and third-largest males in the entire study. This fact lends extra impetus to the need to more fully investigate the role of body size in flight performance of *Ae. aegypti*, and more generally indicates that males are capable of much greater flights than previously thought.

The second unusual result was that the specific transition from light to dark greatly increased flight activity. Although this trend was observed only in the short-term, it runs contrary to the established nature of *Ae. aegypti* being a 'day-flying' mosquito. Therefore, it would be worth investigating whether the sudden onset of darkness also stimulates other

behaviours which in the wild tend to have their peaks in daylight hours, for example mating and (in females) oviposition. If so, the ability to artificially initiate such behaviours would be most applicable to the egg production component of a suppression programme, where light intensity (and its rate of change) is entirely under researcher control. For example, there is anecdotal laboratory evidence that sudden darkness can be used to artificially initiate oviposition when small numbers of eggs are immediately required for experimental purposes (Oxitec internal communication). A scaling-up of this principle in order to synchronise oviposition could be very useful in the context of the large-scale egg production required for a RIDL programme.

The analyses presented in this chapter only used three values from each male's flight: total distance flown, total time spent in flight, and average speed over the entire flight. With the exception of the experiment on light intensity, no analysis was attempted on the patterns of activity within a flight. For example, the number and amplitude of peaks of flight activity, or how many hours it takes mosquitoes to fly the first 80% of their total distance. The improved resolution of the novel flight mills used in these experiments (measurements were recorded automatically every five seconds; previous mills have almost exclusively had measurement intervals of 30 seconds) adds to the potential rewards of interrogating such a dataset. Also not attempted was a formal analysis of the *distribution* of values within each measure of flight performance (data are summarised in Figure 9-2, Appendix); such analysis can be used to reveal important information regarding the position of certain thresholds within the data, for example distances beyond which the majority of males are unlikely to disperse (Winskill et al., 2015).

Flight mills are not used as a means of estimating field dispersal distances. Rather, they enable a more complete measurement of maximum flight capacity. The system used in these experiments represents a step towards using flight capacity as a means of routine or semi-routine quality control (QC) for RIDL mosquitoes. The flight mill system was modular in design, relatively inexpensive, and simple to produce. A system like the one used in these experiments could be provided to a mass production facility within the country of release, enabling QC of male performance to take place in-situ alongside releases. This is important; the geographical and temporal proximity of QC to the actual production and release facilities ensures that QC data is as applicable as possible to release cohorts and conditions. Otherwise, QC data may be compounded by a variety of discrepancies between QC

conditions and field conditions; for example: composition of rearing water, temperature, humidity, and staff skill level, among others. As described in Chapter 3, this principle was evident in the failure of the coffee cup release devices during the Cayman Islands suppression trial in 2010, which had performed well in laboratory conditions up to that point. Additionally, although a flight mill study involves a degree of complexity in terms of equipment and data analysis (when compared with, for example, a longevity study), it has the advantage that the performance of a single male can be assessed within 24 hours, rather than its entire lifetime. This could enable batch-specific examinations of males and so a more timely identification of any potential reduction in male quality.

However, barriers remain to using flight capacity as a method of QC. Subtle differences in insect fitness may simply not manifest as detectable differences in flight ability. For example, in Chapter 5 it was shown that 3 and 4 days of sugar provision result in detectably different survival times; however an equivalent difference in the flight capacity of these two groups was not detected. Perhaps this comparison is unfair, as longevity studies are known to be sensitive to very subtle changes in physiological state (Briegel et al., 2001a), and flight is a more complex behaviour. Nonetheless, with equipment and methods as described in this study it is currently unfeasible to use a flight mill system as a means of routine or semi-routine male mosquito QC. However, this study represents a progressive step towards doing so, and outlines those areas that are in need of optimisation.

7 Mating Performance of Male OX513A

7.1 Introduction

Population suppression through release of RIDL insects depends on a lethal construct being successfully passed on to the offspring of WT females. To this end, the ability of OX513A males to mate with WT females, whilst in competition with WT males, is the single most important aspect of their biology to understand. Previous studies have shown that OX513A males are capable - at least to a certain extent - of achieving WT matings whilst in competition with WT males, whether in the laboratory (Bargielowski, 2010; Harris et al., 2011; Massonnet-Bruneel et al., 2013; Patil et al., 2015), semi-field environment (Lee et al., 2013b), or full-field releases (Harris et al., 2011, 2012; Carvalho et al., 2015; Gorman et al., 2016). This chapter aims to investigate whether the mating ability of OX513A males can be improved by alteration of their size and nutritional status.

The mating challenges faced by field-released OX513A males are significantly more severe than those faced by males in laboratory experiments. The estimated average survival time of a released OX513A male is much shorter than in the laboratory; estimates range from 1.4 days (Carvalho et al., 2015) to 2.3 days (Neira et al., 2014). Therefore, in order to ensure that there is a sizeable 'standing crop' of transgenic males to mate with wild females, the males are generally released several times per week (Harris et al., 2012; Carvalho et al., 2015; Gorman et al., 2016). Females are generally monandrous (Craig, 1967; Spielman et al., 1967), and so all males (released and wild) are in direct competition with one other to obtain a limited number of matings. Therefore, large numbers of males are released to maximise the chances of an available female encountering and being mated by an OX513A male rather than a wild male (e.g. 10 released males per 1 wild female; Alphey et al., 2010; Facchinelli et al., 2013). This 'overflooding' with OX513A males means that the majority of OX513A males will never mate at all. It is therefore vital that the mating performance of the males is maximised, to improve the effectiveness of those few that do encounter mating opportunities. Indeed, in historical releases of mosquitoes with the aim of population suppression, a common reason for failure was the poor competitiveness of the released males (Benedict and Robinson, 2003).

Ae. aegypti is able to mate in aggregations (Hartberg, 1971; Cator et al., 2011) or in single pairs (Roth, 1948), with individual males able to intercept a female to mate (Downes, 1969). However, swarming behaviour increases mating activity, with the number of mating events being positively correlated with the size and duration of the swarm (Cabrera and Jaffe, 2007). Furthermore, males have been demonstrated to release aggregation pheromones which can stimulate the flying activity of females at a distance and will attract other individuals (Cabrera and Jaffe, 2007). Further analysis chemically identified these pheromones (Fawaz et al., 2014) and confirmed that while females do rely on these olfactory cues to locate males, as per Roth (1948), males rely on acoustic signals emitted by the females. However, the males' ability to acoustically identify females is limited to a range of 2 cm (Hoy, 2006); thus, the aggregation pheromones are used to bring together males and females over longer distances, with acoustic signals being used at close range immediately prior to mating. Once they are within acoustic range, a male and female mosquito will each alter their flight-tone frequency such that they harmonise; this is known as 'harmonic convergence' (Cator et al., 2009). Successfully converging pairs are more likely to form a copula; conversely non-convergence is more likely to result in the female rejecting the male (Cator and Harrington, 2011; Cator and Zanti, 2016).

Male size is already known to be an important determinant of mating success in several mosquito species. Larger male *An. freeborni* mate more often than smaller males (Yuval et al., 1993). In *An. gambiae*, males captured whilst mating are significantly larger than males captured while swarming or resting (Sawadogo et al., 2013), and mid- to large-sized males tend to have a higher mating success than smaller ones (Ng'habi et al., 2005, 2008, Maïga et al., 2012, 2014). Although such size-dependent effects were not observed in separate field studies of either *An. gambiae* (Charlwood et al., 2002), or *An. funestus* (Charlwood et al., 2003), the clustering of successful males' sizes around a central value indicate that male size may play a complex and important role in mating competitiveness in *An. gambiae* (Maïga et al., 2014). In *Ae. aegypti*, larger males have been shown to produce and transfer more sperm than smaller males (Ponlawat and Harrington, 2007, 2009), and are able to inseminate more females before sperm depletion affects egg production (Helinski and Harrington, 2011).

Nutritional status, to the extent it can be separated from male size, is also an important determinant of mating success. Field experiments on male *An. freeborni* have demonstrated

that swarming (for the purposes of mating) consumes approximately half of each male's nightly available calories (Yuval et al 1994). Similarly, in *An. gambiae* 50% of available sugar and glycogen reserves were consumed by 25 minutes of swarming activity (although mated and unmated swarming *An. gambiae* males had similar energetic reserves) (Maïga et al., 2014). Clearly then, for species that always mate in swarms, the energetic cost associated with obtaining mating opportunities is high. In non-obligate swarmers such as *Ae. aegypti*, improved nutritional status can contribute to mating success. In aedines, the erection of the fibrillar hairs on the male's antennae is - like the 180° rotation of the terminalia section of the genitalia - an indicator of sexual readiness and a pre-requisite of mating (Oliva et al., 2014a), as these hairs allow the male to locate females by responding to her acoustic flight signals (Roth, 1948). A study by Chadee et al. (2014) found that at 36 hours after emergence, 100% of sugar-fed *Ae. aegypti* males had extended their fibrillae, while less than 15% of the sugar-deprived males had done so. Although the authors of that study did not state when or if the starved males reached the same 100% excitation rate, their results demonstrate a clear initial benefit of sugar feeding to mating potential.

A more direct benefit of sugar-feeding to mating performance was observed in semi-field experiments comparing the mating competitiveness of fertile and sterile *Ae. albopictus* males (Bellini et al., 2014). Sugar was provided to either the fertile or the sterile males at the point of emergence. When the fertile males were provided with sugar the percentage egg hatch from the females was 54%, but when the sugar was provided to the sterile males instead, this dropped to 40%. As such, the provision of a sugar meal early in adult life significantly contributed to an increase in mating competitiveness. The importance of sugar-feeding to mating success within more open or semi-field environments has also been demonstrated in studies of *An. gambiae* (Gary et al., 2009; Stone et al., 2009). In the former study, sugar-deprived males were only able to match the insemination capacities of their sugar-fed brothers when (i) they were of large body size and (ii) the females were in close proximity, and even then only for the first two days of sexual activity. In large cages or semi-natural greenhouse mesocosms (in which the females were harder to find), the mating capacity of sugar-deprived males was always far lower than those provided with sugar.

The experiments in this chapter, as a continuation of the investigatory themes in previous chapters, sought to establish the nature of the relationships between male body size, nutritional status, and mating performance. Male mating performance was measured in two

ways. The first was **mating capacity**, defined as how many females a single male can inseminate over a set period of time. It is less likely that this measure would be of direct field relevance, because the majority of released males are not expected to mate at all. Although, it is quite possible to envisage a scenario where released males encounter aggregations of multiple females at eclosion, feeding, resting, or oviposition sites (Thornhill and Alcock, 1983; Oliva et al., 2014a), in which case having a greater mating capacity would be of benefit. Nonetheless, mating capacity provides a useful direct measure of mating potential. The second measure is **mating competitiveness**, which is concerned with the proportion of female matings a group of OX513A males can achieve when placed in competition with a group of WT males. This measure of mating performance is commonly used in many insect species as a form of quality control prior to field releases, and represents a more accurate recreation of the challenge faced by OX513A males once they are released. Mating capacity and mating performance are separate but related measures of the mating ability of OX513A males, and a deficit in either area would indicate a potential performance cost for field released OX513A males. Both the mating capacity (Bargielowski et al., 2011a; Massonnet-Bruneel et al., 2013) and mating competitiveness (Bargielowski, 2010; Harris et al., 2011; Lee et al., 2013b; Massonnet-Bruneel et al., 2013; Patil et al., 2015) of OX513A males in the laboratory have been assessed previously, and so the results will be discussed in the context of this prior baseline data. However, the present study is the first to investigate the effects of body size and nutritional status on the mating performance of male OX513A *Ae. aegypti*.

7.2 Mating Capacity

7.2.1 Introduction

Dipteran semen is primarily composed of (i) spermatozoa, produced by spermatocysts in the testes, and (ii) secretions from the accessory glands, notably seminal fluid proteins (SFPs) (Jones and Wheeler, 1965). After mating, spermatozoa are stored by the female in organs called spermathecae (Figure 7-1), with a single mating event able to provide her with enough spermatozoa to fertilise her lifetime's output of eggs (Jones, 1968; Rogers et al., 2008; Pascini et al., 2012). In *Drosophila melanogaster* SFPs induce a wide range of effects in the female (Wolfner, 2007), and in female *Ae. aegypti* one such effect is refractoriness to further mating (Craig, 1967; Fuchs et al., 1969). Furthermore, the presence of SFPs has been shown to be essential to fertilisation itself; *Ae. aegypti* females inseminated by males with no accessory glands laid only infertile eggs, even though spermatozoa had still been transferred (Adlakha and Pillai, 1975).

A female's spermathecae can be dissected and visually examined for the presence or absence of spermatozoa. There are both daily and lifetime biological limits to how many females a male can inseminate, dictated by the quantity of spermatozoa and SFPs he can produce. Males can only hold a certain volume of each, and so semen is depleted during periods of sexual activity, and replenished during periods of inactivity (Foster and Lea, 1975). Large males have been shown to produce (Ponlawat and Harrington, 2007) and transfer (Ponlawat and Harrington, 2009) more spermatozoa than smaller males. Interestingly, a recently field-collected strain had more spermatozoa per unit body size than a historical laboratory strain (Ponlawat and Harrington, 2007), and wild males were found to transfer more sperm than laboratory males of similar size (Ponlawat and Harrington, 2009). This latter observation may be a result of shorter copulation duration in dense laboratory cages where competition and physical disturbance is high; alternatively the high frequency of female encounters in laboratory cages may select for those males that spread their finite reproductive potential amongst a greater number of mates.

In the experiments in this chapter, mating capacity was measured as the number of individual females a single male could transfer spermatozoa to, over a given period of time. This system of measurement has been previously used to estimate the mating capacity of

Ae. aegypti males under a variety of experimental protocols, although none investigating the role of body size and nutritional status. For example, under quite intensive mating conditions (single males being presented with a series of females in rapid succession), male *Ae. aegypti* mated an average of around 6 females before becoming depleted of spermatozoa and accessory gland secretions (Jones, 1967; Dapples et al., 1974; Foster and Lea, 1975). When presented with further series of females (interspersed with periods of replenishment), males that survived to the fourth series mated an average of 17.6 and a maximum of 21 females (Foster and Lea, 1975). Under less intensive mating conditions (daily periodic mating access) OX513A males mated an average of 6.6 females over their lifetime, less than the average of 11.5 females inseminated by WT males under the same conditions (Bargielowski et al., 2011a). Similar estimates were obtained by experiments in which males had constant access to females for either one or several continuous days, in each case 5-6 females per day were mated by both OX513A and WT strains (Massonnet-Bruneel et al., 2013). Other insemination estimates include three females per day (Gwadz and Craig, 1970), or 8-9 over the course of a lifetime (Youngson et al., 1981).

Two experiments were conducted to investigate the mating capacity of male OX513A *Ae. aegypti*: one in which males had limited access to females, and one where access to females was continuous. As such each experiment had a different emphasis, as described below; a similar two-pronged approach was adopted in tests of *Ae. aegypti* insemination rates performed by Chadee et al. (2014).

