Role of semiochemicals in oviposition and mating behaviour of *Aedes aegypti* (Diptera: Culicidae)

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Abstract

Aedes aegypti (Diptera: Culicidae) is the major vector of a number of arboviruses including dengue. Understanding how semiochemicals might mediate the key behaviours of mating and oviposition should lead to a better understanding of the biology of *Aedes aegypti* and might also lead to practical vector control applications. The results of behavioural investigations into putative egg associated oviposition pheromone have been contradictory and incomplete in that they failed to control numbers of eggs in their experimental design. A previous study found behavioural evidence for a male produced sex pheromone, but an upwind anemotactic response of females was not tested and it could be argued that the experimental design was not robust. For this thesis no-choice and choice bioassays were carried out to test the oviposition response of gravid females to both specific numbers of con-specific eggs and methanol extract of eggs. An olfactometer was designed to test the upwind anemotactic response of virgin females to male headspace volatiles with and without host odour cues. These male odours were also used to bait BG-sentinel traps in the field. While no evidence was found that was consistent with an egg associated oviposition pheromone in *Aedes aegypti*, results from both laboratory and field based experiments suggest that males might emit a pheromone in response to host odour that attracts virgin females. Results from experiments in which males were not exposed to host odour suggest that host odour might be required to stimulate pheromone release.

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List of abbreviations

d.f. degrees of freedom

FEP Fluorinated Ethylene Propylene

GC-MS Gas Chromatography – Mass Spectrometry

HPLC High-performance liquid chromatography

IRS Indoor Residual Spraying

ORN Odour Receptor Neuron

ppm parts per million

PTFE Polytetrafluoroethylene

SEM Standard Error of the Mean

SPME Solid Phase Micro extraction

χ^2 Chi-squared

Chapter 1 – General Introduction

1.1 Introduction to *Aedes aegypti*

1.1.1 Taxonomy of *Aedes aegypti*

Aedes aegypti Linnaeus 1762 (Diptera: Culicidae) is a member of the subgenus *Stegomyia* and comprises two subspecies; the original feral form *Aedes aegypti formosus* and the domestic form *Aedes aegypti aegypti* although some authors also recognise a third peridomestic form (Foster and Walker 2002). The terms “feral”, “domestic” and “peridomestic” refer to the extent to which they are associated with human habitation. The recognition of *Aedes aegypti aegypti* and *Aedes aegypti formosus* as two subspecies was first proposed by Mattingly (1957) based on the fact that in East Africa *Aedes aegypti* populations in and around human dwellings were paler than those ovipositing in natural containers such as tree holes. While it has been demonstrated that there are no reproductive barriers between these subspecies (Moore 1979), it is thought that in areas where the two subspecies exist sympatrically the behavioural and morphological distinctions between them are maintained as a result of restricted gene flow between them due to their different habitat preferences (Trpis and Häusermann 1978).

1.1.2 Distribution of *Aedes aegypti*

It is likely that *Ae. aegypti* is of African origin and has been spread throughout the tropical and subtropical world by human activity (Brown *et al.* 2011; Christophers, 1960). *Ae. aegypti formosus* is only found in sub-Saharan Africa

whereas the domestic form *Ae. aegypti aegypti* is found throughout the tropics and subtropics.

Currently, *Ae. aegypti* is found as far north as the southern USA and as far south as Queensland in Australia (Rogers *et al.* 2006). Evidence suggests that the area where *Ae. aegypti* is found has been expanding leading to an increase in dengue infection with an especially marked expansion in the Americas since the 1970s (Kyle and Harris 2008). It is thought that the main means by which *Ae. aegypti* has been spread around the world is by utilising human transportation systems (Soper 1967).

1.1.3 Importance of *Aedes aegypti* as a disease vector

Ae. aegypti is a vector of a number of arboviruses including dengue, yellow fever and chikungunya (WHO 2012; Tomori 2004; Ligon 2006). While a vaccine exists for prevention of yellow fever there are currently no licensed vaccines available to prevent dengue or chikungunya. Several potential dengue vaccines as well as anti-viral drugs that target dengue are currently being developed with several dengue vaccine candidates at the clinical trial stage (Schmitz *et al.* 2011). Chao *et al.* (2012) have recently used a mathematical model to determine the vaccination strategy that would need to be adopted in order to interrupt local dengue transmission should a vaccine become available and suggest that this would require vaccination of adults as well as children. This suggests that even if a vaccine for dengue does become available, the challenge of ensuring high vaccination rates among at risk populations means vaccination will only be effective in combination with an effective vector control programme.

Among viruses transmitted by *Ae. aegypti,* dengue is currently of most concern with the incidence of the disease rapidly increasing during the last decades and 2.5 billion people now at risk mostly in tropical and subtropical areas (WHO 2012). *Aedes albopictus* (Diptera: Culicidae) is also a vector of dengue and chikungunya in some areas of Southeast Asia and the Indian subcontinent, but globally is not thought to be as important a vector as *Ae. aegypti* (Rogers *et al.* 2006). With the exception of some remote areas of South-east Asia and West Africa dengue does not have any animal reservoir and thus efforts to reduce transmission must focus entirely on the vector.

1.2 Control of *Aedes aegypti*

1.2.1 Current control methods

Current efforts to control mosquitoes tend to involve environmental management to reduce potential breeding sites and chemical control.

1.2.1.1 Environmetal management

Environmental management strategies include removal of items likely to hold rain water, frequent emptying and scrubbing of water storage containers, covering water storage tanks and efforts to reduce human-vector contact (WHO 2013). Although *Ae. aegypti* females tend to distribute their egg batch over multiple containers (see section 1.5), it has been suggested that any increase in oviposition in remaining containers should lead to an increase in density dependent larval mortality (Focks and Alexander 2006). Environmental management strategies are logistically difficult and often require cultural change. For example, many potential breeding sites may be on private property and thus strong community participation is necessary for success. Although a recent study

in Cuba found that resistance to change can hinder efforts to promote community participation in *Ae. aegypti* control (Perez 2013), other recent studies in Thailand (Kittayapong *et al.* 2012), Myanmar (Wai *et al.* 2012) and Tamil Nadu, India (Arunachalam *et al.* 2012) reported a reduction in the pupae per person index following community based intervention programmes. However, Morrison *et al.* (2008) have argued that community participation is only high during epidemics and campaigns to encourage community participation only temporarily influence the behaviour of community members.

1.2.1.2 Chemical control

Chemical control strategies include both the use of larvicides which target immature stages and adulticides that target adults. Larvicides can be applied to potential breeding sites although the wide range of habitats in which *Ae. aegypti* are able to breed means that treatment of many breeding sites, especially indoor breeding sites such as plant pots can be impractical. Adulticides can be used for indoor residual spraying (IRS) inside buildings and ultra-low volume spraying or thermal fogging using aircraft and trucks outdoors. Since *Ae. aegypti* routinely rest indoors the efficacy of outdoor spraying of insecticides using trucks and aircraft has been questioned (WHO 1997; Morrison *et al.* 2008) and both the knockdown and killing effect of insecticides was shown to be greater when they are applied inside houses (Mani *et al.* 2005). However, getting access to private property to carry out indoor residual spraying may not always be practical.

Chemical control whether targeted at immature or adult stages of the insect is expensive, labour intensive and in many endemic countries there has only been a willingness to commit resources after a disease epidemic has been declared

(Morrison *et al.* 2008). The efficacy of chemical control against *Ae. aegypti* is also increasingly threatened by resistance to currently used insecticides with levels of resistance tending to be much higher in *Ae. aegypti* than *Ae. albopictus* (Zaim and Guillet 2002; Vontas *et al.* 2012). Attempts have been made to develop new types of insecticides with a distinct biochemical mechanism from current insecticide classes, but the development of new insecticides is slow, costly and subject to regulatory approval in the countries where they are to be used. Furthermore, when Paul *et al* (2006) tested the toxicity of six novel insecticides against *Ae. aegypti,* they found that none of them exceeded the toxicity of a currently used insecticide (permethrin) suggesting that even if new insecticides do become available they may not be as effective as those currently available. Increasing resistance to insecticides in *Ae. aegypti* therefore highlights the need for both improved monitoring and surveillance to enable prompt detection of insecticide resistance as well as the development of alternative control methods that can reduce reliance on insecticides. The use of semiochemicals that alter mosquito behaviour has the potential to aid in both population monitoring and the development of alternative control methods.