Experiment A - Males with Limited Access to Females ('Periodic Mating')

Individual males were presented with a small number of females for a limited time each day. This experiment investigated the daily mating abilities of OX513A males, with periods of rest in between daily mating opportunities. The mating arena was large enough that there was effort required on the part of the male to seek out the females within the environment. This, when combined with the restricted mating window, provided a test of the OX513A males' courtship abilities as well as their absolute capacity to inseminate multiple females. In order to successfully mate, males were required to actively court females to obtain matings, and to do so relatively quickly. This experiment was designed to be a more rigorous, all-round assessment of mating capacity, more akin to the challenging mating conditions that the males may encounter in the field.

Experiment B - Males with Continuous Access to Females ('Continuous Mating')

Individual males were presented with a larger number of females than in Experiment A, with which he continuously cohabited for several consecutive days. As such, this experiment investigated the 'lifetime' mating capacity of OX513A males, with continuous mating access and no enforced periods of rest. The mating arena was one-eighth the volume of that used in Experiment A, and so less effort was required on the part of the male to seek out the females within the environment. The smaller mating arena combined with unrestricted mating opportunities therefore was less of a test of the OX513A males' courtship abilities, and more a test of their maximal insemination capacity when access to females was relatively easy. This experiment was not designed to imitate field challenges at all; instead it was designed to completely deplete males of their sperm.

As in previous chapters, the effects of each of (i) male size and (ii) sugar concentration were investigated on the mating capacity of OX513A males, within each of the two mating scenarios contemplated by Experiments A and B. For the male size variable, 'large' males (the product of rearing at 1 larva ml^{-1}) were tested, as well as the 'small' and 'medium' males (products of rearing at 5 and 3 larvae ml^{-1}, respectively) used in previous chapters. The introduction of an additional size class was to increase the likelihood of detecting an effect of male size on mating capacity, should one exist. For the variable sugar concentration, the same treatments as had been used in previous chapters (0%, 1%, and 10% sucrose solution) were used in the mating capacity experiments.

7.2.2 Materials and Methods

For each of Experiments A and B, OX513A males were only permitted to mate on four consecutive days of adult life as this is a generous estimate of their life expectancy post-release, which is approximately two days (Lacroix et al., 2012; Neira et al., 2014; Carvalho et al., 2015; Winskill et al., 2015). Experiments A and B were run side-by-side, using a common pool of OX513A males and WT females. OX513A males were reared at three densities: 1, 3, and 5 larvae ml^{-1}. By feeding all trays equally (see Section 2.2.2), larval food was in varying supply, which produced males of differing size, respectively termed 'large', 'medium', and 'small' (males of each size class, and the WT females, were all significantly different in size from one another; see Figure 9-4, Appendix). Each of these size classes were sustained on three different sucrose concentrations: 0% (water), 1%, and 10%. The three levels within each factor resulted in a total of nine treatments (Table 7-1). WT females to be mated were always reared in trays at 3 larvae ml^{-1}. OX513A insects were reared on tetracycline, while WT insects were reared off tetracycline.

Table 7-1. Male treatments used in the mating capacity experiments.

Treatment	OX513A Male Size	Sugar %
1	small	0
2	small	1
3	small	10
4	medium	0
5	medium	1
6	medium	10
7	large	0
8	large	1
9	large	10

Experiment A - Periodic Mating

Single male pupae were allowed to eclose individually into large mating arenas (30 x 30 x 30 cm cages), with constant access to sugar of the relevant concentration. Adult males were held in isolation for two days to provide sufficient time to reach sexual maturity. On each of

the following four days (days 3-6), five virgin WT adult females were introduced into each male's cage, where they were allowed to be mated for 90 minutes, before being removed and their spermathecae dissected to check for the presence of spermatozoa (Figure 7-1). Introduced adult females were always four days old, as this is the age at which virgin *Ae. aegypti* are most likely to be inseminated (Williams and Berger, 1980). Introduced females were always from the same rearing tray, but were picked on four consecutive days to ensure all females were the same age on introduction to the males. Throughout, the male had constant access to sucrose solution of the relevant concentration.

Experiment B - Continuous Mating

WT females were eclosed in groups of ten into small mating arenas (15 x 15 x 15 cm cages), with constant access to 10% sucrose solution. Single males were allowed to eclose individually into small tubs and had constant access to sucrose solution of the relevant concentration. Once the adult males were two days old and the adult females were four days old, the individual males were introduced into the mating arenas (containing the ten virgin females), and were then left undisturbed for four days. At the same time, the 10% sucrose in the mating arena was replaced with one of relevant concentration to the male. After four days had elapsed, the females were removed and their spermathecae dissected to check for the presence of spermatozoa (Figure 7-1).

A limiting factor in designing Experiments A and B was the number of spermathecae dissections that could be performed per day. A preliminary experiment found that a single OX513A male did not mate more than five females per day, and not more than ten over a four-day period. Therefore, these numbers of females were used in Experiments A and B respectively (as described above). This allowed the males to mate to their maximum capacity, without requiring an unnecessarily large number of dissections. Three repeats of each experiment were conducted, one after the other (i.e. rearing, mating, and dissection for repeat 1 of each of Experiments A and B were started and finished, then repeat 2, then repeat 3). Finally, all males (n=27 for each experiment) had their wing lengths measured, as well as a sample of females (n=86, drawn from both experiments).

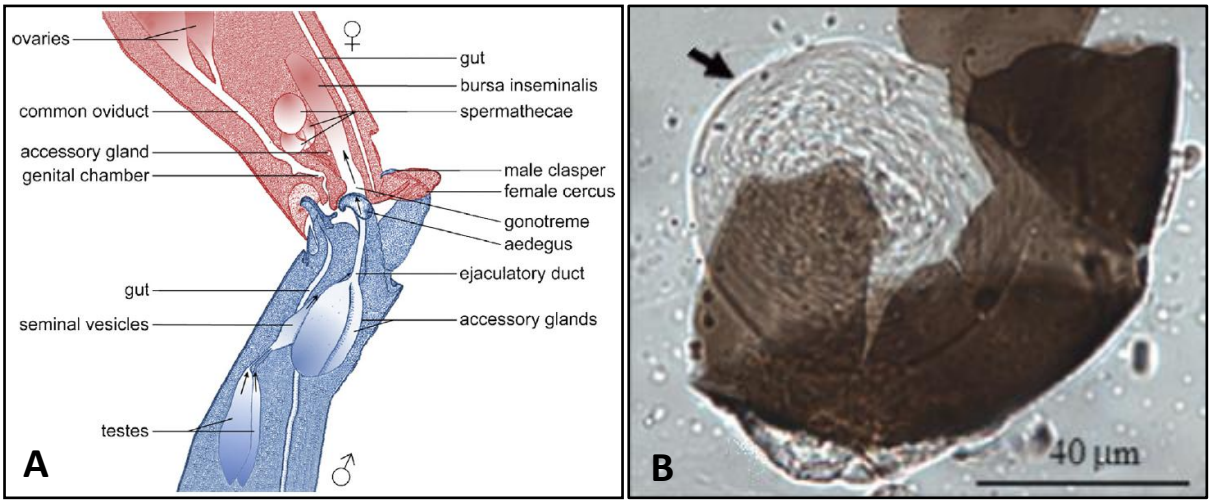


Figure 7-1. Path and destination of spermatozoa in aedines. (A) Reproductive systems of female (red) and male (blue) aedines. Spermatozoa start in the male testes and during mating take the path indicated by the arrows, before finally being stored by the female in the spermathecae. Female aedines have three spermathecae, one large and two small. Figure modified from Oliva et al. (2014a). (B) Dissection of a single spermatheca from a mated female *Ae. aegypti*, which has been broken open to reveal a bundle of spermatozoa inside (arrow). Figure modified from Pascini et al. (2012).

Statistical Analysis[29]

In Experiment A (periodic mating), the range of possible daily mating performance was quite small (between zero and five females mated). Although this is limited by the biological capabilities of the insect, it nonetheless represents rather poor experimental resolution. As such, it was not appropriate to try and discern statistical differences between treatments from day-to-day of the experiment (although the data are still presented in Figure 7-2). Instead, statistical analyses were performed on the total number of females mated at the end of the four-day duration of the experiment. This also allows a more like-for-like comparison with Experiment B (continuous mating), where only total - and not daily - data were collected.

[29] On occasion, it was impossible to determine the mating status of an individual female (usually in the case of a failed dissection). This represented a significant loss of information, as the total number of females available to be mated was only five (Experiment A, daily) or ten (Experiment B). The most statistically robust approach would usually be to simply discard any repeat where information was lost. However, given that practical constraints (number of possible dissections per day) limited the number of repeats to just three, this was not an option. Instead, the proportion of females known to be mated was applied to the total number of available females. For example, if five females were introduced to a male, three were positive for sperm, one negative, and one unknown, then that was treated as three positives out of four (not out of five). Three out of four = 75%, and this proportion was then applied to the original number of females: 75% of five females = 3.75 females mated out of five.

Experiments where the data had approximately normal distributions and equal variances were analysed using ANOVA (one-, two-, or three-way), with Tukey HSD pairwise comparison post-hoc tests. When examining daily matings in Experiment A, distributions were heavily right-skewed as males rarely mated more than one female per daily 90-minute mating window. In this case transformation proved ineffective, and so a Kruskal-Wallis test was used to compare the number of females mated on each day of the experiment (across all the treatments), followed by a pairwise comparison test[30] to identify differences between pairs of treatments (Siegel and Castellan, 1988).

[30] R function `kruskalmc` within package *pgirmess*.

7.2.3 Results and Discussion

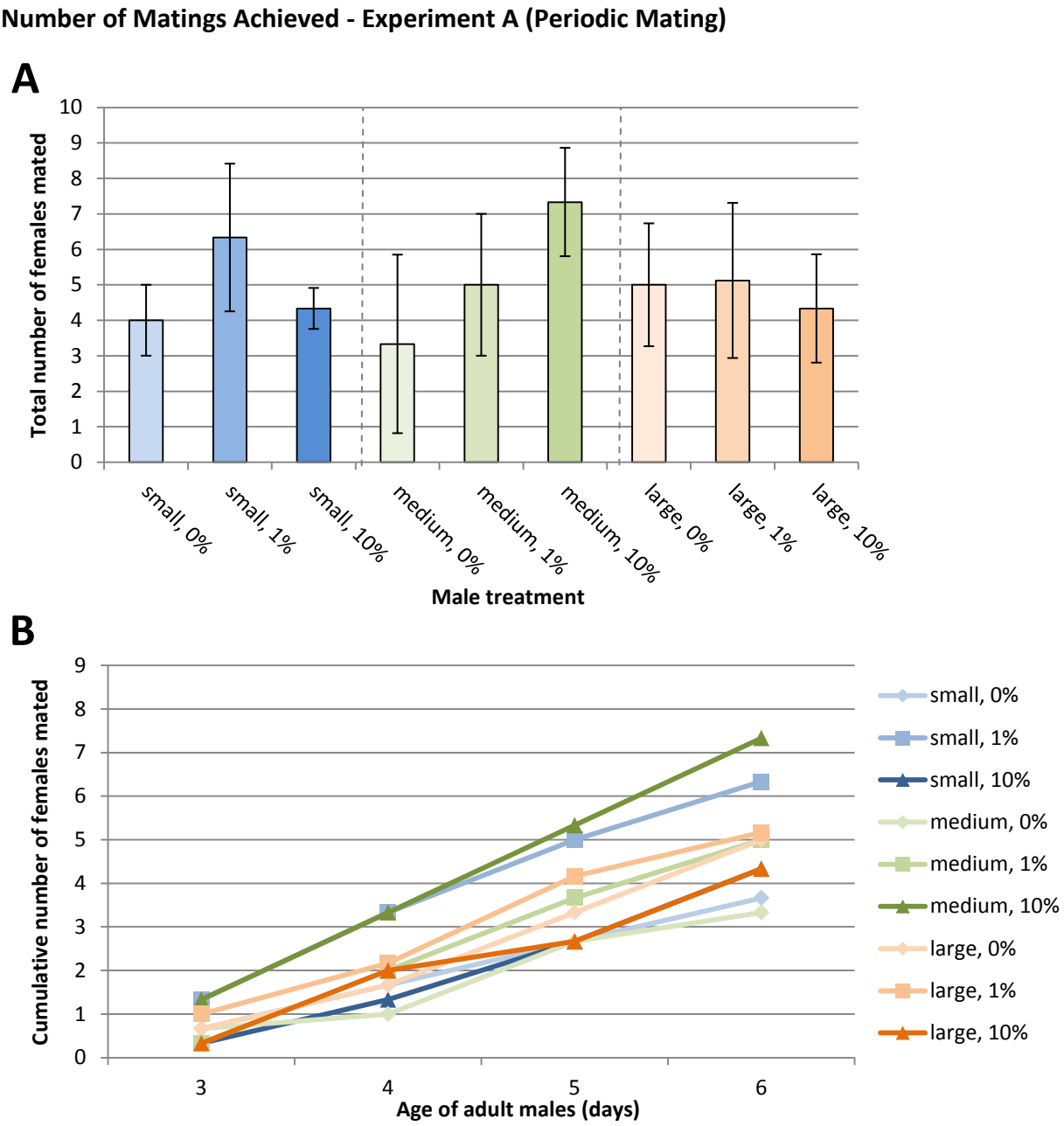


Figure 7-2. Experiment A - Periodic Mating. (A) Total, and (B) cumulative numbers of females mated under limited mating access conditions. Treatments are plotted individually. Each male had access to five virgin WT females for 90 mins on each of four consecutive days. Data plotted are means (n=3). In (A) error bars = sd, in (B) error bars are omitted for clarity.

Under periodic mating conditions, neither male size ($F=0.132$, $p=0.877$) nor sucrose concentration ($F=1.616$, $p=0.226$) had a significant effect on the total number of females mated by each male, nor were there any interaction effects (two-way ANOVA with interactions).

Number of Matings Achieved - Experiment B (Continuous Mating)

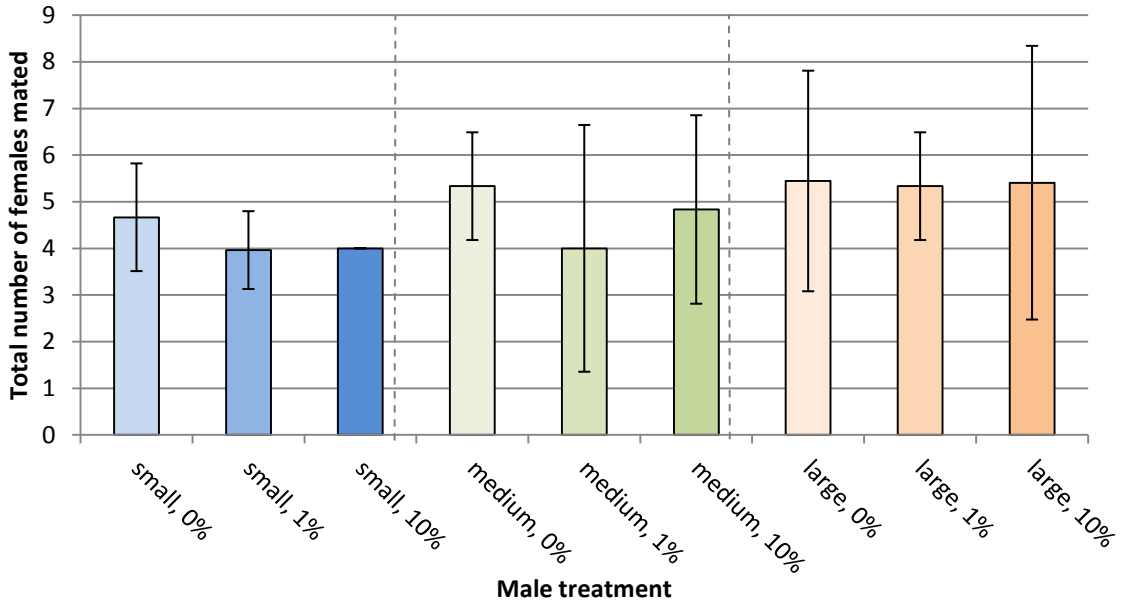


Figure 7-3. Experiment B - Continuous Mating. Total number of females mated under continuous mating conditions. Treatments are plotted individually. Each male had constant access to ten virgin WT females for four consecutive days. Data plotted are means (n=3), error bars = sd. N.B. treatment 'small, 10%' has no error bar as all three repeats gave identical results.

Under continuous mating conditions, neither male size ($F=0.838$, $p=0.467$) nor sucrose concentration ($F=0.306$, $p=0.745$) had a significant effect on the total number of females mated by each male, nor were there any interaction effects (two-way ANOVA with interactions).

In both Experiments A and B, it had been expected that a difference in mating capacity between the most 'advantaged' (large, 10%) and least 'advantaged' (small, 0%) males would be observed. It was anticipated that such an outcome would have been the result of the inherent fitness costs of being smaller and nutritionally deprived (as seen in the longevity experiments, Chapter 5), combined with possible differences in their maximum initial mating capacity and their ability to replenish spermatozoa. Spermatozoa depletion occurs after around 5-6 females have been mated (Jones, 1973; Foster and Lea, 1975). However, in Experiment A, five out of the nine treatments had not mated a total of five females until the penultimate or last day of the experiment; while the other four treatments never reached that total at all (Figure 7-2). Similar mating totals were seen in Experiment B (Figure 7-3). This meant that spermatozoa depletion, if it occurred at all, only occurred towards the end of the four-day experiment. As replenishment of spermatozoa potentially takes several days

(Jones, 1967; Dapples et al., 1974; Foster and Lea, 1975), there was insufficient time left in the experiment for this to occur. With depletion and replenishment therefore not contributing factors, all males were able to mate approximately the same number of females. Having a sample size of only three also likely further masked any differences.

A study published after these experiments had been performed (Chadee et al., 2014) demonstrated - conversely to the results presented here - that sugar-fed *Ae. aegypti* males had a clear advantage over starved males in terms of the numbers of females they were able to inseminate within the early part of their adult life. Interestingly, however, this advantage was highly dependent on the size of the mating arena. In 30 x 30 x 30 cm (27-litre) cages (the same size as used in Experiment A), both sugar-fed and starved males showed very similar insemination rates of approximately 70-75% of all females by day 3. However by day 4, while sugar-fed males continued to inseminate females, most of the starved males had died, and those that were still alive showed no extension of antennal fibrillae and had not inseminated any females. In larger (60 x 60 x 60 cm / 216-litre) cages, this divergence in insemination rate occurred almost immediately; sugar-fed males inseminated at the same rate as they had been observed to do in the smaller cages, but starved males were never able to inseminate more than 5% of the females. Although Chadee et al. (2014) did not comment on this stark difference between the results observed in the two sizes of cage, it seems apparent that the smaller cages permitted both sugar-fed and starved males to seek and inseminate females (until mortality limited starved male performance from day 4). However the larger cages - being eight times the volume of the smaller cages - presented a search area of such size that starved males simply did not have the energetic capital required to seek out and mate with females. Making mating attempts (Bargielowski et al., 2011a) and swarming (Yuval et al., 1994; Maïga et al., 2014) are both known to be very substantial drains on energetic resources. The sugar-fed males, being adequately equipped with these resources, enjoyed insemination success in the larger cages to the same degree as they had in the smaller cages, ultimately inseminating 91% of females. Similar results have also been reported in *An. gambiae* (Gary et al., 2009): sugar-deprived males were able to match the insemination capacities of their sugar-fed brothers in small (38-litre) cages, but in large (80-litre) cages or very large (21,443 litres) semi-natural greenhouse mesocosms, the mating capacity of sugar-deprived males was always much lower than those provided with sugar.

The contrasting fortunes of starved males in small and large mating arenas highlights the close interaction between nutritional status and dispersal ability - as explored in the previous chapter - with mating success. Furthermore, it may indicate that the sizes of mating arenas used in Experiments A and B (30 x 30 x 30 cm and 15 x 15 x 15 cm, respectively) were likely insufficient to allow a nutritional status-dependent difference in mating capacity to become apparent, even at the most basic starved (0%) vs. sugar-fed (10%) level.

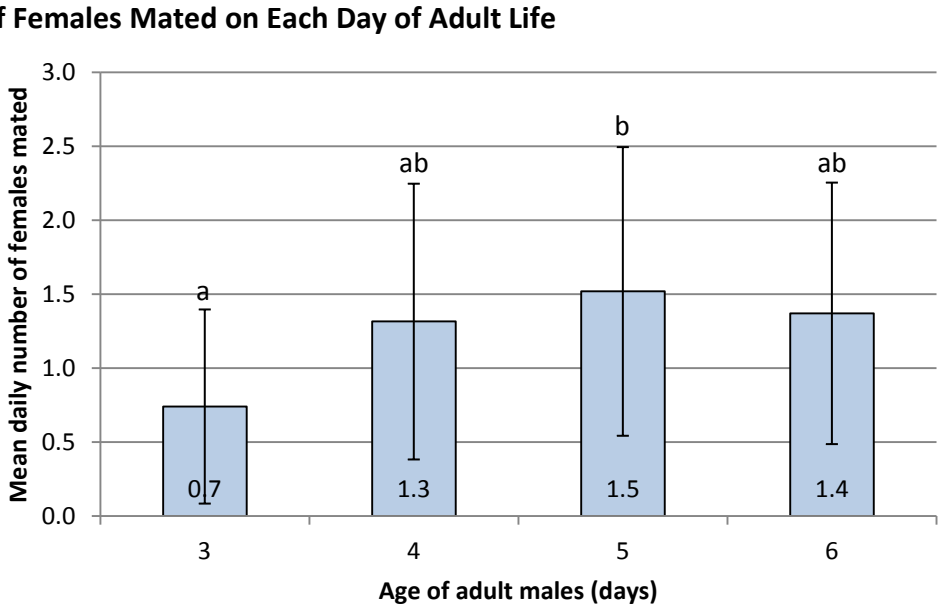


Figure 7-4. Mean number of female matings achieved versus age of adult males. Data are from Experiment A (periodic mating), and are means of males of all treatments and from all repeats (n=27). Error bars = sd. Letters indicate statistically different groups. N.B. Although statistical differences were calculated using non-parametric (ranked data) methods, mean values are presented for the sake of clarity.

From the Experiment A data, it was possible to calculate the average number of females mated in the 90-minute mating windows on each of days 3-6 of male adult life, across all male treatments (Figure 7-4). Performance differences over the first few days of life are of potential importance because they could inform the optimum day on which to release males. Males were found to mate significantly fewer females on day 3 than on day 5 (Kruskal-Wallis test, χ^2 =11.93, p=0.0076). Adult males cannot generally mate (although they may attempt to) before the terminalia part of their genitalia has rotated 180°, which in *Ae. aegypti* takes 15-24 hours post-eclosion (Roth, 1948; Klowden, 1990). Accordingly, no

swarming or copulation is observed in males less than 24 hours old (Cabrera and Jaffe, 2007). Foster and Lea (1975) stated that quantities of spermatozoa and accessory gland products reach their maximum at two days old; however, a later study showed that the maximum may not be reached until day 10 or even later (Ponlawat and Harrington, 2007). The same study indicated that quantities increase most sharply between days 1 and 5; a trend which if applied to this study implies that on day 5 male *Ae. aegypti* have significantly more reproductive material at their disposal than on day 3. This may therefore be a contributing factor to the higher overall number of matings on day 5 than on day 3. This finding may have relevance to the timing of field releases, as the average mating capacity of the release population could potentially be improved by keeping the males until they are 5 days old before releasing them.

Comparison Between Periodic and Continuous Mating

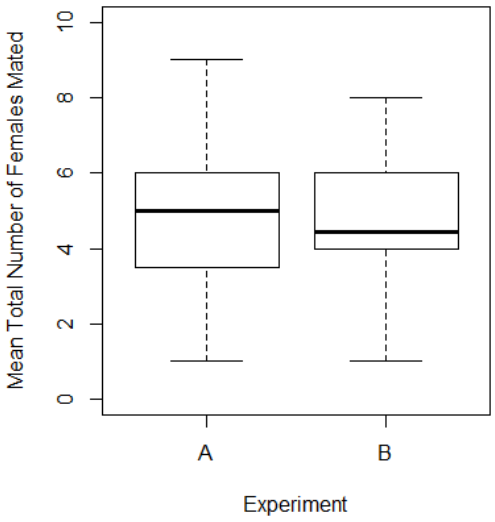


Figure 7-5. Total number of female matings achieved by all males from Experiment A (periodic mating) and Experiment B (continuous mating), n = 27.

Males with limited access to females inseminated on average mated 5.0 (±1.9) females, and males with continuous access to females mated on average 4.8 (±1.6) females. There was no significant difference between these totals (one-way ANOVA, F=0.175, p=0.677). These results were highly consistent with previous studies, both in terms of the absolute number of females inseminated, and the lack of effect on insemination rate of periodic versus continuous mating. For example, in a comparison of OX513A and WT strains, males of both strains mated approximately five females, whether they had access to the females for a single day or several days (Massonnet-Bruneel et al., 2013). Under conditions similar to

Experiment A males mated on average 6.6 females (Bargielowski et al., 2011a), slightly higher than observed here. However, this average was over a male's entire lifetime, whereas Experiment A was over only four consecutive days.

On initial examination, the total numbers of inseminations achieved by OX513A males in this and the previous mating capacity studies (Bargielowski et al., 2011a; Massonnet-Bruneel et al., 2013) appear to be indications of lower mating capacity than that previously reported, where males mated with 18 females over the course of their lifetime (Foster and Lea, 1975). However, these two sets of results are actually quite similar. Firstly, as discussed above it seems likely that by the end of Experiments A and B the males were either depleted or near-depleted. The average total number of inseminations prior to first depletion was therefore around 5, not dissimilar to the 5.8 reported by Foster and Lea (1975). The higher lifetime totals (average 18, maximum 21) in that study were achieved subsequently, after several rounds of depletion and replenishment, and are averages achieved by the minority (8%) of males which maintained normal mating function through to the fourth and final series of matings.

In Experiment A, it was repeatedly observed that only females in flight were able to be mated (as per Spielman, 1964; Hartberg, 1971; Moore, 1979; Cator et al., 2011), and that males were unable to coerce resting females into flight in order to mate with them (both behaviours were also observed in additional small tests with WT males). OX513A males did exhibit the characteristic figure-of-eight 'patrolling' of the airspace in the mating arena, as is observed during mating in the wild (Hartberg, 1971). However, apart from during the introduction and removal of the females - when the mating arena was disturbed - females were effectively unavailable to be mated. This issue was also mentioned by Foster and Lea (1975), who agitated their mating arenas to induce flight and subsequent mating. However, they also stated that mating performance was best when the male and female needed little or no inducement to fly. Therefore, the experimental approach taken in the present study was to introduce the females to the mating arenas, after which they were left undisturbed until the time came for the females to be removed. This minimal-intervention method had the advantage of being straightforward to standardise across all replicates. Furthermore, it provided an element of additional mating challenge for the males by testing their ability to initially coerce females to mate. In hindsight this was an unnecessary additional challenge, and clouded the primary question of insemination capacity. Furthermore, females in the

wild are less likely to require coercion to fly, by virtue of being in a more dynamic environment with multiple external stimuli. By periodically agitating the mating arenas, the females would have been similarly stimulated to fly and would therefore have been available to be mated. Agitation of mating arenas was therefore subsequently incorporated into the mating competitiveness experiments described in the next Section 7.3.

Indeed, the issue of females making themselves unavailable for mating could have been eliminated entirely from the experiment using methods of tethered or induced mating (first described by McDaniel and Horsfall (1957) and then developed specifically in *Ae. aegypti* (Spielman, 1964) and *Ae. nigromaculis* (Miura, 1967, 1969)). However, these experiments have been shown to give quite different results to those using free-mating. For example, induced matings can last twice as long as free-matings (Jones and Wheeler, 1965), likely as the female is prevented from forcibly ending the copulation event at a time of her choosing. Consequently, under induced mating the male may be able to transfer more sperm than he might otherwise during free-mating (Oliva et al., 2014a), which would in turn reduce the number of females he could inseminate overall. Practically speaking, induced mating experiments are also very labour intensive, as every female to be mated has to be individually tethered, which may have prohibitively reduced the number of replicates.

7.3 Mating Competitiveness

7.3.1 Introduction

The ultimate goal of released transgenic males is to successfully mate with wild-type females, in competition with - and to the exclusion of - wild-type males. This ability is termed mating competitiveness, and is the single most aspect of male vigour used to determine the fitness of a transgenic insect, and its appropriateness for further development and use. Generally speaking, an assessment of a strain's mating competitiveness can be obtained by co-habiting transgenic males, WT males, and WT females, to see what proportion of females is mated by each male type. If transgenic males are present in equal numbers to the WT males, yet mate significantly less than 50% of the WT females, this would be indicative of a performance penalty that may reduce the chances of the control programme achieving suppression. As such, mating competitiveness experiments are an important and widely-used part of quality control of sterile insects (FAO/IAEA/USDA, 2003).

In the experiments on mating capacity in the previous section, males never interacted with one another; they were always individually housed with virgin females. While this permitted assessments of individual male mating capacity, it was not representative of a field situation where males are always likely to be in direct contest competition with other males to obtain mating opportunities. Although released males are also in competition with each other, in the context of population suppression the competition interaction of most importance is that between the released males and their wild counterparts. As such, mating competitiveness experiments are used to provide an indication of how well transgenic males are able to directly compete with wild-type males. OX513A has previously been subjected to these assays, which showed that its mating competitiveness was not significantly different to that of wild-type strains, in laboratory (Harris et al., 2011; Massonnet-Bruneel et al., 2013; Patil et al., 2015) and semi-field (Lee et al., 2013b) environments (although see Bargielowski (2010) for an indication of inferior laboratory competitiveness). As well as these studies, several other assays of OX513A's mating competitiveness have been made as part of routine strain characterisation and maintenance, and/or in preparation for field releases (Oxitec internal data). However, the present study is the first to investigate the effects of body size and nutritional status on OX513A mating competitiveness.