1.2.2 Use of semiochemicals in monitoring and control

1.2.2.1 Use of semiochemicals for baiting traps

As noted by Copps (1984) sampling methods used for adult mosquitoes have long included traps baited with carbon dioxide or live hosts such as mice. Odour baited traps are more effective if they are “active” (i.e. incorporating some form of suction device such as a fan) rather than “passive” (Copps 1984). A trap now increasingly used for trapping adult *Ae. aegypti* in the field is the BG Sentinel

trap (Biogents AG, Germany) which can be baited with synthetic kairomone blends such as the BG lure (Biogents AG, Germany). The BG Sentinel trap has recently been used and evaluated for trapping *Ae. aegypti* and other mosquito species by a number of authors. For example, Williams *et al* (2007) have trialed the use of BG Sentinel traps for population sampling of *Ae. aegypti* in north Queensland, Australia. This trap is essentially an "active" baited trap which incorporates a fan and it is claimed by the manufacturers that the convection currents produced by the fan mimic those created by a human body. Although the trap is designed for use with synthetic kairomone blends, additional or alternative attractants can be used (Biogents AG 2011).

A disadvantage of baiting traps designed for haematophagous insects with kairomones is that response to host odours can be common across several haematophagous species that feed on similar hosts and thus traps utilising host odours will not be specific to the target species. Pheromones on the other hand are species specific and therefore pheromone baited traps should be more specific in luring the target insect. A more species specific trap should make monitoring more cost effective as a reduced number of non-target insects being caught in traps should lead to less time needed to sort and identify the insects that had been caught and therefore less manpower would be required. Indeed, the use of pheromones in trapping agricultural pests is widespread. For example, Bentz (2006) has described the use of pheromone baited traps to sample the population of mountain pine beetles, *Dendroctonus ponderosae* (Coleoptera: Curculionidae), in central Idaho, USA. More recently Tillman and Cottrell (2012) have described the placement of pheromone baited traps within trap crops (sorghum) for control of the brown stink bug, *Euschistus servus* (Hemiptera: Pentatomidae) in cotton. Sex

pheromone baited traps can be a reliable means of enabling early detection of the adult population (Mitchell 1977) although they do not provide information about immature stages that do not respond to sex pheromones.

Interest in the use of pheromone baited traps for haematophagous insects is evidenced by the development of pheromone baited traps for the New World sandfly, *Lutzomyia longipalpis* (Diptera: Psychodidae). Morton and Ward (1990) described the use of a pheromone baited sticky trap for catching *Lu. longipalpis* females and more recently Bray *et al.* (2009) have described the use of the synthetic pheromone (+)-9-methylgermacrene-B to bait mechanical light traps as well as sticky traps in the field. Bray *et al.* (2009) were able to develop dispensers that released pheromone at a similar rate to that of aggregating males. More recently Bray *et al.* (2010) have trialled the use of pheromone baited sticky traps in chicken sheds for catching *Lu. longipalpis* and found that yellow sticky traps baited with pheromone caught significantly more males than traps not baited with pheromone.

Sex pheromone baited traps may also aid in the control of agricultural pests. In fact use of sex pheromones for mass trapping has long been a useful tool for the control of many agricultural pests. For example, Taschenberg *et al* (1974) described the use of a female produced sex pheromone for the mass trapping of the redbanded leafroller moth, *Argyrotaenia velutinana* (Lepidoptera: Tortricidae). More recently Aurelian *et al* (2012) have investigated the use of a female produced sex pheromone in combination with a source of kairomones for the mass trapping of the apple clearwing moth, *Synanthedon myopaeformis* (Lepidoptera: Sessiidae). For agricultural pests it is often both the males and females that can

damage crops and so aggregation pheromones that attract both males and females are more useful than pheromones which only attract one sex (Mitchell 1977). However, in the case of mosquitoes, a trap baited with a male produced sex pheromone that only attracted females should be adequate to interrupt disease transmission since male mosquitoes do not blood feed and so do not play a role in disease transmission.

Traps can also be baited with attractants that attract gravid females. Indeed, volatile attractants have already been used to increase the attractiveness of ovitraps to *Culex quinquefasciatus* (Diptera: Culicidae). For example, Barbosa *et al*. (2007) describe the use of the synthetic oviposition pheromone erythro-6-acetoxy-5-hexadecanolide which along with 5 to 20 day old grass infusion was found to increase the attractiveness of the ovitrap, named “BR-OVT” which they had developed. Meanwhile, sticky ovitraps baited with attractants from organic infusions have recently been evaluated in Wuhan Province, China for the surveillance of *Ae. albopictus* (Zhang and Lei 2008). Prakash *et al.* (2007) have also patented the use of n-heneicosane as an oviposition attractant for baiting ovitraps for controlling or monitoring *Ae. aegypti.* In a field trial, water tanks that were treated with 1% w/v n-heneicosane contained *Ae. aegypti* larvae after 15 days, but adjacent water tanks that were not treated did not, suggesting that n-heneicosane displaced oviposition from taking place at adjacent water tanks by attracting those gravid females in the vicinity. The patent includes the use of n-heneicosane in combination with an insect growth regulator, such as diflubenzuron, that acts as a larvicide. Bhutia *et al* (2010) have tested the mammalian toxicity of n-heneicosane in combination with diflubenzeron, with a view to the development of n-heneicosane baited lethal ovitraps and were unable

to find any significant toxic effects on rodents. This demonstrates how advances in understanding the chemical ecology of *Ae. aegypti* can lead to the development of new and promising control strategies.

1.2.2.2 Other uses of semiochemicals in control

Sex pheromones can also be used to control insect populations by mating disruption whereby the release of the pheromone over a large area prevents mate location. For example, Roelofs *et al.* (1976) describe the use of this technique for control of both redbanded leafroller moth, *Argyrotaenia velutinana* (Lepidoptera: Tortricidae) and the grape berry moth, *Endopiza viteana* (Lepidoptera: Tortricidae) in grape vineyards. More recently Cocco *et al.* (2013) have tested mating disruption using sex pheromones to control the tomato leafminer, *Tuta absoluta* (Lepidoptera: Gelechiidae) in greenhouse tomato crops and found that this technique significantly reduced the percentage of fruits damaged by this pest.

The push-pull or stimulo-deterrent diversionary strategy is another way in which pheromones could be exploited for control of insects. r Pickett *et al.* (1997) have summarised this strategy as the use of repellent semiochemicals to “push” insects away from their target host or oviposition site coupled with the induced aggregation by semiochemicals of the insects in some form of lethal trap containing insecticide or insect growth regulator. For example, both ovipositional repellents and attractants could be utilised in a “push-pull” control strategy whereby the attractiveness of traps is further enhanced by the use of repellents in the normal oviposition sites of the mosquito as discussed by Cook *et al*. (2007). Xue *et al*. (2005) have shown that in *Ae. albopictus* forced egg retention over a prolonged period can reduce both the fecundity and fertility of mosquitoes

suggesting that even on their own, repellents could potentially reduce population size over time. However, Navarro-Silva *et al*. (2009) urge caution with regard to this approach to ensure that use of repellents does not result in population dispersal leading to introduction of the mosquito and its associated diseases in previously unaffected areas.

1.2.3 Novel control technologies and the need to better understand insect behaviour

Some recent research has focused on developing transgenic mosquitoes with the aim of population suppression or population replacement (e.g. Lacroix *et al.* 2012; Harris *et al.* 2011; Mathur *et al.* 2010). Population suppression relies on the release of transgenic sterile males which have been genetically engineered so that their offspring are not viable in the field, because they contain a lethal transgene which unless it is repressed by the presence of tetracycline (i.e. when they are being mass reared in the laboratory) causes death at the late larval or early pupal stage. Open field trials with the mass release of these males have recently been conducted in the Cayman Islands (Harris *et al.* 2011) and Malaysia (Lacroix *et al.* 2012). Harris *et al* (2011) found that genetically modified males successfully mated with and inseminated wild females in the field although Lacroix *et al.* (2012) found that genetically engineered males dispersed shorter distances on average compared with a laboratory strain that was not genetically modified. Population replacement would rely on the mass release of insects genetically engineered to be refractory to the virus which ideally would eventually replace the wild type population (Huang *et al.* 2007; Mathur *et al* 2010) although this technology is still in its infancy.