In the mating capacity experiments in the previous section, if differences between treatments did exist they were impossible to detect given the small sample size (n=3). Given that variation within OX513A mating competitiveness studies tends to be high (Oxitec internal data), it was necessary to increase the number of replicates per treatment. To this end, sugar concentration was removed as a variable from the experiment. There were also other reasons for this. Firstly, standard Oxitec mating competitiveness experiment protocols required a mating opportunity of 48 hours. Over this short period of time it was judged that any effects of the concentration of available sugar would be negligible. However, not providing sugar at all might unfairly disadvantage the smaller males (the longevity study showed that when no sugar is provided, smaller males start to die from day 4 of adult life onwards, i.e. within the timeframe of this experiment). Therefore all males and females were provided with 10% sugar for the duration of the experiment.

As well as removing sugar concentration as a variable, 'medium' sized males were not used in this experiment, only 'small' and 'large'. Again, this was done to reduce the number of treatments (enabling more replicates to be performed), and also to increase the likelihood of detecting an effect of male size on mating competitiveness, should one exist. The densities used to rear 'small' and 'large' males (5 and 1 larvae ml^{-1}, respectively), represent reasonable extremes of rearing densities for a field release programme.

7.3.2 Materials and Methods

Performing a mating competitiveness experiment where both groups of males are the same strain (e.g. small OX513A males vs. large OX513A males) is impossible, as the paternity of the progeny cannot be determined either by larval fluorescence profile or PCR. Therefore OX513A males were always in competition with WT males to mate with WT females. Each combination of large/small and OX513A/WT was performed (Table 7-2). Males of both strains were reared at 1 and 5 larvae ml^{-1}, with equal food in each, to produce 'large' and 'small' males respectively (see Section 2.2.2). WT females were reared at 3 larvae ml^{-1}. All insects were reared on tetracycline.

Table 7-2. Male treatments used in the mating competitiveness experiments.

Treatment	OX513A male size	WT male size
1	small	small
2	large	large
3	small	large
4	large	small

Mating arenas (30 x 30 x 30 cm cages with 10% sucrose solution) were set up as follows: 15 OX513A and 15 WT male pupae were allowed to eclose into each mating arena. Adults were held for three days to provide sufficient time to reach sexual maturity, and also to replicate the typical duration adults are held prior to field release. On the fourth day, 15 WT virgin females were introduced into each mating arena. These females had been provided with 10% sucrose solution for four days as adults. Therefore, at the time of introduction all males were four days old and all females were five days old. Males and females were left together in the mating arenas for 48 hours, which were periodically agitated to stimulate flight and to ensure all females were available to be mated. After 48 hours the females were removed, blood fed (on defibrinated equine blood), and put into individual tubes containing soaked cotton wool as an oviposition substrate. Eggs were hatched and larvae given Liquifry No. 1 (Interpet Ltd., UK) to allow them to grow to late L2 stage; each tube contained one 'larval family', i.e. the larvae from the eggs of a single female. Each larval family was screened for the presence of the fluorescent marker protein DsRed2, the expression of which is integrated into the OX513A construct. Larval families were scored as follows: (i) all larvae in the family were fluorescent (indicating they had an OX513A father); (ii) no larvae were fluorescent (indicating they had a WT father); or (iii) larvae were a mixture of fluorescent and non-fluorescent (indicating that the female had mated with at least two males - a WT and an OX513A - referred to as a 'multiple mating'). In this way the proportion of females from each cage mated by OX513A and WT males was determined. Finally, approximately 32 males of each treatment had their wing lengths measured, along with 40 WT females.

Fourteen repeats of each treatment were completed, two repeats at a time. Repeats 1-4 used a 10:10:10 OX513A male: WT male: WT female ratio, which was increased to 15:15:15 for repeats 5-14. Not all females introduced into the mating arenas produced offspring

(primarily due to death prior to either blood feeding or oviposition)[31]. As a result the mean number of larval families produced from mating arenas with 10 and 15 females was 10.0(±1.0)[32] and 13.0(±1.8) respectively.

Statistical Analysis

For each mating arena[33], the proportion of matings by OX513A, WT, or multiple fathers was calculated. Chi-square comparisons were performed, where the null hypothesis was that the proportion of OX513A matings was equal to 0.5. Comparisons were only made within treatments, between OX513A and WT mating proportions.

[31] For a discussion of how these incidents were dealt with statistically, please see Appendix 9.3.2.

[32] This average is >10 because the number of females introduced into each mating arena in repeats 1-4 was occasionally slightly >10.

[33] For a discussion of an alternate means of data aggregation, please see Appendix 9.3.2.

7.3.3 Results and Discussion

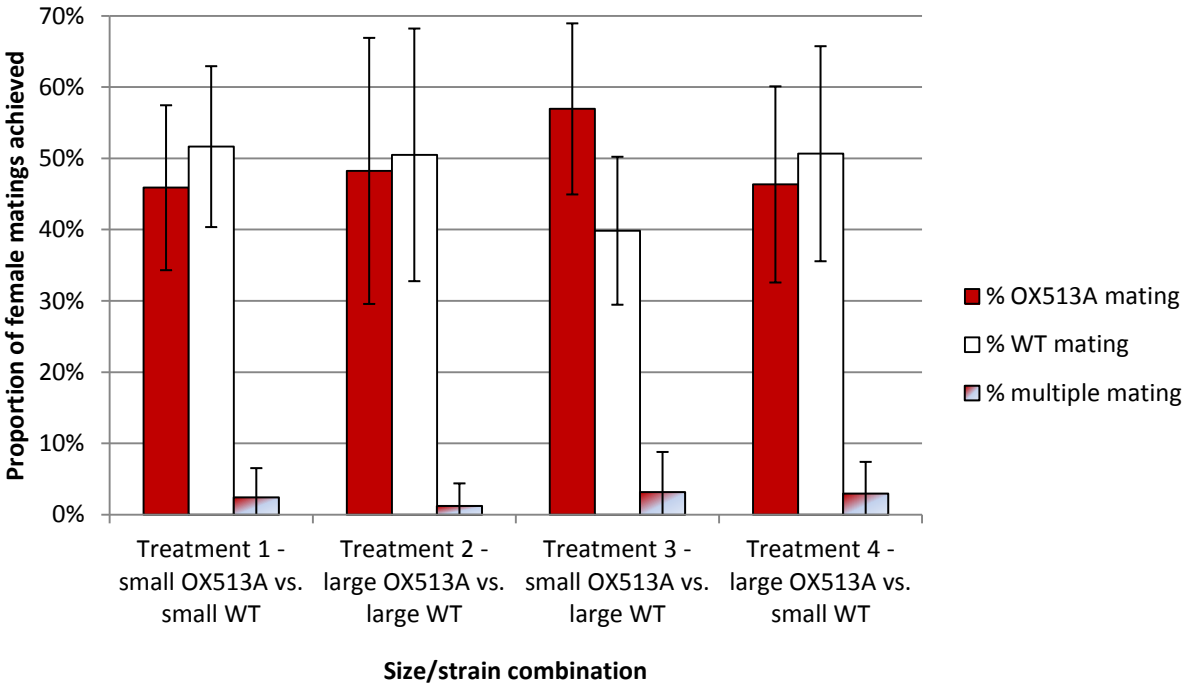


Figure 7-6. Mating competitiveness of small and large OX513A males and WT males. Each mating arena yielded a percentage value describing the proportion of females mated by OX513A males, WT males, or both ('multiple matings'). For each treatment, the value plotted here is a mean of these percentages (n=14), error bars = sd.

In each of treatments 1, 2, and 4, WT males achieved a slightly higher proportion of matings than OX513A males, although this was not statistically significant (χ^2 = 0.302, 0.011, 0.027 and p=0.582, 0.915, 0.869 respectively for treatments 1, 2, and 4). However, in the case of small OX513A males competing with large WT males (treatment 3), the OX513A males were able to achieve significantly (χ^2 = 13.369, p=0.0003) more matings than the WT males: 57.0(±12.0)% OX513A fathers to only 39.9(±10.4)% WT fathers.

The outcome seen in treatments 1, 2, and 4 was consistent with the published studies of OX513A mating competitiveness (Harris et al., 2011; Lee et al., 2013b; Massonnet-Bruneel et al., 2013; Patil et al., 2015), all of which found that OX513A had non-significantly different competitiveness with the WT strain being used as a comparator. However, none of these studies included male body size as a variable, and so the result of treatment 3 - that small OX513A experienced a mating advantage over large WT males - was novel to the present study.

In a variety of mosquito species, mating success appears to be partly determined by the ability of males and females to successfully display harmonic convergence, whereby each sex modulates its flight tones relative to the other (Cator et al., 2009). For example, in both sexes of *An. gambiae*, larger individuals have higher flight tones, and are able to converge earlier and more quickly towards other individuals with higher flight tones (Cator et al., 2010). This suggests that in a busy swarm comprised mostly of males (Hartberg, 1971; Yuval, 2006; Cator et al., 2011) larger males would be more likely to mate first, a considerable mating advantage given that female *An. gambiae* (like *Ae. aegypti*) are generally monandrous (Tripet et al., 2003). Cator and Zanti (2016) recently investigated convergence and mating in combinations of small and large males and female *Ae. aegypti*[34]. In encounters between pairs of the same size class (i.e. a small male meeting a small female, or a large male meeting a large female), convergence increased the subsequent chances of copula formation to a greater extent than in meetings of pairs of different size classes. As large males were only slightly smaller (in absolute terms) than small females in that study, this would suggest the presence of an optimum size differential between the male and the female in each pair. Within this range of size difference, convergence increased the chance of copula formation, but this mating advantage was not present in pairs that were either too similarly- or differently- sized. In the present study the females were all of one size class (being reared identically). The large WT males were the largest male type, and so it is possible that they were not sufficiently different in size from the females, and so convergence did not increase their chances of copula formation. The small OX513A males - being the smallest male type - may have been sufficiently different in size (but not too different), and so did experience the described mating advantage. This advantage was most apparent when placed in competition with the least-advantaged class, the large WT males. Differentials in mating success between other combinations may have been present, but following this principle would have been less pronounced and so were undetectable in this experiment. The strength of this assertion could be tested by repeating the experiment with multiple size classes of females; the apparent mating disadvantage of the large WT males in the present study would presumably disappear when mating with females that were sufficiently large enough to re-establish the optimum size differential.

[34] Here 'small' and 'large' are relative terms within each sex; large males were still smaller in absolute terms than small females.

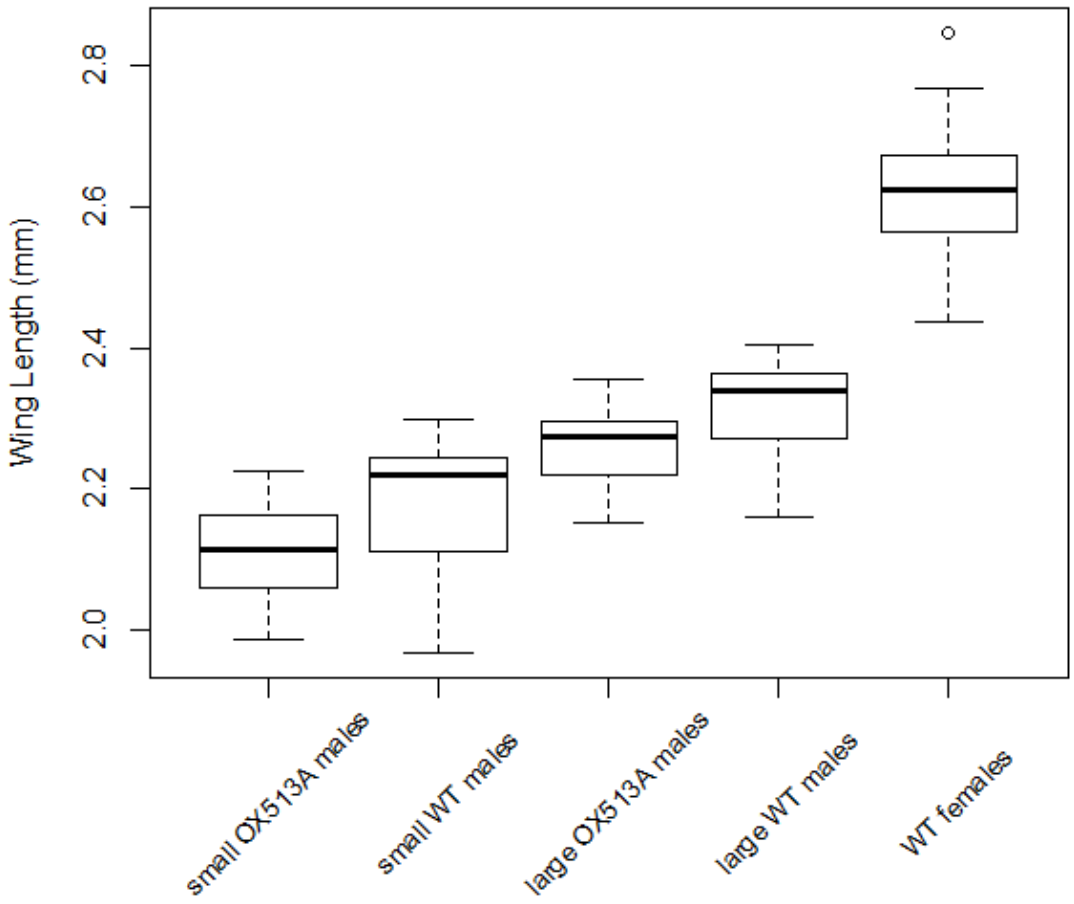


Figure 7-7. Wing lengths of males and females used in mating competitiveness experiment. For male groups n≥31, for females n=40. Each of the five groups was statistically different from all other groups (one-way ANOVA with Tukey HSD post-hoc tests).

However, in the study of harmonic convergence discussed above (Cator and Zanti, 2016), a male's size did not outrightly effect the likelihood of his convergence with either size of female, or his probability of subsequent mating. Male size may therefore have been exerting an effect on mating success in this study via a different mechanism. In *An. gambiae*, intermediate-sized males were the first to obtain a mate significantly more often than both small and large males, with that mating advantage being most pronounced against the large males (Ng'habi et al., 2008). The authors of that study posited that small males are more agile in flight and so are more likely to be the first male to make contact with a female. The larger males are less limited by their energetic reserves and so can more easily sustain swarming activity, which is of considerable energetic cost (Yuval et al., 1994; Maïga et al., 2014). Therefore the intermediate males, possessing a balanced mix of these traits, emerge with the greatest overall mating success. *An. gambiae* are obligate swarmers, and in the study by Ng'habi et al. (2008) did so continuously for 40-45 mins, with no sugar provided.

This would have used up a significant portion of each male's energetic reserves; in a separate study of *An. gambiae* 50% of available sugar and glycogen reserves were consumed by only 25 minutes of swarming activity (Maïga et al., 2014). In the present study, although the mating period was much longer (48 hours), sugar was provided throughout. Furthermore the males were not swarming continuously; indeed from anecdotal observation most mating took place in single pairs immediately following periodic agitation of the mating arena to interrupt resting behaviour. Therefore, the energetic pressure on the small *An. gambiae* was likely not acting on the small *Ae. aegypti* in the present study. However, if the proposed agility benefits of being smaller (as has also been observed in other swarming dipterans; Neems et al., 1998; Crompton et al., 2003) still applied, then the smallest males would have experienced the greatest mating advantage over the largest males, as indeed was observed.

Multiple Mating

In each larval family, a mixture of fluorescent and non-fluorescent larvae indicated that the female had mated with at least two males - a WT and an OX513A - referred to as a 'multiple mating'. It is assumed that multiple mating actually occurs at approximately double the observed rate; multiple matings can occur in four combinations (OX513A-OX513A, WT-WT, OX513A-WT, or WT-OX513A) of which only the latter two produce offspring of mixed fluorescence[35]. Multiple matings occurred significantly less often than single matings (by either strain), at a mean observed rate of 2.5%, equating to an assumed rate of 5%. This rate of polyandry is in line with previous studies of *Ae. aegypti*, although the range is considerable. For example, other authors have variously noted multiple mating in the laboratory occurring at rates of: 0% (Weidhaas and Schmidt, 1963); 0.6% (Massonnet-Bruneel et al., 2013); 1.8% (Bargielowski, 2010); and 18% (Patil et al., 2015). It is often assumed that rates of multiple mating in the field will be lower than that in a laboratory setting, due to high densities in the laboratory resulting in more frequent copulation.

[35] Assuming that females do not mate more than twice, and that there is no additional bias in a female's choice of second partner as compared to her first. When analysing the results, multiple matings can be treated in two ways. Females that were observed to be multiply mated can be excluded from the analysis, and paternity calculated as a proportion of the single-father families only. Alternatively, a multiple mating can be considered a 'success' for both the OX513A and WT males (i.e. when calculating percentage matings, the number of 'multiple matings' is added to both the 'number fathered by OX513A male' and the 'number fathered by WT male'). Statistical significance or lack thereof was consistent across both methods; all statistical values reported were calculated using the second method, where multiple matings are a 'success' for both male types. However the graph of results (Figure 7-6) shows multiple matings as a separate bar, so that for each treatment all bars sum to 100%.

However, rates as high as 14% were observed in semi-field enclosures (Helinski et al., 2012), and the first evidence of polyandry in a wild population was reported at 6.3%, including one female that contained sperm from three different males (Richardson et al., 2015). It should be noted that in those studies which recorded multiple matings using sperm-labelling methods, it is possible that the female contained sperm from multiple males but only one male's was actually used, in which case she could be said to be reproductively monogamous if not sexually monogamous. However, in the present study a mix of genotypes was observed in a single female's offspring, confirming that she had actually used sperm from more than one male. Currently, there is no evidence of sperm competition in mosquitoes (as there is in *Drosophila*; Price et al., 1999), and the way in which a multiply-mated female might use sperm from different males is not yet understood (Oliva et al., 2014a).

Relevance to Field Releases

The results of this experiment suggest that small OX513A males have a mating advantage over large WT males. Does it follow that rearing smaller rather than larger OX513A males would be advantageous to a release programme? Certainly over millions of released males, it is appealing to envisage that even a relatively small shift in mating advantage would have a cumulatively large impact on the numbers of wild females mated by OX513A males. However, in reality this may not be the case, as discussed below.

One of the proposed explanations for the observed result was the potential presence of an optimal size differential between the males and females (into which range the small OX513A males fell but the large WT males did not), leading to improved mating success via harmonic convergence. If such an optimum size range did exist, then a similar trend may well follow in the field. A mating advantage of small OX513A males would be of great benefit to a suppression programme, as smaller males are the product of higher rearing densities (in this experiment, small OX513A males were reared at five times the density of large OX513A males). Even with the increased difficulties associated with rearing and harvesting mosquitoes at higher densities, this would represent a significant increase in production capacity from a mass rearing facility of certain size. However, the size of wild females varies with environment (Gillies and Meillon, 1968; Fish, 1985; Briegel, 1990; Schneider et al., 2004), and so rearing procedures would need to be tailored based on the size of the females being targeted. Another more significant impediment to the release of apparently mating-

advantaged smaller males is the resultant loss of male vigour in other areas, notably longevity. In order to maintain a sufficient 'standing crop' of transgenic males in the field, releases would likely need to be made more frequently if using shorter-lived males. Although the increased production capacity (by virtue of the higher rearing density) may permit production of the required greater number of insects, more frequent releases also incur more frequent demands (for example on staff, equipment, and community tolerance) which may preclude releases from occurring sufficiently often.

The second proposed explanation of the improved mating success of the smaller OX513A males was their greater agility resulting in increased likelihood of being first to mate. If this was indeed the underlying mechanism, then it is uncertain that such an advantage would translate to an improvement in field release efficacy. It seems likely that the agility benefit may only be present in situations where external energetic resources (i.e. sugar) are in ample and proximal supply (as was the case in the present study), as this mitigates the energetic disadvantage of being smaller in size. Given that sugar is likely to be less readily available to a released OX513A male than a caged one, the agility benefit would likely be subsumed by the more pressing energetic needs of survival and dispersal.

Presuming that there is indeed an 'ideal' size of male mosquito (specific and relative to the sizes of the WT females in the release area), then released males that were significantly outside of this ideal size range may suffer a mating disadvantage. This would likely be exacerbated by the fact that the WT males and females would have experienced the same densities and food availabilities during larval development, and as such would presumably be closer to the 'ideal' proportional size. Moreover, in this study there also seemed to be an inherent size differential between the OX513A and WT strains. Both were reared under identical conditions, yet within each size class WT males were significantly larger than OX513A males (on average 3.2% and 2.5% bigger, for small and large classes respectively; Figure 7-7). This trend of WT males being larger than OX513A males was also found by Bargielowski (2010). It is possible that the inherently smaller size of OX513A males could be a product of being subjected to rounds of mass rearing, which may select for the earlier pupating, smaller males. There may also be an effect of incomplete suppression of the transgene, causing sub-lethal fitness effects that manifest as smaller body size.

Depending on the nature and strength of the relationship between body sizes (of both male and females) and mating success, it may be prudent to undertake an assessment of the body sizes of the target mosquito population. Such information could then be used to inform the rearing procedures of the transgenic release cohorts, such that an optimal body size is targeted. Such an optimum would likely vary by location and season; however body size surveys are relatively quick and easy to perform. However, the selective breeding of males with the aim of fostering certain traits (being of small or large size, for example), should be treated with caution. As was seen in the case of the melon fly in Japan (Hibino and Iwahashi, 1989, 1991; Koyama et al., 2004), there is potential for the introduction of a trait by which wild-type females are able to recognise and avoid transgenic males (assortative mating), which would have severe implications for the effectiveness of a RIDL programme (Robinson et al., 2009).

In addition to laboratory assessments of OX513A male mating competitiveness (Bargielowski, 2010; Harris et al., 2011; Massonnet-Bruneel et al., 2013; Patil et al., 2015), equivalent tests have also been performed in semi-field conditions. In these, OX513A males demonstrated equal mating competitiveness with a WT strain in a mock-up of a typical apartment in Kuala Lumpur (Lee et al., 2013b). The number of individuals used (ten each of OX513A males, WT males, and WT females) was low given the large volume of the mating arena (≈85 m^3). This was a deliberate choice based on observed numbers of *Ae. aegypti* in an actual domestic setting (Getis et al., 2003), and presented a substantial but quite realistic challenge for the males in terms of locating females and obtaining matings. Performing mating competitiveness experiments in realistic approximations of field conditions is expensive in terms of time, space, and resources, and is therefore usually preceded by experiments similar to the present study. However, history demonstrates that male competitiveness in laboratory cages does not always hold true in the field (Benedict and Robinson, 2003). A likely contributing factor is the constraining of natural mating behaviours by high density and/or close proximity of individuals in small mating arenas, which may disguise shortcomings in the males' ability to seek, locate, and mate with free-flying females (FAO/IAEA/USDA, 2003). As such, local semi-field assessments of mating competitiveness, such as that performed by Lee et al. (2013b), are an important intermediate step between laboratory mating competitiveness studies and full-field releases (Benedict et al., 2008;

Facchinelli et al., 2013). It would be very interesting to see if the results of the present study hold true in a more realistic and challenging mating environment.

7.4 Conclusions and Further Work

Mating Capacity

The total numbers of females inseminated by OX513A males was consistent with previous studies, and did not depend on male size, nutritional status, or even length of time spent with access to females. It appears that the volumetric constraints of the mating arenas did not allow the energetic advantage of the larger or sugar-fed males to manifest as greater mating capacity, as males did not need to expend much energy in mate-seeking. Therefore, the possibility of nutritionally-disadvantaged males having a lower mating capacity remains. In addition, significantly more males were mated on day 5 than earlier in the experiment, indicating that males may be equipped with the highest quantity of spermatozoa and accessory gland secretions at this age.

As a result of the large numbers of males released as part of a suppression programme, most will never get the chance to mate. As such, the mating capacity of a single male is unlikely to be the limiting factor to his maximum reproductive capacity. However, reduced mating capacity of males of a certain size, nutritional status, or age would be an indication of reduced mating ability overall, and so would warrant further investigation. In the case of the present study, a good next stage of investigation would be to repeat the experiment in larger laboratory or semi-field cages, in order to allow the mating advantages of certain males to become apparent. Eliminating the 'middle' treatment level in each variable (medium-sized males, 1% sugar meals) would help to generate more measurable effects while at the same time increasing the number of replicates that could be run and thus the discriminatory power of the study. Where possible, care should be taken to mirror the methods used by Chadee et al. (2014) in their study comparing two sizes of mating arenas, such that the results could be used to compare the relative effect of size and nutritional status on OX513A males and the recently field-collected males used in their study.

Mating Competitiveness

The proportion of matings achieved by small OX513A males (57%) was significantly larger than that achieved by large WT males (40%). Proportions achieved by the other three possible combinations of small and large OX513A and WT males were not significantly different. This may have been a result of size-specific harmonic convergence leading to an increase in mating success and/or the potentially greater agility of the smaller males.

As in the case of the mating capacity experiments, the recommended next stage of investigation would be to repeat the present study in larger laboratory or semi-field cages, in order to assess whether the mating advantage observed in the small OX513A males persists in more challenging mating conditions. Semi-field evaluations of the mating competitiveness of irradiated *Ae. albopictus* have been successfully carried out (Oliva et al., 2012; Madakacherry et al., 2014), and the mating ability of another transgenic *Ae. aegypti* RIDL strain was assessed in semi-field conditions in Mexico (Facchinelli et al., 2011, 2013). The latter study is of particular significance, as it exposed a mating disadvantage that, while significant, had not been detected in smaller laboratory cage tests (Wise de Valdez et al., 2011). This highlights the ability of such larger-scale tests to reveal fitness traits that were not observable in more artificial conditions (a recurring problem within genetic control of mosquitoes; Benedict et al., 2009b), and thus the importance of performing such experiments prior to large-scale releases (FAO/IAEA/USDA, 2003).

Final Conclusions

The evaluation of male mating ability in the laboratory is recognised as a valuable step in the transition to field releases (Sinkins and Gould, 2006; Harris et al., 2011). However, there are numerous examples of promising laboratory performance being followed by disappointing mating aptitude in the field (Asman et al., 1981; Benedict and Robinson, 2003; Gould and Schliekelman, 2004; Scott et al., 2006). Despite a decade or so of concerted effort into furthering our understanding of male mating biology (Ferguson et al., 2005, 2010; Lees et al., 2014; Oliva et al., 2014b), at present our knowledge of natural mating behaviour and outcomes remains poor (Oliva et al., 2014a). Conditions inside a laboratory cage are in no way representative of conditions in the field, with the latter being far more challenging. Laboratory males do not have to contend with the host of environmental factors present in a heterogeneous field environment: wind, precipitation, and fluctuations in temperature and

humidity may all negatively impact male survival, dispersal and mating behaviours. In the field, females are likely to be both more distant and less densely aggregated than in laboratory mating competitiveness experiments. All of these factors seem likely to reduce the effectiveness of transgenic males when compared to their performance in a laboratory environment. Indeed, while the mating competitiveness of OX513A has been shown to be equal to wild-type strains in laboratory (Harris et al., 2011; Massonnet-Bruneel et al., 2013; Patil et al., 2015) and even semi-field (Lee et al., 2013b) tests, mating competitiveness estimates from full-field releases are significantly lower (Harris et al., 2011, 2012; Carvalho et al., 2015; Gorman et al., 2016). A similar trend was observed in classical SIT releases (for example Patterson et al., 1977). A greater understanding of natural mating behaviours, combined with knowledge of how the mating performance of the released males may be optimised, is essential for the success of future large-scale suppression programmes based on male release.

8 Overview and Conclusions

8.1 Introduction

The mosquito *Ae. aegypti* is the principle vector of dengue fever, a disease that is thought to infect around 390 million people per year (Bhatt et al., 2013). Although mortality from dengue fever is low, the potential severity of symptoms combined with the disease's prevalence causes a great deal of human suffering worldwide. Current methods aimed at controlling the vector have so far failed to prevent the spread of dengue fever, and no fully-effective vaccine exists. As such, there has recently been a renewed research effort into genetics-based control strategies. One of these is RIDL® (Release of Insects carrying a Dominant Lethal gene, Thomas et al., 2000), which is a variant of the classical sterile insect technique (SIT), and relies on the release of large numbers of 'sterile' individuals. The RIDL strain OX513A contains a late-acting dominant lethal genetic element, which can be repressed by the presence of tetracycline in the diet of the insect. At its core, a control system using this strain relies on the ability of released male mosquitoes to mate with the females in the population that is targeted for suppression. The offspring of such pairings inherit the lethal genetic element, and as tetracycline is not present in the natural environment, they die before they can reach the adult stage. As the strain is self-limiting rather than self-propagating, continued male releases are required to reduce the population, and maintain suppression thereafter.

This thesis is primarily concerned with assessing and improving the vigour of those released OX513A *Ae. aegypti* males. Chapter 3 investigated the ways in which male OX513A pupae can be stored, allowed to emerge into adults, and then released. Different techniques were tested and optimised in both the laboratory and subsequently the field. In Chapters 5-7, the nutritional status of the males was adjusted by modifying their size (a function of larval rearing conditions), and the type and duration of sugar provision afforded to them. The combination effects of these changes on the subsequent longevity of the insects were measured in Chapter 5. In Chapter 6, the flight ability of the males was measured using a flight mill, and the effects of altered nutritional status were observed. In Chapter 7, the mating ability of the OX513A males was assessed; firstly by mating capacity, and then by placing them in competition with wild-type males. For each of these chapters the primary

results, their implications, and recommendations for future work are discussed. Finally, the place of a RIDL-based control system within the wider context of vector and disease control is discussed.