Another novel technology which has been proposed is the use of *Wolbachia* infection to prevent *Ae. aegypti* from carrying arboviruses. Bacteria of the genus *Wolbachia* are cytoplasmically inherited endosymbionts which are present in the reproductive tissues of arthropods (Werren 1997). *Wolbachia* can cause reproductive modifications in their host insects such as cytoplasmic incompatibility whereby uninfected females are unable to mate successfully with infected males and since this benefits females which are infected, *Wolbachia* infections tend to spread. Strains of *Wolbachia* have been found which can inhibit replication of viruses in the mosquito midgut including both dengue (Bian *et al.* 2010) and more recently yellow fever and chikungunya (van den Hurk *et al.* 2012). *Ae. aegypti* have been transfected with various strains of *Wolbachia pipientis* with the aim of reducing their potential to carry viruses and a recent field trial in Australia found that *Wolbachia* infected mosquitoes were able to successfully invade wild populations (Hoffmann *et al.* 2011). More recently Turley *et al.* (2013) found that *Wolbachia* infected males were able to repeat mate as frequently as uninfected males.

The practical application of these techniques will require a better understanding of the reproductive behaviour of *Ae. aegypti*. For example, sterile males released in the field will be competing with wild type males for access to females and ensuring the sterile males can effectively compete requires an understanding of mating behaviour. Deepening our knowledge of the reproductive behaviour of the insect will also involve greater emphasis on studying males, an area traditionally neglected due to the fact that male mosquitoes do not blood feed and therefore do not transmit diseases. This has been recognised by Ponlawat and Harrington (2009) who looked at factors affecting male mating success in *Ae.*

aegypti. Ponlawat and Harrington (2009) found that older and larger males were more succesful in inseminating females than younger and smaller males. The authors acknowledged that other factors which were not studied might also impact on male mating success and this would include the type of mate finding behaviours that occur in the field. Recently, Lee *et al.* (2013) tested the mating competitiveness of a type of transgenic *Ae. aegypti* (genetically engineered to produce unviable offspring) with that of wild type *Ae. aegypti* under semi-field conditions and did not find any significant difference between the transgenic and wild type strains. Nevertheless, a more comprehensive understanding of the phenotypic traits that affect mating competitiveness and the discovery of their genetic basis might enable the development of transgenic males that are able to out-compete the wild type strain when competing for wild type females in the field.

1.3 Life cycle of *Aedes aegypti*

All mosquitoes have immature aquatic stages as well as a mature terrestrial stage and it is during the terrestrial stage that mating, blood feeding and oviposition take place. Following mating and during a gonotrophic cycle an adult female mosquito will go through several behavioural phases including host seeking, blood feeding, resting, pre-oviposition and oviposition (Clements 1999). Eggs laid by a gravid female mosquito will hatch into larvae which then moult four times before developing into pupae that subsequently emerge as adults (Bates 1949). Eggs of mosquitoes in the subgenus *Stegomyia* such as *Ae. aegypti* are resistant to desiccation and can lie dormant for many months which it seems is an adaptation to the transient nature of the sites used for oviposition by these species (Bates 1949). Unlike *Anopheles* and *Culex* spp., mosquitoes of the subgenus *Stegomyia* are diurnal and so blood feed and mate during the photophase. This

thesis will focus on the regulation by semiochemicals of two important behavioural phases, namely oviposition and mating behaviour.

1.4 Volatile semiochemicals and insect behaviour

The ability of an insect to detect and respond to external stimuli is vital for its survival as well as its ability to reproduce. External stimuli include convection currents emanating from a warm object (Peterson and Brown 1951), light intensity (Chadee and Martinez 2000), visual factors (Sippell and Brown 1953) and various chemical stimuli which will be discussed. As far as the role of external stimuli in host location is concerned, visual factors are thought to be more important when the host is moving while chemical stimuli may play a greater role in the case of a stationary host (Kennedy 1940; Sippell and Brown 1953). Chemicals in the environment together with other environmental stimuli and the internal state of the central nervous system are thought to determine the behaviour of an individual insect (Städler 1984). The physiological state of the insect can also be important in determining how the individual insect responds to stimuli and recently Bohbot *et al.* (2013) have shown that prior to 24 hours post-emergence *Ae. aegypti* do not respond to either carbon dioxide or carbon dioxide plus octenol whereas after 24 hours post-emergence they respond to both.

It is thought that often one stimulus may make an insect more receptive to another stimulus and this can lead to the development of a behavioural momentum that may for example lead to the location of a vertebrate host by a haematophagous insect (Lehane 2005). In the case of host location by haematophagous insects the development of this behavioural momentum has been divided into three phases: i) appetitive searching whereby the insect

engages in behaviour that increases its chance of coming into contact with host derived stimuli (this behaviour is regulated by the internally programmed circadian rhythm of the insect and its physiological state), ii) activation whereby the insect responds to host derived stimuli by actively seeking to locate the host and iii) attraction whereby specific host derived stimuli are used to guide the insect into the immediate vicinity of the host (Sutcliffe 1987).

The mechanism by which an insect is able to sense a chemical compound in the environment will depend on whether it is air borne and the degree of volatility of that compound under physiological conditions. Volatile air borne chemical stimuli termed olfactory stimuli play an important role in modulating insect behaviour and it is these air borne chemical stimuli that this thesis will focus on. Chemicals capable of inducing a behavioural response are known as semiochemicals and are often divided into pheromones that act on members of the same species and allelomones that act on members of different species (Sbarbati and Osculati 2006). Allelomones can in turn be divided into allomones, kairomones and synomones based on whether they are advantageous or disadvantageous to either the producer or receiver. Allomones are advantageous to the emitting species while being disadvantageous to the receiving species, kairomones are disadvantageous to the emitting species while benefiting the receiving species and synomones benefit both the emitter and receiver. (Sbarbati and Osculati 2006).

This thesis focuses primarily on pheromones. The term “pheromone” was first proposed by Karlson and Butenandt (1959) to differentiate those substances which mediate communication between different individuals of the same species from hormones which mediate communication within an individual organism.

Insect pheromones are normally hydrocarbons which may contain double bonds, have varying functional groups and may form either aliphatic branched chain or cyclic structures (Luntz 2003). Their structural variation in addition to geometrical and optical isomerism aids species specificity. Geometrical isomerism can also affect the bioactivity of pheromones (Mori 2007). For example, in the case of the sex pheromone produced by the female olive fruit fly *Dacus oleae* (Diptera: Tephritidae), one enantiomer affects males while the other enantiomer affects females (Haniotakis *et al*. 1986).

In Diptera, straight chain aliphatic alcohols, acetates and aldehydes have been identified as sex pheromones. The molecular weight of volatile pheromones is limited by the need for them to have low boiling points so they evaporate under environmental conditions despite compounds with higher molecular weights being able to contain more information. (McCall and Cameron 1995). As can be seen from Table 1.1, many pheromones have a molecular weight of between 200 and 400 which must be a balance between the need to evaporate under environmental conditions and the need to carry adequate information. However, the volatility of a compound may not be the only factor determining its rate of diffusion into the environment. For example, the larval origin compound thought to act as an oviposition pheromone in *Ae. aegypti*, heneicosane (molecular weight: 297; C21), has a boiling point of 100°C, but is non-polar and so will form a thin film over the surface of water thereby increasing its surface area and facilitating more rapid diffusion of the pheromone into the environment (Seenivasagan *et al*. 2009).

Table 1.1 – Examples of compounds that act as volatile pheromones and their molecular weights

Pheromone Compound	**Molecular weight***	**Species**	**Reference**
neryl (S)-2-methylbutanoate	238	*Frankliniella occidentalis*	Hamilton *et al.* 2005
n-heneicosane	297	*Aedes aegypti*	Ganesan *et al.* 2007
erythro-6-Acetoxy-5-hexadecanolide	312	*Culex quinquefasciatus*	Lawrence and Pickett 1982
(Z)-9-tricosene	323	*Musca domestica*	Carlson *et al*. 1971
(R)-lavandulyl acetate	196	*Frankliniella occidentalis*	Hamilton *et al.* 2005
(S)-9-methylgermacrene-B	218	*Lutzomyia longipalpis; Lutzomyia cruzi*	Hamilton *et al.* 1996

*Molecular weights obtained from “The Pherobase” (El-Sayed 2012)

1.5 Oviposition behaviour

The domestic form of *Ae. aegypti*, *Ae. aegypti aegypti* shows a remarkable flexibility in the types of habitats it will oviposit in although it is especially known for ovipositing in manmade containers such as those shown in Figure 1.1. Oviposition sites can include any area of standing rain water such as uncovered water storage tanks, discarded tyres and discarded food and drinks containers and thus the increasing spread of this mosquito along with the viruses it transmits has often been associated with the rapid urbanization taking place in many tropical and subtropical countries (Orozco 2007) where these containers are increasingly common. On the other hand *Ae. aegypti formosus* tends to oviposit in natural containers such as treeholes rather than man made containers (Mattingly 1957) although in some domestic situations in Africa it has adapted to oviposit in rain filled containers (Foster and Walker 2002).