8.2 Field Release Experiments

8.2.1 Summary of Results

Two types of adult mosquito release device (RD) were designed and tested in the laboratory: 'coffee cup' RDs and 'cassette cage' RDs. After optimisation by the addition of inserts separating the eclosed adults from the surface of the water, >90% pupa-to-adult survival was observed at densities of 800 males per RD. The cassette cage RDs were similarly promising, with the percentage of males able to disperse after 'release' being not significantly different to that of control males kept in 'ideal' conditions. This parity was maintained throughout a pre-release containment period of ten days, far longer than required. However, the cassette cage RDs were relatively costly to produce, whereas the coffee cup RDs required very little modification from cheap and commercially available disposable coffee cups. As such, the coffee cup RD design was chosen for use in the upcoming suppression trial to take place in the Cayman Islands in 2010.

From the first field releases of male OX513A, it became clear that the high survival and dispersal observed in the laboratory testing was not being achieved in the field. A series of four changes was implemented in an effort to improve survival and dispersal of the released mosquitoes: (i) males were stored in the RDs for less time prior to release; (ii) the density of the males in the RDs was halved; (iii) an insert component (that had proved beneficial in laboratory testing) was removed; (iv) an anti-fungal agent was added to the sugar feed provided to the males prior to release. After implementation, on-site laboratory experiments showed that all four of these changes were beneficial to the survival of the males, and the quality of the insect output of the trial was improved.

8.2.2 Recommended Future Work

Despite the initial difficulties encountered during the 2010 trial in the Cayman Islands, the project was a success, reducing the wild *Ae. aegypti* population within a portion of a small town by 80% relative to control areas (Harris et al., 2012). A subsequent trial in suburban Brazil achieved and maintained a reduction in the population of 81-95% over the course of a year (Carvalho et al., 2015). Finally, in Panama a reduction in the wild population of up to 93% was attained (Gorman et al., 2016). In the case of the Cayman Islands and Panama, reduction in the wild population was achieved without the aid of ongoing conventional control methods (such as deployment of larvicides to breeding sites). Thus, the RIDL technique is demonstrably able to significantly reduce wild populations of wild *Ae. aegypti*.

However, in all three trials to date, the treatment areas in which OX513A males were released (and suppression was achieved) were relatively small (<20 ha). The next challenge is to successfully demonstrate the efficacy of the technology on a larger scale, by controlling a mosquito population across an entire city or region. Indeed, following the success of the field trials in the Cayman Islands and Brazil, larger-scale rearing and release is currently underway in both counties. The programme in Brazil is now particularly large; current releases cover an area in which 60,000 people live[36], and a newly-opened production facility has the further capacity to produce up to 60 million male OX513A per week, which would protect 3 million people[37].

However, the mass rearing systems and equipment currently used to produce large numbers of male mosquitoes (Carvalho et al., 2014) and the methods used by the original practitioners of classical SIT in *Ae. aegypti* (Trembley, 1944; Ansari et al., 1977) are, in principal, identical (Curtis, 2006; Benedict et al., 2009b; Dame et al., 2009). Although the scale of production has increased, mosquitoes are still produced in a batch-wise rather than continuous manner, and the rearing activities themselves (setting up rearing trays, harvesting pupae, populating RDs, etc.) are conducted entirely by hand with no automation. The means by which mass rearing operations may be scaled-up to meet demand is a topic beyond the scope of this thesis, but a move from batch to continuous processing would seems likely to be an area in which dramatic improvements could be made (Benedict et al., 2009b). For example, systems incorporating circulating larval rearing water and automatic feeding have been demonstrated, albeit only on a small scale (Feldmann et al., 1989). More recently, a concept was put forward for a pupa-larva separator for continuous mechanical separation (Benedict et al., 2009b). Advancements in these areas will necessitate the enlistment of expertise from other disciplines such as process engineering, mechanical engineering, and hydroculture.

Nonetheless, with the requisite knowledge, equipment, and manpower the production of millions of sterile (Bailey et al., 1980; Savage et al., 1980) or transgenic (Carvalho et al., 2014) males per day is currently possible. Unfortunately, the systems for the actual release of

[36] Oxitec press release, 6 September 2016: http://oxitec.com/oxitec-piracicaba-city-hall-start-release-friendly-aedes-additional-10-downtown-neighborhoods/

[37] Oxitec press release, 26 October 2016: http://oxitec.com/oxitec-opens-large-scale-mosquito-production-facility-brazil/

these millions of insects have evolved to an even lesser extent than those for mass rearing. Releases of OX513A in Brazil are still made by hand, using RDs which are fundamentally identical to the coffee cup RDs described in this thesis (Carvalho et al., 2015). However, for releases into urban areas, ground releases by hand may continue to be appropriate (Dame et al., 2009). Although they are labour-intensive, such activities allow the fine-tuning of release points in a very heterogeneous environment while also contributing to other important requirements of a suppression programme such as visibility and community engagement. However, for larger and/or more rural areas with poorer road access, a less labour-intensive system may be required. As is often the case with mosquito releases, useful insight may be drawn from the current large-scale releases of sterile agricultural pests such as the Mediterranean fruit fly (*Ceratitis capitata*). The adults of this species are robust enough to be immobilised by chilling, and then packed together and released directly into the air from a specially-modified plane. This method is capable of releasing millions of insects per flight (de Longo et al., 2000; Dowell et al., 2000), and for large scale releases can be cheaper than ground releases (Pereira et al., 2000). Following calls for increased research into this area (Dame et al., 2009), the International Atomic Energy Agency (IAEA) held a consultation on the matter (Herrero et al., 2014), and have recently demonstrated a prototype system for adult releases from drones[38]. Such multidisciplinary projects are commendable and significant further effort should be put into the design and testing of aerial release systems for mosquitoes. Concurrently, vigilance will be required to ensure that the physical demands placed on the organisms during an aerial release process are not overly detrimental to their subsequent field performance.

[38] IAEA press release, dated 2 March 2016: https://www.iaea.org/newscenter/news/new-iaea-supported-drone-places-4th-in-international-drones-for-good-competition

8.3 Longevity of Male OX513A

8.3.1 Summary of Results

The longevity of OX513A males of various size and nutritional status was investigated in two experiments, one with water provided post-'release' and the other without. In a post-release scenario devoid of water, the number of days of sugar provision prior to release was the most important determinant of subsequent longevity. When a water source was introduced post-release (removing dehydration as a limiting factor), males lived longer overall and concentration of sugar meal became the most important factor, followed by male size, with medium-sized males overall living longer than their smaller counterparts. Therefore the resources available to males after release may be determining factors in formulating an optimal release strategy. A balance must be struck between holding the males in release devices for sufficient time to allow them to obtain a benefit from sugar-feeding, without holding them too long such that excessive damage occurs from being kept at very high adult densities. The decision of when to release will be further informed by the observation that in small males, sugar feeding prior to release was not sufficient to compensate for their lack of teneral reserves relative to their larger brothers.

There was a significant correlation between wing length and longevity. Of males dying on day 7 and older, average wing length increased incrementally with each subsequent day of death. To survive for more than 14 days, males on the poor-quality sugar diets (0% or 1%) needed to be larger; conversely, those males on 10% sugar were capable of surviving for this length of time even if they were much smaller than average size. However, males dying on days 4-6 were unexpectedly large given the early stage of the experiment.

It should be emphasised that the improvements to survival times observed in the longevity experiments will not be directly reflected in a release cohort. Average longevity in Experiment 2 was 11.4 days; in the field this drops to approximately two days (Lacroix et al., 2012; Neira et al., 2014; Carvalho et al., 2015; Winskill et al., 2015). Rather, the idea is that through laboratory experimentation the methods by which longevity can be most improved are investigated, before these methods are then applied to release cohorts. Although the absolute benefit will likely be much smaller in the field, even a marginal increase in longevity - when multiplied over thousands or millions of released insects - is a valuable increase to the reproductive potential of release cohort as a whole.

8.3.2 Recommended Future Work

An important piece of information that is still missing is the extent to which released males actually imbibe any sugar solution provided to them prior to release; no attempt to quantify this was made in the present study. Given the positive general effect of sugar provision on mosquito life-history characteristics (Foster, 1995; Briegel, 2003; and results herein), it is important to understand and maximise the extent of sugar uptake in release cohorts. An assessment of (i) the proportion of males that imbibe, and (ii) the volume imbibed by each, would facilitate a greater understanding of the beneficial effects of sugar provision at the level of the entire release cohort, and not just the individual. Factors that may influence sugar uptake include density of males within the release device, and sugar concentration (Ignell et al., 2010; Gouagna et al., 2014). In addition, such an experiment would be greatly complemented by quantification of the total caloric contents of members of a release cohort by biochemical assay, using the cold anthrone reaction (Van Handel, 1972, 1985).

As well as sugar feeding behaviour prior to release, little is known about the water-drinking and sugar feeding behaviours that occur in the wild (Foster, 1995). In the present study, the relative importance of the three investigated determinants of male longevity (body size, sugar concentration, and length of sugar provision) was found to be dependent on the availability of water in the post-release environment. Therefore, the propensity of males to utilise any naturally-available water or energetic resources will be an important piece of information in determining an optimal release strategy. A possible method was recently demonstrated during a study in Ecuador, in which coloured sugar solutions were sprayed onto local vegetation to identify sugar-feeding behaviour of wild *Ae. aegypti* (Qualls et al., 2016). Of the subsequently captured females more than half were detectably sugar-fed, but of the captured males not a single individual had sugar-fed. If OX513A males were found to have similarly low rates of sugar feeding in the field, then this would further increase the impetus to maximise the proportion of males that are supplied with a sugar meal prior to release.

A study of field-collected *Ae. aegypti* females indicated that the relationship between body size and longevity may be more complicated than previously thought (Juliano et al., 2014). This has also been hinted at by a mark-release-recapture study which found that while larger males lived for longer, the same did not apply to females (Maciel-de-Freitas et al., 2007). In

the present study, although the relationship between male wing length and age at death was largely linear, males dying on certain days early in the experiment were larger than expected. This observation may be an indication of a similarly more complex relationship in males. The primary aim of the experiments in this chapter was not to perform a full assessment of wing length and longevity, and so measurements were only taken from a sample of males. It is suggested that a more complete study of wing length and longevity should be undertaken, with an effort made to produce a wider and more even spread of male wing lengths, in order to ascertain whether the unusual observation in the present study was symptomatic of an underlying feature of the relationship between size and longevity.

An investigation into the effects of sugar provision on the field performance of irradiated sterile male *Ae. albopictus* was recently undertaken by Bellini et al. (2014); that study found that sugar provision extended lifespan (as it is known to do in the laboratory), and also improved mating competitiveness. While these results are not particularly surprising, they do represent probably the first demonstrable improvement of the performance of *released* males by direct alteration of their nutritional status. It is strongly recommended that a similar experiment be undertaken with OX513A males. In addition, the introduction of higher sugar concentrations, such as 20% or 25%, would provide valuable information regarding the extent to which sugar concentration (an easily modifiable element of the rearing and release procedures) can improve male performance. Certainly, higher concentrations have been shown in females to be of greater nutritional benefit by allowing reserves to be accumulated more quickly and to a greater extent (Briegel et al., 2001a). In the experiment by Bellini et al. (2014), male *Ae. albopictus* imbibed equally from 10% and 20% sucrose solution, indicating that individual males did not (or were not able to) discriminate between the two concentrations. Equally however, those same males did modulate their sugar-feeding behaviour directly in response to temperature and humidity, with high temperature and low humidity both being positively correlated with increased sugar uptake. This may indicate that sugar provision is of most value under these conditions.

8.4 Flight Capacity of Male OX513A

8.4.1 Summary of Results

The flight performance of male OX513A mosquitoes was measured on a flight mill; to the author's knowledge this was only the second ever flight mill study to be performed in *Ae. aegypti* males (after Bargielowski et al., 2012). Flight mill number initially had a significant effect on distance flown and time spent in flight, so a post-hoc data correction was applied to equalise the performance of each mill. After this, none of the three measures of flight performance (distance flown, time spent in flight, average speed) were significantly affected by male size class or number of days of sugar provision (although male size classes were found not to be distinct). The mean values for distance flown, time spent in flight, and average speed were in line with the few other studies that have been performed on male aedines (Nayar and Van Handel, 1971; Yee and Anderson, 1995; Bargielowski et al., 2012). Also as expected, most flights were relatively short (<2 km), although two males displayed exceptional flight performance. However, the observed flight distances achieved in this study will not be representative of actual or even potential flight performance in the field; rather they provide a controlled means of assessing how males are able to convert their nutritional status into physical performance.

In a separate smaller-scale experiment, the flight speed of the males was found to be modulated by the ambient light intensity, where a switch to darker conditions immediately stimulated faster flight.

8.4.2 Recommended Future Work

The two males which exhibited exceptional flight performance were members of the treatment with the expected greatest nutritional benefit, and were in fact the largest and third-largest males in the entire study. As such, it would be worthwhile to see whether the apparent alignment of conditions that produced their substantial flight capacity could be replicated. It is supposed that the novel flight mills used in this study, being more lightweight than previous equipment (Rowley et al., 1968; Bargielowski et al., 2012; Kaufmann et al., 2013a; and others), permitted flight performance that was to a previously-unobserved extent. Notably, both exceptional flights took place on those mils that appeared to offer the least mechanical resistance. Therefore, a good next step would be to construct a new set of mills according to the same design but to a more exacting specification, in order to eliminate the between-mill variation that was seen in the present study. Following which,

one could compare the flight performance of (for example) very large males provided with a high-quality sugar meal, with that of small males provided with a low-quality sugar meal. The latter group would establish a baseline minimum flight performance, while the flights of the former group would hopefully emulate the flights of the two exceptional males in the present study. This experimental design would also remedy the lack of data produced by the present study of the effect of male size on flight performance. It seems likely that such a relationship does exist in males, given that it has already been clearly demonstrated in females (Briegel et al., 2001a; Kaufmann et al., 2013b).

Finally, as was also suggested in the previous section on longevity, biochemical assays of on-board reserves can be conducted in tandem with flight mill studies (Nayar and Sauerman, 1971a, 1973; Nayar and Van Handel, 1971; Briegel et al., 2001a, 2001b; Briegel, 2003; Kaufmann and Briegel, 2004; Kaufmann and Brown, 2008; Bargielowski et al., 2012; Kaufmann et al., 2013b). In the case of the proposed experiment described above, such analyses would allow a more quantitative assessment of the energetic composition of males capable of achieving significant flight performance.

Presuming that male flight performance was demonstrably improved by significant increases in size and improvements to nutritional status, the application of these improvements to field release cohorts may still prove difficult. A field study in Brazil showed that although larger males did travel further, this was not statistically significant (Maciel-de-Freitas et al., 2007). Similarly, Valerio et al. (2012) found that in the field larger males travelled further, but this effect was only detectable in a single experiment that had nearly double the statistical power of the others. Both of these studies indicate that larger males likely disperse further in the field; nonetheless, the obfuscation of this trend by the host of other variables that exists in the field may make it quite challenging to demonstrate any value of increased body size to OX513A field dispersal.

The effects of the light intensity experiment were compelling and deserve further investigation. In the first instance, the experiment should be repeated with a greater number of replicates in order to better establish the pattern of behaviour. Secondly, an attempt could be made to confirm the hypothesis that the increased flight activity resulting from sudden darkness is an attempt to seek a safe resting site. In order to do so an experiment with an element of directionality could be used. For example, a choice chamber

containing at one end sources of sugar and/or mating cues[39], and at the other end resting sites. The expected outcome would be that following a sudden switch to darkness, males would lose interest in feeding and mating and would become preferentially attracted to the resting sites. Such an experiment would require that the males be free-flying, and so tracking software and equipment (such as that used by Boyer et al. (2013) to track *Ae. albopictus* activity patterns in the laboratory) would be required in the place of flight mills. Unfortunately, the increased flight activity observed after a sudden reduction in ambient light intensity is difficult to exploit, as the light intensity of the field conditions into which males are released cannot be directly controlled.

[39] Mating cues could comprise virgin females and/or mating aggretation pheromones (Cabrera and Jaffe, 2007; Fawaz et al., 2014).

8.5 Mating Performance of Male OX513A

8.5.1 Summary of Results

From the perspective of male-release control strategies such as RIDL, the mating performance of the released male insects is probably the single most important aspect of their life history to understand. The mating performance of OX513A *Ae. aegypti* males was assessed by two separate assays: mating capacity, and mating competitiveness. In the former, males were from one of three size classes, and given access to three sugar diets of varying quality. Neither of these variables had a significant effect on the number of females each male was able to inseminate, either when given periodic access to the females, or continuous access. Furthermore, when comparing these two experimental conditions overall, the average number of females mated in each was not significantly different. On average, the same males mated more females on the fifth day of adult life than on the third day.

The mating competitiveness assay was concerned only with the effects of male size. OX513A and wild-type males, reared to be of 'small' and 'large' sizes, were placed in competition with each other to mate with wild-type females. In three out of the four mating scenarios, the wild-type males had a small, consistent, but statistically non-significant mating advantage over the OX513A males. However, when small OX513A males were in competition with large wild-type males, the OX513A males had the mating advantage, fathering 1.4 times as many larval families.

8.5.2 Recommended Future Work

Mating Capacity

It is hypothesised that there were two reasons for the lack of detectable difference in mating capacity between males of different size and nutritional status. The first is that males only became sperm-depleted towards the very end of the experiment, if at all. Therefore, if the larger and/or nutritionally-advantaged males did possess an advantage in terms of their absolute quantities of sperm or accessory gland secretions, this was not apparent. In order to detect this advantage, complete depletion was required, which could have been ensured by presenting single virgin females to each male until he was depleted (as per Foster and Lea, 1975), or by using tethered or induced mating techniques (McDaniel and Horsfall, 1957;

Spielman, 1964; Miura, 1967, 1969). However, the removal of the mate-seeking and acquisition elements of mating - which are energetically very expensive for the male (Yuval et al., 1994; Bargielowski et al., 2011a; Maïga et al., 2014) - would instead change the emphasis of the study to a quantification of the absolute volume of sperm and accessory gland secretions possessed by males of each treatment. While such a study would by no means be without merit, the excising of behavioural aspects of mating from the experimental design would reduce the field-applicability of the results.

Therefore, instead of imposing intensive mating conditions in order to ensure sperm depletion, it is suggested that the experiment be altered to address the second proposed reason for the lack of detectable advantage of larger or sugar-fed males. Chadee et al. (2014) demonstrated that improved nutritional status only manifested as improved mating capacity in larger mating arenas. In mating arenas the same size as those used in the present study, the authors of that study did not detect any difference between the mating capacity of starved and sugar-fed male *Ae. aegypti* (at least not until the starved males actually died). By imposing a more challenging (and also more 'natural') mating environment, one in which mate-seeking and acquisition requires a more substantial investment of energy, the advantage possessed by the larger and/or nutritionally-advantaged males would likely become measurable experimentally.

Mating Competitiveness

The first suggested reason for the mating advantage experienced by small OX513A males over large WT males was the presence of an optimum size differential between the female and her mate. In this case, the large WT males were of too similar a size to the females, whereas the small OX513A males were sufficiently smaller. The strength of this hypothesis could be tested by repeating the mating competitiveness experiment, but with the introduction of female size as a variable; the apparent mating disadvantage of the large WT males in the present study would presumably disappear when mating with females that were sufficiently large enough to re-establish the optimum size differential. It would also be of benefit to perform more replicates than were performed in the present study, in order to increase statistical power.

The second hypothesis for the greater proportion of matings achieved by small OX513A males when in competition with WT males is that the smaller males were more agile in flight

and so were more likely to be the first to mate. Given that the mating arenas were relatively small, the energetic burden of mate seeking and acquisition was not great enough to disproportionately reduce the mating success of those smaller males (which possess more limited on-board reserves). Similar to the proposed further work in the mating capacity experiments, it is suggested that the present study be repeated in larger mating arenas, in order to expose smaller males to a more realistic energetic challenge during mating. Indeed, to understand the likely effect of male size on mating competitiveness in the field, a similar experiment to the present study should be conducted in semi-field enclosures or walk-in mesocosms. A previous study of another RIDL strain (Facchinelli et al., 2013) starkly demonstrated that disparities in mating competitiveness may only be detectable in these kinds of environments, where the mating challenge is greatest and most similar to that which will be experienced in the field.

8.6 The Place of RIDL Within Vector Control

The recent positive results of trials of a dengue fever vaccine (Capeding et al., 2014; Villar et al., 2015) represent a notable step in the fight against this mosquito-borne disease. However, the vaccine in its current form is of limited effectiveness, and even after its further development the control of the primary vector *Ae. aegypti* is anticipated to remain a primary focus of dengue control efforts (WHO, 2012; Alphey et al., 2013). However, there are significant problems associated with traditional mosquito control methods. They can have undesirable off-target effects, such as the accumulation of chemicals in the environment or food chains, or mortality of non-target species. Resistance development is a perennial problem for insecticides, and bed nets are of limited use against day-biting mosquitoes. Furthermore, effectiveness may be limited by the ecology of the insect itself. For example, larval control of *Ae. aegypti* is particularly difficult given its preference for breeding in small disaggregated bodies of water (such as blocked gutters, discarded tyres, and general debris). Finding and treating a sufficient proportion of these breeding sites to establish control is often impractical, especially given that such sites are often located on private property. The overall inadequacy of traditional control methods is demonstrated by their failure to prevent outbreaks of dengue fever in Singapore, despite being implemented within a well-equipped, efficient, and long-standing programme (Ooi et al., 2006; Egger et al., 2008).

By contrast, systems based on the release of 'sterile' males circumvent these problems neatly. Releases of transgenic mosquitoes do not involve the input of chemicals into the environment, and released males are only able to mate with wild females of their own species. Therefore, the environmental footprint of such a system is low, and species-specificity is high. However, it should be noted that species-specificity is not always an advantage, for example when multiple species transmit the same pathogen, as indeed is the case for dengue fever which is transmitted by both *Ae. aegypti* and *Ae. albopictus*. Due to the ability of released males to actively disperse and seek out wild females, a male-release solution can further be said to be 'homing' or 'target-seeking'. This makes it especially appropriate for control of insects with dispersed or cryptic breeding habits, such as *Ae. aegypti*, and/or for targeting the final remnants of a wild population that may permanently remain out of the reach of conventional control methods. An advantage over the SIT that is specific to releases of transgenic males is the ability to determine the specific time of death.

Mosquito larvae grow under highly density-dependent conditions, and so the alleviation of this density-dependent pressure can severely limit the overall control effect. By contrast, the OX513A construct results in 'late-acting' lethality, meaning that larvae which inherit the construct will survive long enough to maintain this density-dependent larval pressure on their wild conspecifics. This has the effect of suppressing the wild population more quickly and reducing the overflooding ratios required to achieve eradication, a significant advantage over traditional SIT (Barclay, 2005; Atkinson et al., 2007; Phuc et al., 2007; Yakob et al., 2008; White et al., 2010).

To date, RIDL has been used to control populations of mosquitoes in the Cayman Islands (Harris et al., 2012), Brazil (Carvalho et al., 2015), and Panama (Gorman et al., 2016). These studies were successful in reducing wild mosquito populations in relatively small areas (<20 ha) and were conducted largely as proof-of-principle. However, operations in the Cayman Islands and Brazil have since been significantly scaled up to provide protection for a much larger number of people inhabiting a wider area[40]. As RIDL methods begin to demonstrate their utility for control over larger areas, the pertinent question becomes how to implement this new weapon in the most impactful way. It is generally accepted that RIDL is of greatest effectiveness when used as part of an area-wide, integrated pest management (AW-IPM) approach (Coleman and Alphey, 2004; Hendrichs and Robinson, 2009). Despite their drawbacks, current control methods do have some advantages over genetically-engineered control methods, and it seems likely that an optimal strategy would comprise a blend of the best elements of each (Alphey et al., 2013). For example, conventional control methods are capable of significant short-term control against a large wild population; whereas male-release methods become proportionately more efficient against smaller wild populations (as greater overflooding can be achieved with the same number of released males). Therefore, an initial knock-down of a target population by means of insecticides, followed by releases of transgenic males, makes most effective use of the strengths of each control type. Once the population is reduced in this way beyond a relevant threshold (for example, one below which disease transmission does not occur; Anguelov et al., 2012; Dumont and Tchuenche,

[40] Oxitec press release, 28 July 2016:
http://oxitec.com/gm-mosquito-release-commences-grand-cayman/
Oxitec press release, 6 September 2016:
http://oxitec.com/oxitec-piracicaba-city-hall-start-release-friendly-aedes-additional-10-downtown-neighborhoods/

2012), continued lower-level releases of transgenic males should be able to maintain the population at its current suppressed level. The low adverse environmental impact of suppression based on transgenic release makes it ideal as the continuing long-term element of a mixed-approach control strategy. Following this integrative principle, as and when control measures that specifically target the disease (such as vaccines) become viable tools for widespread use, these can be further incorporated in a similarly optimal way.

There are a number of key challenges that must be overcome in order for genetic control strategies to be successful on a truly 'area-wide' scale. The first of these challenges is that of male performance. Genetic control strategies make direct use of an insect species' reproductive ability in order to reduce the size of a particular population. Therefore, the success of these techniques largely depends on a good understanding of the target species' reproductive biology. Although efforts are being made - including the work presented in this thesis - to improve the level of knowledge in this area, significant gaps remain (Oliva et al., 2014a). Of the many areas that require extensive further investigation, the highest priority are those concerning the specific mechanics of mating behaviour and the factors that may influence mate choice. Although laboratory studies in these areas lay essential groundwork, field studies of these behaviours in wild mosquitoes are currently lacking and are absolutely essential in order to optimise the chances of released males being successful in their goal.

The second challenge is one of scaling-up. As more fully discussed in 8.2.2, the processes of mass rearing and release require continued multi-disciplinary development in order to scale them up to be of utility for truly area-wide control. In particular, a move is required from batch-processing (which is only adequate and cost-effective for laboratory and limited field trial use) to continuous processing. This change applies equally to both the rearing and release of mosquitoes. In the case of the latter, aerial release presents a particular obstacle (Dame et al., 2009), which if overcome would revolutionise the delivery of mosquitoes as part of sterile-male programmes. In addition, genetic control programmes require the development of standardised quality control measures applicable across multiple mass-rearing facilities in different territories, as per those used to regulate the production of fruit flies (FAO/IAEA/USDA, 2003). While these quality control measures do not directly measure the competitiveness of the released males, they provide an excellent indication of the consistency of production (Benedict et al., 2009b). As part of a control method that involves releases of insects over many months or even years, consistency of output may be more

important than absolute quality, particularly with regard to surveillance and determination of entomological end-points (Morrison et al., 2008; Carvalho et al., 2015).

Truly successful use of genetic control methods on a large scale will also necessitate investment into the area of project management. It is a truism to state that area-wide control programmes usually fail not because of technical or biological inadequacy but because of poor management and lack of public support (Dyck et al., 2005). The production and release of millions of insects, often occurring in a developing country, is a formidable technical and logistical undertaking even without considering the biological criteria that must also be met. The biologists charged with delivering an area-wide control solution must be willing to concede that they are not the ones best placed to meet these challenges, and seek expertise from those in other fields (for example industrial mass-production, business, and project management). This allows the insight possessed by the scientists to be fully utilised, without the distractions posed by those operational issues secondary to production and release (such as issues concerning human resources, facilities, budgets, etc.), which are handled by more qualified others. Careful management at the lower levels of staff is also required. Staff responsible for the day-to-day rearing and release of the insects should not also be tasked with the development of supporting technology and/or dealing with issues that arise mid-programme (Dame et al., 2009). While communication between these two staff groups is imperative, merging these roles results in loss of objectivity and efficiency. As well as expertise from other disciplines, formalised management systems such as the Program Evaluation and Review Technique (PERT) have been shown to be of tremendous use in ensuring a structured and successful management approach (Rao, 1974).

Finally, the emergence of new genetic control methods have resulted in a concurrent prioritisation of community engagement as an essential component of AW-IPM (Curtis and Reuben, 2007; Vasan, 2009, 2010; El Zahabi-Bekdash and Lavery, 2010; Morrison et al., 2010). Community engagement is especially important in genetic control methods as compared to traditional control, as the conceptual difficulty inherent to male-release programmes can be difficult to overcome. Local residents are accustomed to control methods designed to kill mosquitoes, and so actively releasing mosquitoes can provoke confusion or outright hostility (Lowe et al., 1980). Where local vector control institutions are engaged as partners to deliver a control programme involving male release, the severity of such adverse reactions may be exacerbated by pre-existing feelings toward that institution,

such as their perceived lack of effectiveness to date and/or ties to government (author's anecdotal observation during the adult releases described in Harris et al., 2012).

To conclude, genetic control strategies such as RIDL show tremendous promise and possess a number of key advantages over currently-available mosquito control methods. However, these novel methods face a number of significant challenges in order to be successful on a globally significant scale. Nonetheless, with strategic application of funding and research effort, these challenges are by no means insurmountable. The presence of the pre-existing framework for area-wide integrated pest management will considerably aid the realisation of the potential of such genetic control programmes.

8.7 **Concluding Statement**

The success of male-release control techniques such as RIDL depends greatly on the released insects being of sufficient quality. The recent emergence of strains such as OX513A as viable candidates for such control techniques has highlighted the lack of knowledge concerning how male insect quality may be effectively assessed and improved. The work in this thesis goes some way to increasing this knowledge base, by demonstrating the relationships between physiological traits and insect performance. The primary performance measures used - longevity, flight, and mating - were chosen based on their relevance to behaviours that released males are required to display in order to successfully achieve their goal of mating with wild-type females. The identification of which factors are most important to male performance is an important step towards improving the efficacy and viability of mosquito control methods based on male releases.