Figure 1.1 – Examples of man-made containers used as oviposition sites by *Aedes aegypti*: Any area of standing water may serve as an oviposition site for *Ae. aegypti* including; **A:** buckets filled with water, **B:** uncovered water storage tanks and **C:** discarded rubbish that can hold rainwater [Photo: Burhani 2011]

Based on mark recapture experiments, *Ae. aegypti* is thought to oviposit over a number of sites in a process known as “skip oviposition”, whereby eggs from the same batch are disseminated over several containers (Corbet and Chadee 1993; Reiter 2007). The term “skip oviposition “ was first proposed by Mogi and Mokry (1980) after studying the oviposition behaviour of *Wyeomyia smithii* (Diptera: Culicidae) which they found laid eggs from the same batch among more than one pitcher plant. Skip oviposition behaviour has also been reported in

Ae albopictus (Trexler *et al.* 1998), but other mosquito species such as those in the *Culex* genus lay their entire egg batch together. More recently laboratory studies by Snell *et al.* (2010) suggest that the rockpool mosquito *Opifex fuscus* (Diptera: Culicidae) may also exhibit skip oviposition behaviour. Skip oviposition although requiring greater energy expenditure is thought to enable gravid females to reduce sibling competition and distribute risk (Harrington and Edman 2001).

Once a female mosquito has taken a blood meal, its behaviour switches from host seeking to pre-oviposition behaviour. Pre-oviposition behaviour can be defined as the behaviour of gravid females leading to arrival at potential oviposition sites and the analysis of their suitability (Clements 1999). For example, Davis (1984) showed that Odour Receptor Neurons (ORNs) sensitive to the host odour lactic acid are down-regulated following a blood meal. A number of environmental factors can influence the attractiveness of a potential oviposition site and their effect can be species specific. For example, Metge and Hassaine (1998) found eggs of *Aedes caspius* (Diptera: Culicidae) at higher densities in soils with a low salinity to organic matter ratio while the reverse was true for *Aedes detritus* (Diptera: Culicidae). Navarro *et al*. (2003) have since found a negative correlation between oviposition in *Ae.* a*egypti* and salinity with oviposition ceasing to occur at salinities greater than 12%. More recently Vonesh and Kraus (2009) have found evidence that pesticide contamination may impact selection of oviposition sites with pools contaminated with carbaryl insecticide receiving 12-fold more *Culex* eggs although no such impact was observed in the case of *Anopheles* spp. It is not clear whether this reflects a behavioural response to the insecticide itself or if gravid females were actually responding to lower numbers of conspecifics and/or predators due to pools containing insecticide also containing

less conspecifics and/or predators (due to the insecticide being lethal to conspecifics and potential predators).

The selection of oviposition sites may also be affected by biological factors including the presence of predators and cues of future competition (Edgerly *et al.* 1998; Munga *et al.* 2006). For example, there is evidence that gravid *Ae. aegypti* avoid oviposition sites containing larvae parasitized by *Plagiorchis elegans* (Trematoda: Digenea), thereby reducing the risk that their progeny will be parasitized (Lowenberger and Rau 1994). As reported by Wang *et al* (2008), learned behaviour modulates oviposition preference in some phytophagous insects and McCall (2002) has reviewed some studies suggesting a similar ability in haematophagous insects.

1.5.1 Oviposition pheromones

Egg associated oviposition pheromones were first suggested in mosquitoes by Osgood (1971) who showed that gravid *Culex tarsalis* (Diptera: Culicidae) were attracted to concentrated ether washes of *Cu. tarsalis* eggs. Ether washes of *Cu. tarsalis* eggs were also attractive to *Culex pipiens* (Diptera: Culicidae), but were not attractive to *Ae. aegypti*. Starratt and Osgood (1972) found that an active fraction of extract of these eggs contained a mixture of 1,3-diglycerides which when subjected to methanolysis yielded methyl esters of mono- and dihydroxy fatty acids. Since then, studies have suggested evidence for oviposition pheromones in other Diptera including sand flies (El Naiem and Ward 1991; El Naiem and Ward 1990). The oviposition pheromone present on the eggs of the sand fly *Lu. longipalpis* was isolated using HPLC fractionation by Dougherty *et al.*

(1994) and identified as dodecanoic acid using gas chromatography-mass spectrometry (GC-MS) by Dougherty and Hamilton (1997).

Compounds of both larval origin (Mendki *et al.* 2000; Seenivasagan *et al*. 2009) and egg origin (Ganesan *et al.* 2006) have been identified which affect oviposition behaviour in *Ae. aegypti.* However, it is not clear how concentrations of these compounds tested in bioassays relate to numbers of eggs or larvae found in the field and thus at the concentrations at which they exist in the field these compounds might not have the same effect on oviposition behaviour. The fact that a compound is biologically active at a certain concentration in the laboratory may not necessarily mean that it acts as a pheromone in the field. For example, despite an oviposition pheromone having been identified in *Lu. longipalpis,* there is currently no field based evidence for aggregated oviposition in this species and Wasserberg and Rowton (2011) failed to find any significant effect of conspecific eggs on oviposition by *Lu. longipalpis* in the absence of old sand fly colony remains.

1.5.2 Egg aggregation

Given that *Ae. aegypti* distribute their eggs over many sites the ability of gravid females to oviposit in the vicinity of pre-existing conspecific eggs might be evolutionarily advantageous as it would reduce the risk of isolation of individual eggs and therefore of larvae and thus might be expected to provide easy access to mates for the emerging adults. Nevertheless, as acknowledged by McCall and Cameron (1995) there is a trade-off between the potential advantages and disadvantages of egg aggregations since larger aggregations of larvae will result in greater competition for resources. *Ae. aegypti* often oviposit in small containers

which may not contain resources to sustain many developing larvae and thus high larval densities can be a major cause of larval mortality in container breeding mosquitoes such as *Ae. aegypti* whereas for other mosquito species that tend to breed in larger ground pools predation is a greater risk (Washburn 1995). It therefore might be expected that egg aggregation and conspecific attraction would be less advantageous in container breeding mosquitoes such as *Ae. aegypti* and that presence of conspecifics might even deter/repel oviposition.

However, evidence for aggregation of conspecific eggs at oviposition sites is contradictory although a study by Williams *et al.* (2008) suggests it may be density dependent with 11-38 pre-existing eggs eliciting a positive oviposition response while other densities of pre-existing eggs had no effect. Interestingly Chadee *et al.* (1990) also suggested that *Ae. aegypti* might be able to distinguish between conspecific eggs and eggs they had laid themselves; however although gravid females laid eggs in a higher percentage of ovipots containing conspecific eggs than eggs they had laid themselves this was not actually significant. Nevertheless the suggestion by Chadee *et al.* (1990) elicits the question as to whether kin selection plays any role in *Ae. aegypti* oviposition site selection and whether the survival and successful development of eggs yet to be oviposited takes precedence over the survival of progeny from eggs laid previously. Determining the oviposition response to different numbers of conspecific eggs might help to shed light on this question.

A clearer understanding of how pre-existing eggs at an oviposition site influence subsequent oviposition by gravid *Ae. aegypti* females would also help

elucidate whether egg associated compounds identified by Ganesan *et al.* (2006) are likely to function as pheromones at the ecological level.

1.6 Mating behaviour

Copulation in *Ae. aegypti* tends to occur in flight (Bates 1949). Once a female mosquito has been inseminated it becomes refractory to further mating which in *Ae. aegypti* can occur within only 10 seconds following successful insemination (Clements 1999). Male *Ae. aegypti* mate repeatedly during their lifetime but it was previously assumed that females *Ae.aeggypti* tended to be refractory to mating following their first insemination due to insertion of male accessory gland proteins (Dickinson and Klowden 1997); however recent evidence suggests that a small but significant proportion of female *Ae. aegypti* may contain sperm from more than one male (Helinski *et al.* 2012) indicating a low level of polyandry in this species.

While convergence of male and female flight tones is known to be responsible for sexual recognition in *Ae. aegypti* (Cator *et al.* 2009), it is thought that male *Ae. aegypti* are only able to distinguish the flight tone of a virgin female within a distance of 150-300 mm (Clements 1999). This suggests that while auditory cues maybe the most important cues when male and female mosquitoes are already within less than 300 mm of each other, other cues are required to bring male and female mosquitoes into close enough proximity to detect auditory cues.

Since *Ae. aegypti* is a diurnally active species vision might be expected to play a greater role in facilitating mating than in a nocturnally active species.

However, while *Ae. aegypti* is very sensitive to light it has been shown to have poor visual acuity (Muir *et al.* 1992). Nevertheless, Kawada *et al.* (2005) showed that complete darkness during the daytime deactivates the host seeking activity of both *Ae. aegypti* and *Ae. albopictus* females, which the authors suggested was due to the indispensable role of visual cues in host-seeking behaviour. It is therefore possible that visual cues may also play a role in mating behaviour although this has not been investigated to date.

Host location cues may play a role in facilitating mating in *Ae. aegypti* since this species has been reported to mate in the vicinity of vertebrate hosts (Hartberg 1971) and males have also been shown to be attracted to human bait (Nelson *et al.* 1978). This contrasts with other species of mosquito such as *Ae. iriomotensis* and *Culiseta inornata*, where males remain near the emergence site and mate with newly emerged females (Miyagi and Toma 1981). Indeed field observations in East Africa have shown that between 12 and 30% of landing catches consisted of males suggesting the attraction of both male and female *Ae. aegypti* to vertebrate hosts may be the main way by which the sexes are brought together to facilitate mating (Hartberg 1971). Attraction of male and female mosquitoes to vertebrate hosts involves both visual and chemical cues and thus these cues could be argued to facilitate mating. Any involvement of host odour cues in facilitating mating would be similar to the situation with regards to phytophagous insects where chemicals from host plants were found to enhance response to sex pheromones (Landolt and Phillips, 1997; Reddy and Guerrero, 2004). This also might explain why unmated blood fed mosquitoes continue to engage in host seeking behaviour as host seeking behaviour is likely to increase their chances of finding a mate (Fernandez and Klowden 1995).

1.6.1 Swarming Behaviour and its Role in Facilitating Mating

Swarming involves the aggregated, co-ordinated flight of insects at specific times where individual members fly in loops or zig-zag within a limited space (Clements 1999). Copulation in mosquitoes has often been associated with male swarming (Bates 1949) and at the beginning of the twentieth century Knab (1906) described observations of swarming behaviour in *Culex spp* in the field. More recently Gibson (1985) analysed video recordings of swarming by *Culex quinquefasciatus* and described how males in the swarm responded to the wing beat of females. Recently Assogba *et al.* (2010) have identified males from swarms of two subspecies of *An. gambiae s.l.* and found that swarms mostly contained males of the same subspecies. Most reports of mosquito swarming in the field noted that swarms tended to form around a stationary visual marker suggesting that visual cues are important in swarming behaviour. For example, Charlwood *et al.* (2002) found that *An. gambiae* in São Tomé formed swarms 2-3m above markers exhibiting horizontal contrast such as between grassy areas and footpaths or bushes.

Field observations by Hartberg (1971) and laboratory observations by Cabrera and Jaffe (2007) suggest that in the case of *Ae. aegypti* swarming takes place in response to host cues although it is not clear whether olfactory or visual cues emanating from the host are of more importance. Field studies by Gubler and Bhattacharya (1972) suggest that host cues may also be an important trigger for swarming in other species in the subgenus *Stegomyia* and Cabrera and Jaffe (2007) have suggested that swarming around a host rather than a stationary marker is one of the factors that differentiates the type of swarming that occurs in *Stegomyia spp.* from that which occurs in other mosquito species. Cator *et al.*

(2011) noted that male *Ae. aegypti* appeared around a human observer within 5 minutes and swarms comprising mostly males formed within 1m of the human observer's legs; however this study focused on the role of auditory cues within the swarm rather than chemical cues.

It is possible that in the field mating in the subgenus *Stegomyia* is mostly associated with the formation of male swarms around vertebrate hosts in which individual males attempt to intercept females that are attracted by odours emanating from the vertebrate host. Indeed Cabrera and Jaffe (2007) reported that in the laboratory only males formed swarms and field observations of *Ae. albopictus* by Gubler and Bhattacharya (1972) suggest males may form swarms around a human host prior to the first females arriving.1.6.1 Role of pheromones in mating behaviour

In many insects mate attraction is mediated by sex pheromones (Rafaeli 2009). The first Dipteran sex pheromone was identified in *Musca domestica* (Diptera: Muscidae) as (Z)-9-tricosene by Carlson *et al*. (1971) and since then sex pheromones have been identified in many more species of Diptera. So far no sex pheromones have been identified in mosquitoes although behavioural evidence for both contact sex pheromones (Nijhout and Craig 1971; Lang and Foster 1976) and volatile sex pheromones (Cabrera and Jaffe 2007) has been presented.

1.6.1.1 Evidence for contact sex pheromones in mosquito mating

Evidence for a contact sex pheromone in *Ae.* aegypti comes from a study by Nijhout and Craig (1971). When a female *Ae. aegypti* mosquito was suspended in a cage with males of *Ae. albopictus,* the males were attracted when the female *Ae. aegypti* beat her wings, but upon contact flew away. Nijhout and Craig (1971)

therefore suggested that species recognition in *Aedes* spp. is mediated by a female produced contact sex pheromone, although there are no reports in the literature of such a contact pheromone being subsequently identified.

There have also been studies suggesting a role for contact sex pheromones in other species of mosquitoes although again the contact pheromones were never subsequently identified. For example, Lang and Foster (1976) while failing to find evidence for a volatile sex pheromone in *Culiseta inornata*, nevertheless did find that contact with female legs resulted in male copulation attempts. Lang and Foster (1976) concluded that stimuli from female legs was tactile and chemical rather than visual since dried leg sets did not have the same effect.

It is possible that if contact sex pheromones are important in facilitating mating then epicuticular hydrocarbons may be responsible. While most of the interest in epicuticular hydrocarbons in mosquitoes has centred around their role in age determination (e.g. Desena *et al*. 1999), it is possible they might also play a role in mating behaviour since apart from their physical waterproofing properties they have been shown to act as sex pheromones during courtship behaviour in *Drosophila melanogaster* (Ferveur 1997). Horne and Priestman (2002) have attempted to chemically characterize the epicuticular hydrocarbons of *Ae. aegypti* and while the qualitative results were similar to those previously reported for other mosquito species, there were significant quantitative differences. Cuticular hydrocarbons may also play a role in signalling to males whether a female has already mated since Polerstock *et al*. (2002) have already shown that mating

alters the cuticular hydrocarbons in females of both *Ae. aegypti* and *An. gambiae sensu stricto*.

1.6.1.2 Evidence for volatile sex pheromones in mosquito mating

Cabrera and Jaffe (2007) reported behavioural evidence for a male produced pheromone in *Ae. aegypti* that might facilitate aggregation of males and females for the purpose of mating. When virgin females were exposed to air blown over virgin males there was a significant increase in the proportion of virgin females flying for more than 60 seconds. The paper by Cabrera and Jaffe (2007) is the only published study that has specifically addressed the question of whether or not volatile pheromones modulate mating behaviour in in any species of mosquito and there have not been any papers published since investigating their findings. There is thus a need to confirm whether male *Ae. aegypti* emit a pheromone that attracts female *Ae. aegypti* mosquitoes as asserted by Cabrera and Jaffe (2007). If such an assertion could be confirmed by olfactometer based experiments then it would be a major contribution to our understanding of the mating behaviour of *Ae. aegypti* and no doubt lead to a renewed interest in the possible existence of volatile sex pheromones in other mosquito species. Knowing whether it is males that actively select females or females that actively select males might give an indication as to whether male-produced sex pheromones or female-produced pheromones are more likely. However, as acknowledged by Ponlawat and Harrington (2009) it is not currently clear whether it is males or females that are the main selecting sex in *Ae. aegypti.* Nevertheless given the fact that swarms of *Ae. aegypti* are comprised mostly of males (Hartberg 1971; Cator *et al.* 2011) it might be concluded that a male produced pheromone would be more likely since it would increase the chances of virgin females entering the swarm.