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9 Appendix

9.1 Longevity

9.1.1 Wing Lengths of Males in Experiments 1 and 2

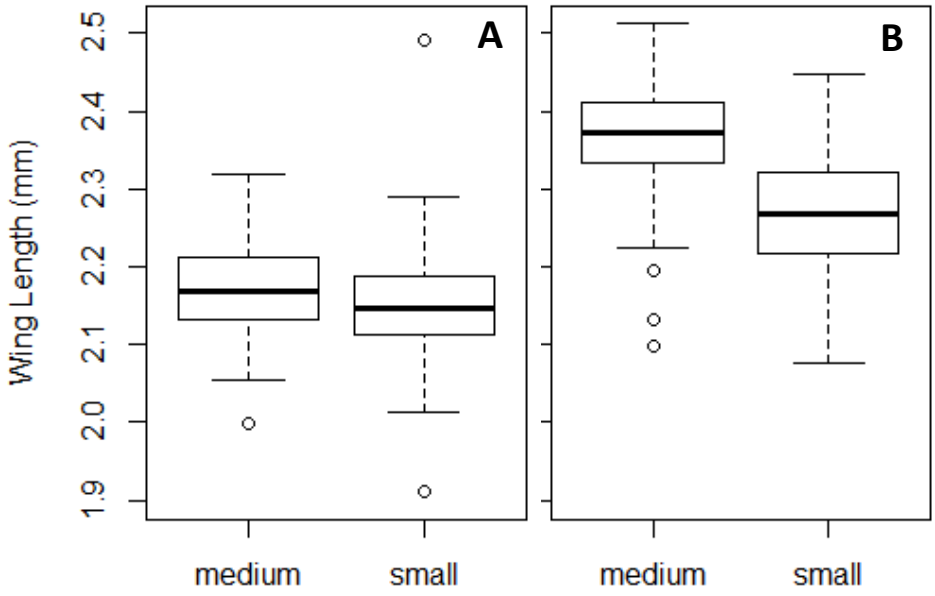


Figure 9-1. Box plot showing wing length distributions of males from (A) Experiment 1, and (B) Experiment 2. For Experiment 1, n=97 and n=100 for medium and small males respectively; for Experiment 2, n=181 and n=171 for medium and small males respectively. In both experiments, small and medium males were significantly different in size, although in Experiment 1 this difference was marginal ($p=0.042$ and $p<0.0001$ for Experiments 1 and 2 respectively, one-way ANOVA).

9.2 Flight Capacity

9.2.1 Distributions of Flight Performance Measures

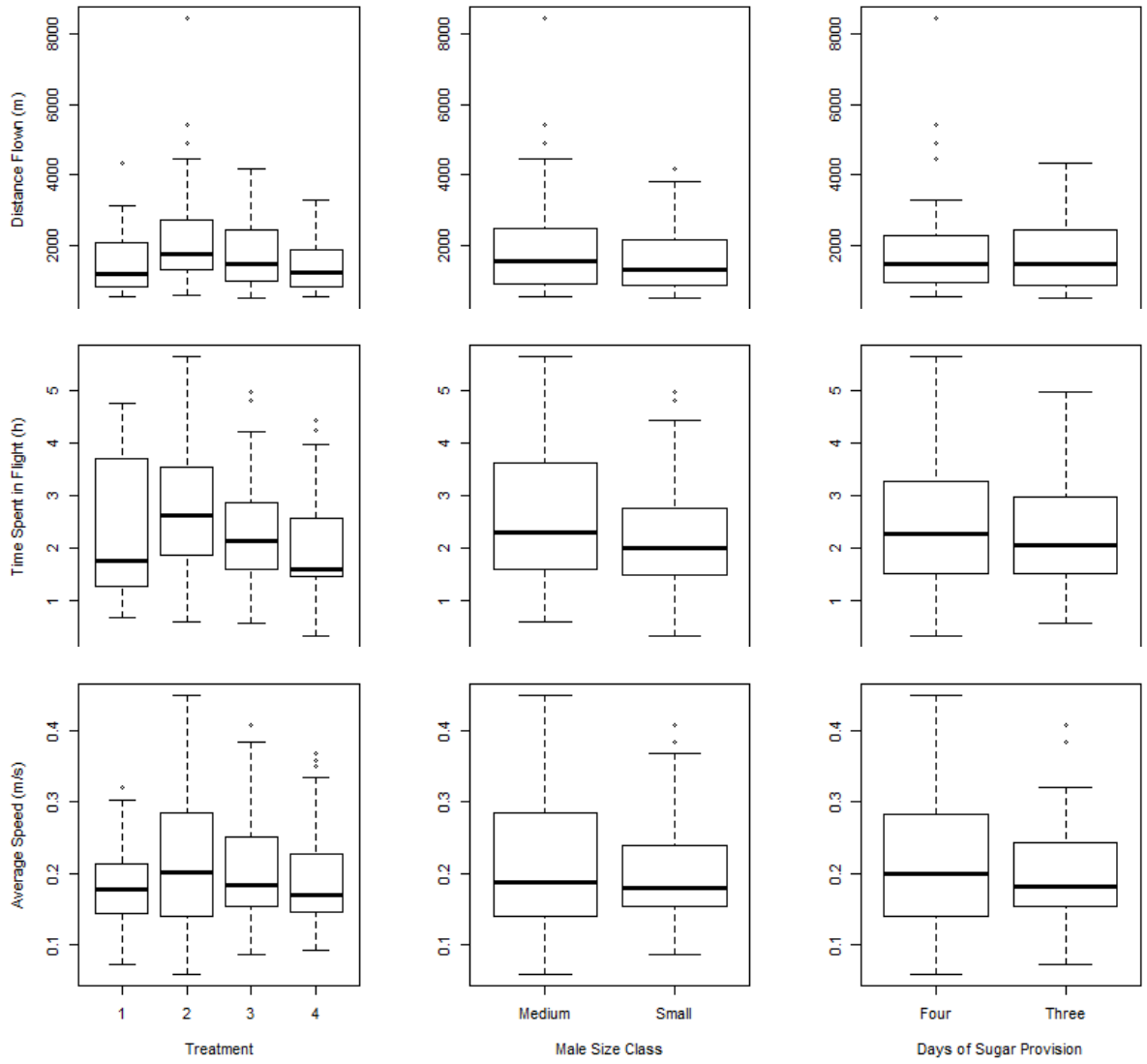


Figure 9-2. Distributions of flight performance measures in flight capacity experiments. Rows (top-bottom) show distance flown, time spent in flight, and average speed (all values are post-correction). Columns (left-right) show data split by treatment, male size class, and days of sugar provision.

9.2.2 Wing Lengths of Male Size Classes

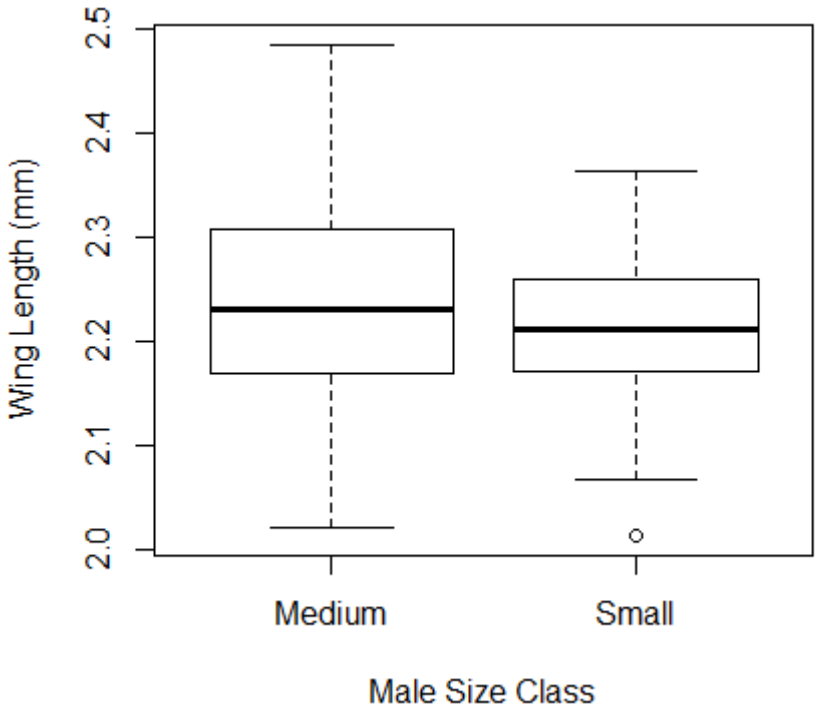


Figure 9-3. Wing lengths of males used in flight capacity experiments (n=103[41]). 'Medium' and 'small' males were reared at 3 and 5 larvae ml^{-1} respectively. Despite the different rearing densities, there was no significant difference in wing length between medium and small males (one-way ANOVA, F=2.62, p=0.109).

[41] 105 flights by inidividual males were used in the final statistical analysis of flight capacity. Of these, two male corpses were accidentally destroyed during attempted subsequent wing length analysis.

9.3 Mating Performance

9.3.1 Mating Capacity

Mating Capacity Experiments - Wing Lengths

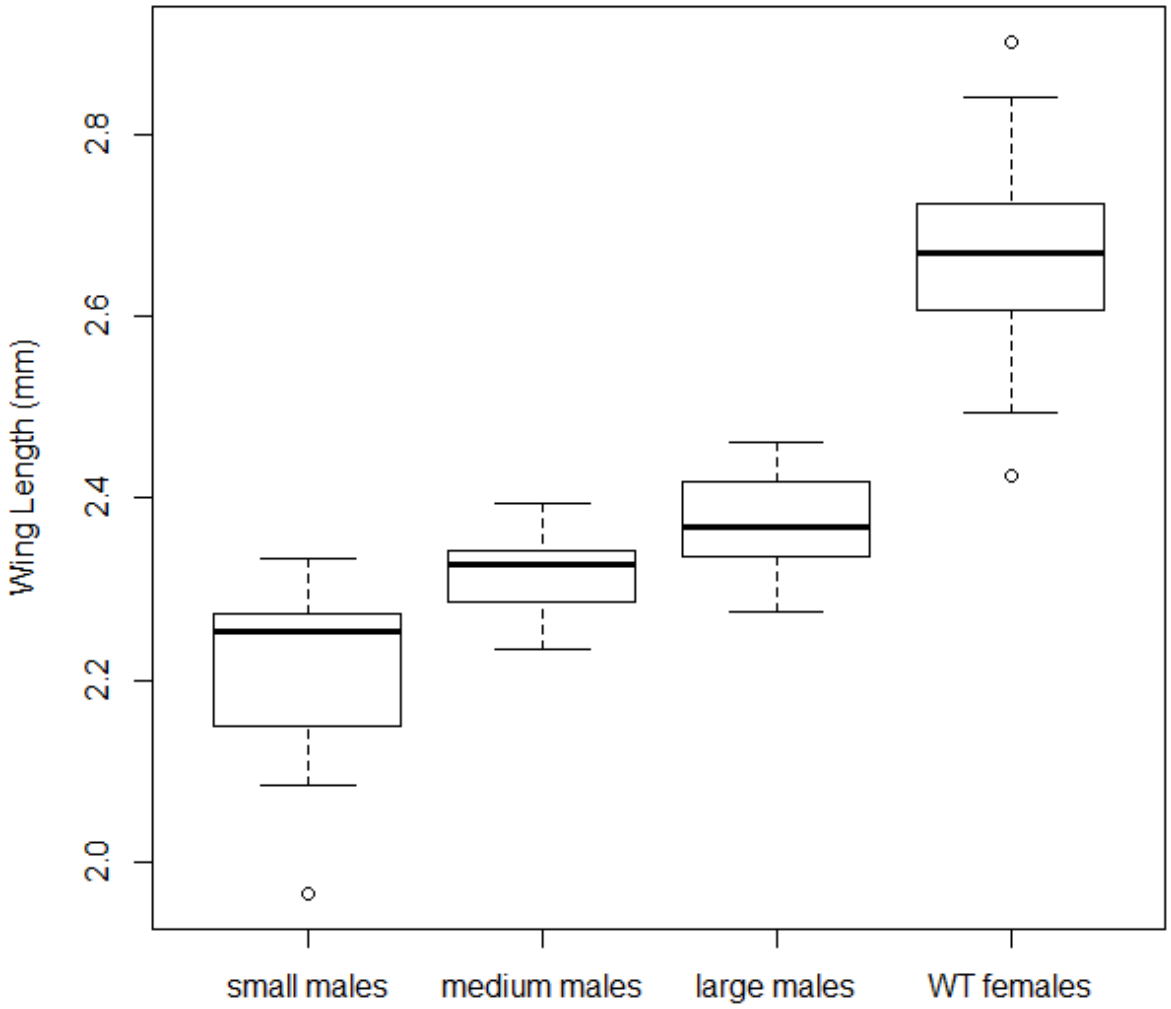


Figure 9-4. Wing lengths of all males and females used in mating capacity experiments. For male groups n≥16, for females n=86. All groups were significantly different from one another (one-way ANOVA with Tukey HSD post-hoc tests)[42].

[42] In actual fact the difference between medium and large males was given as non-significant (p=0.24), but the author attributed this to lack of statistical power, having only n=16. Analysing non-parametrically as an alternative (Kruskal-Wallis test with Wilcoxon tests as post-hoc) gave all the differences as significant.

9.3.2 Mating Competitiveness

Data Handling

The loss of data due to female death was less severe than in the mating capacity experiments, due to each repeat having more females (10 or 15, rather than 5). However, there was still a question of whether mating percentages should be calculated as a proportion of females introduced to the mating arenas (10 or 15), or as a proportion of larval families actually produced. The latter was chosen, because for those females that did not produce larvae and so for which no data existed, it is assumed they were mated in the same OX513A: WT ratio as those that did produce larvae[43]. Mating proportions are normally calculated in this way (Lee et al., 2013b).

In the method of data aggregation used, each mating arena (i.e. each repeat) generated percentages of females mated by each male type, e.g. 45% mated by OX513A males, 55% mated by WT males, 0% multiple mating. The means of these proportions are presented in Figure 7-6. However, using this method each female was responsible for a large proportion of the mating ratio within her repeat. For example, in a hypothetical mating arena with ten females, with two that didn't produce offspring, each egg-laying female represented 12.5% of the total achievable matings by either male type. This may have introduced an inherent lack of experimental resolution at the level of individual repeats. As a potential alternative method of analysis, percentages were separately calculated by pooling all females within a treatment, regardless of repeat number. Interestingly, when compared to the primary method of analysis used (where the mating arenas were used as replicates), percentage values were on average only 1% different. Of course, by summing all females and disregarding repeats, there is no standard deviation value to associate with the percentage result. However, an advantage is that it may be possible to achieve the same result using fewer, larger mating arenas, with more insects per arena. This would be a more efficient way of conducting the experiment, as the introduction and removal of females from many mating arenas was quite time-consuming. Larger mating arenas may also represent a slightly more open and therefore 'natural' mating environment.

[43] There is no reason to think that being mated by a male of a particular strain would increase the likelihood of a female not producing offspring. If this were the case, highly skewed mating percentages would exist in only one direction (e.g. if being mated by an OX513A male led to a decrease in ability to produce offspring, then no mating arenas in which OX513A achieved the heavy majority of matings would exist). In actual fact heavily skewed (as high as 80:20) mating bias - in favour of either strain - did occur occasionally.