Interestingly, in the sandfly *Lu. longipalpis* where mating also takes place in the vicinity of a host, it is the males that release a volatile pheromone to attract females and thus this might suggest that a male produced pheromone is more likely to occur in *Ae. aegypti* than a female produced pheromone. Evidence for sex pheromones in *Lu. longipalpis* males was first demonstrated by Ward (1986) who showed that females were preferentially attracted to hosts in the presence of male tergal gland extracts.

The sex pheromones produced by male sandflies may also play a role in facilitating host location as Morton and Ward (1989) found extracts of male pheromone glands enhanced the attractiveness of a host to females. Furthermore, Bray and Hamilton (2007) showed that addition of host odour increased the number of virgin females attracted to sex pheromone. This suggests that if a volatile pheromone is in fact involved in *Ae. aegypti* mating behaviour it is likely to interact either synergistically or additively with host odour. Alternatively (or additionally) host odour might play a role in stimulating production or release of sex pheromone as is the case for certain phytophagous insects (Landolt and Phillips 1997; Reddy and Guerrero 2004).

1.7 Rationale for thesis

The rapid spread of *Ae. aegypti* populations along with the arboviruses they transmit and especially dengue is likely to continue as a result of urbanization and global travel. There is thus a need for improved tools for the surveillance and monitoring of this mosquito as well as alternative control strategies that overcome increasing resistance to insecticides. As discussed, pheromones have already been used successfully in both the monitoring and control of a number of insect

pests and thus identification of pheromones mediating important behaviours in *Ae. aegypti* could be put to good use.

There is evidence in the literature for the existence of compounds of egg and larval origin that mediate oviposition in *Ae. aegypti*, but there is a need to re-examine behavioural evidence for pheromone mediated oviposition to determine whether these compounds are biologically active at the ecological level.

No volatile sex pheromones have as yet been identified from any species of mosquito although Cabrera and Jaffe (2007) have presented some behavioural evidence for their existence in *Ae. aegypti*. However, Cabrera and Jaffe (2007) measured the flight response of virgin females to male odour and thus there is a need to examine the anemotactic attraction of virgin females in an olfactometer to determine whether the postulated pheromone (if it exists) acts as an attractant in addition to stimulating flight.

1.8 Aims of thesis and overview

The aim of this thesis is to explore the role of semiochemicals in both oviposition site choice and mating behaviour with emphasis on re-examining behavioural evidence for the involvement of pheromones. Experiments presented in this thesis were designed to focus on behavioural evidence for pheromones rather than the identification of specific compounds that might act as pheromones.

Chapter 2 outlines methods widely referred to throughout the thesis including the conditions under which mosquitoes were reared. Chapter 3 describes various experiments that were designed to investigate egg aggregation and indirect evidence for an egg associated oviposition pheromone. Chapter 4 describes the development of an olfactometer to investigate mating behaviour in *Ae. aegypti* and Chapter 5 describes evidence obtained through both laboratory

and field based behavioural experiments for a volatile male produced pheromone that increases the attraction of host odour to virgin females. Finally in Chapter 6 overall conclusions are drawn from the results of experiments and further work suggested.

Chapter 2 - General materials and methods

2.1 Mosquitoes

2.1.1 Mosquito strain

Experiments were carried out using the "Liverpool" strain of *Ae. aegypti* since this strain was readily available at Keele. This strain may be of West African origin, but is thought to have been maintained in the laboratory since the 1930s (Kuno 2010).

2.1.2 Insectary conditions

The mosquito colony was kept in an insectary which was maintained at 26°C and a humidifier (Defensor 505; JS Humidifiers Ltd, UK) elevated the relative humidity to 60 – 70%. A 12:12 hour light cycle was maintained using a timer controlled switch; the light source consisted of fluorescent tubes at ceiling level.

2.1.3 Maintenance of stock colony

Eggs were floated in a 16L plastic storage box (43 x 33 x 17cm; Wilkinson Ltd, UK) which was filled with 4L distilled water. 20 drops liquid fish food (Interpet Ltd, UK) was then added as food for newly hatched larvae. A nylon cover was fitted over the storage box to prevent emerging mosquitoes from escaping. Larvae were then maintained on fish food flakes (TetraMin GmBH, Germany). Emerged mosquitoes were removed daily using a aspirator and transferred to a large cage (30 x 30 x 30cm internal diameter; BugDorm-4030F Insect Cage, MegaView Science Co. Ltd, Taiwan). In order to provide mosquitoes with access to sucrose

solution a glass vial was filled with 5ml 10% w/v sucrose solution into which a folded piece of paper towel to act as a wick was inserted and this was placed inside the cage.

Females were fed defibrinated horse blood (TCS Biosciences Ltd, UK) by a Hemotek FU1 membrane feeding system (Discovery Workshops, UK) which maintained the blood at 37°C. Females were allowed to feed for several hours or until fully engorged.

To provide a suitable oviposition substrate, a plastic petri-dish (90mm x 16mm; Barloworld Scientific Ltd, UK) containing cotton wool was filled with water and a filter paper (Whatman No. 1; Whatman International Ltd, UK) was placed on top. This was then placed inside the cage and gravid females laid eggs on the wet filter paper which was kept wet by the water saturated cotton wool underneath. Filter papers were changed regularly and those containing eggs were dried and stored in the insectary ready for use.

2.1.4 Rearing of known-age mosquitoes for use in experiments

Eggs were first hatched under vacuum using a desiccator (Desiccator vacuum with stopcock, 250mm; Fisher Scientific Ltd, UK) attached to a small vacuum pump (Welch 2511 dry vacuum pump; Gardner Denver Thomas, Inc., USA). Approximately 200 larvae were then transferred per plastic tray (33 x 25 x 4cm) containing 1L distilled water. 10 drops liquid fish food (Interpet Ltd, UK) were added to each tray. However, when insectary space was more limited larvae were reared under the same conditions as for the stock colony.

Pupae were removed daily and placed in polystyrene cups filled with water which were then placed in a cage (18 x 18cm) with access to 10% w/v sucrose

solution. Cages used consisted of nylon pouches with a sleeve opening and were tied to a steel frame as depicted in Figure 2.1. Cages were kept on plastic trays (33 x 25 x 4cm) to keep the glass vial containing sucrose solution upright.

In order to ensure the ages of mosquitoes were known, the polystyrene cups containing pupae were transferred to a fresh empty cage each morning and the cage containing mosquitoes that had already emerged was labelled with the date so that each cage contained mosquitoes which had emerged within less than 12 hours of each other. The age of mosquitoes in a cage was calculated as the number of days since the pupae container(s) were removed from the cage. This meant that the age of mosquitoes was known within about half a day.

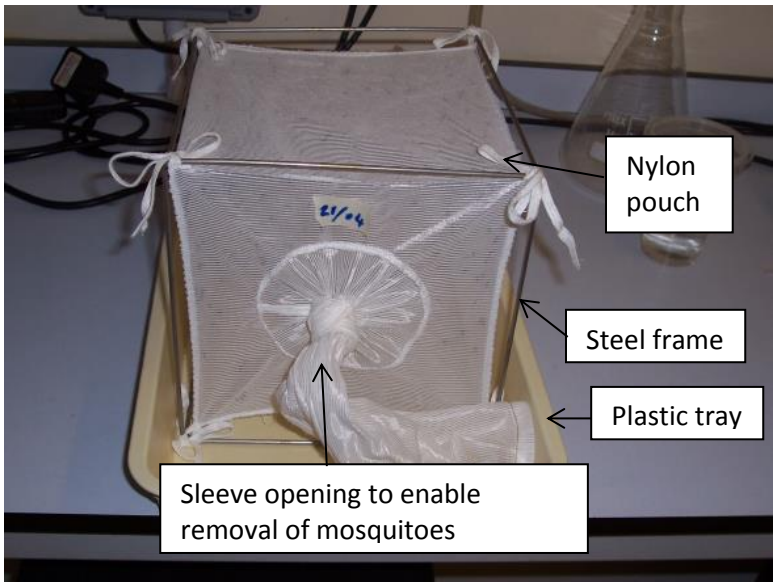

Figure 2.1 – Cage consisting of nylon netting tied to steel frame: Cages were places on plastic trays to keep the glass vial containing sucrose solution upright.

2.1.5 Rearing of unmated mosquitoes for mating behaviour experiments

Where unmated mosquitoes were required, pupae were removed with the aid of a Pasteur Pipette (3ml non-sterile; Fisher Scientific Ltd, UK) and males and females separated by examination under a dissecting microscope as described by Benedict (2007). Polystyrene cups containing male and female pupae were subsequently placed in separate cages to prevent mating following emergence into adults. Male and female cages were visually checked following adult emergence to ensure they were free from mosquitoes of the opposite sex. If males were found in the female cage or vice versa then the whole cage was discarded and not used for experiments.

2.1.6 Rearing of gravid females for oviposition experiments

Males and females were kept in the same cage to enable mating and females were blood-fed with defibrinated horse blood (TCS Biosciences Ltd, UK) 5 days after emergence as described for the stock colony. Following blood feeding, the abdomens of mosquitoes were inspected visually and those females that did not appear to have blood fed were removed.

2.1.7 Use of aspirators for handling mosquitoes

Two types of mechanical aspirators were used. For experiments where specific numbers of mosquitoes needed to be transferred, an aspirator was assembled by attaching a plastic 10ml serological pipette (FB51888; Fisher Scientific Ltd, UK) to a mini vacuum cleaner (Halloa Enterprise Co Ltd, Taiwan). Where large numbers of mosquitoes needed to be transferred such as for cleaning the stock colony cage, a commercially available mechanical aspirator (Watkins and Doncaster, UK; Barrel size: 50x28mm, Tube diameter 9mm) was used.

2.2 Bioassays

2.2.1 Bioassay room conditions

Laboratory based bioassays were carried out in a room maintained at 25°C. The humidity of the room was monitored using a thermo-hygrometer (DRT880 Digitron Ltd, UK) and found to vary between 15 - 17% throughout the period of experiments. Olfactometer based bioassays were carried out with the main lights switched on. Oviposition bioassays were run over a 24 hour time period and a timer switch socket was used to maintain a 12:12 hour light cycle.

2.2.2 Olfactometer based bioassays

A number of different olfactometer designs were utilised which are described in individual experimental chapters. Compressed air (humidity of 50ppm) was supplied from an air cylinder (BOC Industrial Gases, UK). Different components of the olfactometer were connected by FEP tubing (Chemfluor; 0.25 inch internal diameter; 0.625 inch outer diameter; Sigma-Aldrich Company Ltd, UK) and all joints and connections were sealed with PTFE sealing tape (Z104388; Sigma-Aldrich Company Ltd, UK) to prevent air leakages and maintain constant airflow thorough the apparatus. Air flow rate downstream of the odour source was checked using a bubble-flow meter and adjusted to give an air flow rate of 300 ml min^{-1}.

2.2.3 Protocol for cleaning apparatus

All apparatus used was thoroughly washed to remove any source of odour. Nitrile medical examination grade gloves (VWR International Ltd, UK) were worn during the washing of apparatus to prevent introduction of skin derived volatiles. Glassware was washed with odour free detergent (Teepol L, BDH Laboratory

Supplies Ltd, UK) and hot water and then rinsed with distilled water and acetone (Laboratory Reagent Grade; Fisher Scientific Ltd, UK). Glassware was then kept overnight in an oven (Thermo Electron Corporation, USA) maintained at 180°C. Non-glass components were washed with odour free detergent and hot water and then rinsed in distilled water and left to dry at room temperature. Gloves were worn when handling washed components to prevent re-contamination with skin derived volatiles.

Chapter 3 – Behavioural evidence for oviposition pheromones in *Aedes aegypti*

3.1 Introduction

Gravid female mosquitoes use both nonchemical and chemical cues in their selection of oviposition sites. Nonchemical oviposition cues have long been acknowledged and extensively discussed (e.g. Bates 1949; Williams 1962; Harrington *et al.* 2008; Bernáth *et al* 2012). As noted by Ganesan *et al*. (2006), chemical cues used by gravid female mosquitoes in site selection can include both inter-specific signals and intra-specific signals.

Inter-specific signals include kairomones such as bacterial produced metabolites (Lindh *et al.* 2008; Ponnusamy *et al* 2010). The role of kairomones in modulating oviposition behaviour of *Ae. aegypti* has been well documented and several kairomones as well as the microorganisms from which they emanate have recently been identified by Ponnusamy *et al.* (2008). These kairomones included carboxylic acids ranging from nonanoic acid to octadecanoic acid and several carboxylic acid methyl esters and the authors later found that synthetic versions of some of these compounds (nonanoic acid, tetradecanoic acid, and methyl tetradecanoate) induced a positive oviposition response in *Ae. aegypti* within a narrow range of concentrations.

Intra-specific signals consist of those chemicals emanating from immature stages of the mosquito that act as pheromones and it is the role of these chemical cues in *Ae. aegypti* oviposition behaviour that is the focus of this chapter.

3.1.1 Larval-associated oviposition cues in *Aedes aegypti*

There have been several reports in the literature regarding the response of gravid females of several mosquito species to conspecific larvae and pupae and the earliest such studies which suggested greater oviposition in water that had previously contained larvae or pupae have been reviewed by Clements (1999). Benzon and Apperson (1988) have also shown that microorganisms in the water may affect the response of gravid female *Ae. aegypti* to conspecific larvae and pupae. When Benzon and Apperson (1988) treated water that had held larvae with antibiotics, gravid females laid significantly fewer eggs in this larval holding water compared with a distilled water control, but when water that had contained larvae was not treated to remove bacteria significantly more eggs were laid in this larval holding water compared with a distilled water control. As discussed by the authors, in some studies where water containing conspecific larvae was shown to lead to increased oviposition this may have been due to bacterial contamination. It was therefore suggested that any pheromone associated with larvae is likely to repel or deter oviposition, but that this effect is masked by the much stronger attractive or stimulatory effect of bacteria associated chemical cues. It is likely that these bacterial associated cues may include compounds such as carboxylic acids and methyl esters recently identified from bacteria by Ponnusamy *et al.* (2008). However, results from a recent field based study suggest that the presence of larvae and pupae at an oviposition site increases subsequent oviposition with

gravid females laying more eggs and being more likely to lay eggs in containers that contained conspecific larvae and/or pupae (Wong *et al.* 2011).

Several studies have also shown that the response of gravid females to conspecific larvae is dependent on the relative health of those larvae suggesting that cues from conspecific larvae are used by gravid females to assess the relative suitability of an oviposition site. For example, it has been shown that the response of gravid females to conspecific larvae may depend on how well fed the larvae were. Water which contained larvae that had been starved for 3-5 days was significantly less attractive than water that contained fed larvae (Zahiri *et al.* 1997). Meanwhile, Lowenberger *et al.* (1994) showed that *Ae. aegypti* laid significantly fewer eggs in water containing larvae parasitized with *Plagiorchis elegans*.

Mendki *et al.* (2000) have identified an alkane, n-heneicosane ($C_{21}H_{44}$) from water in which larvae had been kept and Seenivasagan *et al.* (2009) have since shown using olfactometer based bioassays that this compound is attractive to gravid females at 10 ppm, but repellent at concentrations higher than 100 ppm.

3.1.2 Evidence for egg-associated oviposition pheromones in *Aedes aegypti*

Evidence for egg-associated oviposition pheromones in *Ae. aegypti* is contradictory. Published laboratory based studies that have attempted to address this issue are summarised in Table 3.1. Chadee (1990) found that when egg free ovipots were available, gravid *Ae. aegypti* females avoided ovipots containing conspecific eggs, but this study was later contradicted by Allan and Kline (1998) who found that gravid *Ae. aegypti* mosquitoes laid significantly more eggs on strips of oviposition paper containing either pre-existing *Ae. aegypti* eggs or pre-existing

Ae. albopictus eggs. Interestingly Allan and Kline (1998) reported that pre-existing eggs of either species did not influence oviposition choice by gravid *Ae. albopictus* females. Williams *et al*. (2008) found that 11-38 pre-existing eggs elicited a significantly positive oviposition response compared to zero pre-existing eggs, but that at higher densities the oviposition response was *not* significantly different to zero pre-existing eggs. None of these studies tested specific numbers of pre-existing eggs. There were differences between these studies in the age of gravid females used as well as the number of days after blood feeding when they were used which may have contributed to differing results. For example, Williams *et al.* (2008) used 10-20 day old gravid females seven days after blood feeding while Chadee *et al.* (1990) blood fed mosquitoes 3 days after emergence and did not specify how long after blood feeding the females were used for experiments. Meanwhile, Allan and Kline (1998) blood fed mosquitoes 4-5 days after emergence and subsequently used the mosquitoes another 4-5 days after blood feeding.

Table 3.1 – Summary of studies examining the effect of pre-existing eggs on subsequent oviposition activity in *Ae. aegypti*. None of these studies tested specific numbers of eggs, but the study by Williams *et al.* (2006) suggests response to pre-existing eggs might be density dependent

Publication	**Number of pre-existing eggs**	**Oviposition activity in response to pre-existing eggs**
Chadee *et al.* 1990	Not specified	Negative oviposition response
Allan and Kline 1998	145 ± 23.98	Positive oviposition response
Williams *et al.* 2008	11-38 and 39-74	11 – 38 eggs elicited positive response; 39-74 did not

There is also some indirect evidence from field studies suggesting that gravid *Ae. aegypti* females may respond to the presence of pre-existing eggs (Apostol *et al.* 1994; Chadee *et al*. 2009). For example, Chadee (2009) used modified ovitraps to study oviposition behaviour in Trinidad and found that significantly more ovitraps contained less than 30 eggs which they suggest indicates that gravid females prefer oviposition sites initially free from eggs. However, Chadee (2009) also found significantly more positive ovitraps in the >90 eggs category than in the 61-90 eggs category. Chadee (2009) suggested the higher number of ovitraps in the >90 eggs category may have been due to forced

egg retention which can lead to gravid females laying their entire egg batch in a single container, a theory supported by their finding that significantly more eggs were laid in the >90 eggs category during the late dry season when potential oviposition sites would be more limited.

A recent study by Bernáth *et al* (2012) suggested that chemical cues that might have emanated from eggs can mask the effect of polarized light on oviposition site choice by *Ae. aegypti*. When the trays used for oviposition by gravid females were rinsed so as to remove any chemical stimuli (from eggs, gravid females or carcasses of dead females), significantly more eggs were laid in arena exposed to horizontally polarized light, but when the trays were not washed horizontally polarized light did not significantly influence oviposition site choice. Although the authors suggested that cues indicative of conspecifics were responsible for masking the effect of polarized light, their experiment was unable to differentiate between i) egg associated chemical cues, ii) chemicals released by ovipositing females, iii) chemical cues from carcasses of dead females, iv) chemical cues from any defecation by mosquitoes at the oviposition site or v) microbial derived chemical cues from microorganisms. Nevertheless, this study indicates how response of gravid females to one environmental factor can be completely neutralized by a different factor.

3.1.3 Postulated oviposition pheromones in *Aedes aegypti* isolated from egg extracts

Ganesan *et al.* (2006) isolated and identified by GC-MS, compounds that were present on the eggs of *Ae. aegypti* and also tested the behavioural response of gravid females to each of the compounds over a range of three different

concentrations (1, 10 and 100 ppm). They found that some compounds extracted in methanol were biologically active at certain concentrations; eliciting either negative or positive oviposition responses. For example, tetradecanoic acid significantly increased the numbers of eggs laid at 1 ppm and 10 ppm, but not at 100 ppm while dodecanoic acid increased the numbers of eggs laid at all three concentrations tested. While most of the fatty acids induced a significantly positive oviposition response, all the esters identified induced a negative oviposition response. Ganesan *et al.* (2006) did not test the biological activity of the egg extract itself and it could be argued that for a compound to be described as a pheromone it must be shown to be biologically active at an appropriate physiological concentration; for example it is not clear whether the concentrations of 1, 10 and 100 ppm at which these compounds were tested represent the concentrations at which these compounds are likely to be found associated with the eggs or at oviposition sites.

3.1.4 Gaps in current knowledge

As discussed, previous studies investigating the effect of conspecific eggs on oviposition did not test specific numbers of eggs and their results were contradictory in that some studies reported a positive oviposition response to conspecific eggs while other studies reported a negative oviposition response. Although compounds have been identified from methanol extracts of eggs and though these compounds affect ovipositional behaviour at certain concentrations (Ganesan *et al* 2006), the methanol extracts of eggs were not themselves tested for biological activity. Furthermore, some previous studies (Williams *et al.* 2008; Chadee *et al.* 1990) have used bioassay durations of more than 3 days and thus it is possible that towards the end of the bioassay gravid females may have been

responding to eggs they had previously laid rather than those laid by conspecifics. It is also not clear whether differences in the time between blood feeding and duration of exposure to potential oviposition sites affects the behavioural response of gravid females to oviposition cues as this might have been partly responsible for conflicting results between previous studies.

There is therefore a need to more comprehensively test the effect of pre-existing eggs on subsequent oviposition activity. Furthermore, attempts need to be made to determine whether methanol extract of *Ae. aegypti* eggs is itself biologically active. It is understandable that studies have focused on the very practical issue of whether certain compounds modulate oviposition activity. However, there is a need to determine whether such compounds are likely to effect oviposition behaviour at their physiological concentrations in order to assess their importance at the ecological level.

3.2 Aims and objectives

3.2.1 Aims

The main aim of the work described in this chapter was to re-examine the effect of conspecific eggs on oviposition activity and to determine if any effect is density dependent. The other aims were to; i) determine whether methanol extract of *Ae. aegypti* eggs as analysed by Ganesan *et al. (*2006) is itself biologically active, and; ii) examine whether the time since blood feeding had any effect on oviposition site choice or the total number of eggs laid and thus to determine whether this might have contributed to differing results in previous studies.

3.2.2 Objectives

1) To determine by means of a two-choice bioassay whether 20, 100 and/or 200 pre-existing eggs influenced the number of eggs laid compared with no pre-existing eggs (control).
2) To determine by means of a no-choice bioassay whether 20, 100 and/or 200 pre-existing eggs influenced the number of eggs laid. In each case a control consisting of zero pre-existing eggs was run alongside these experiments and test data were compared with this control.
3) To determine by means of both no-choice and two-choice bioassays whether methanol extract of 20 eggs influenced the oviposition activity of gravid females.
4) To examine the difference in oviposition behaviour of gravid females 3 days after blood feeding compared with 4 days after blood feeding.
5) To determine when no eggs were laid if this was due to egg retention or because the mosquitoes were not gravid.

3.3 Materials and methods

3.3.1 Preparation of gravid females

Ae. aegypti mosquitoes were reared as previously described (Chapter 2; General methods). Males and females were kept in the same cage to enable mating to take place and females were blood-fed five days after emerging. Upon visual inspection females that had not blood fed were discarded and those that had were used for experiments 3 days later. In experiment 3, the gravid females were also used 4 days after blood feeding.

3.3.2 Bioassays

A summary of the bioassays carried out is given in Table 3.2. Both choice bioassays where gravid females could choose to lay their eggs in either of two Petri-dishes (one test Petri-dish and one control Petri-dish) and no-choice bioassays where gravid females only had access to one Petri-dish (either test or control) in which to lay their eggs were carried out.

Table 3.2 – Summary of oviposition bioassay experiments. Both no choice and 2-choice bioassays were carried out to test the oviposition response to specific numbers of pre-existing eggs and methanol extract of 20 eggs

Expt.	Bioassay	Eggs or egg extract	Number of eggs*	Number of replicates
1	2-choice	eggs	0, 20, 100, 200	23, 22, 26
2	no-choice	eggs	0, 20, 100, 200	32, 22, 19
3	2-choice and no-choice	extract	extract (20 eggs)	14 - 25

*The number of pre-existing conspecific eggs or extract of equivalent number of eggs.

For these experiments cages consisting of nylon netting supported by a steel frame (20 x 20 x 20 cm) were used. These cages were used rather than larger cages in order to maximise the number of replicates that could be carried out simultaneously. Glass Petri-dishes (5 cm diameter) containing either 3-4 hour old eggs, extract of 3-4 hour old eggs or the appropriate control were placed in each cage; a single glass Petri-dish was placed in the centre of the cage for no choice experiments while two Petri-dishes were placed at opposite corners of the cage for choice experiments. The freshly laid eggs were transferred to the distilled water in the petri-dishes using the tip of a hypodermic needle (40mm; BD Microlance 3; Fisher Scientific Ltd, UK). For no choice experiments, a single glass Petri-dish was placed in the centre of the cage. For choice experiments, two Petri-dishes were placed at opposite corners at the base of the cage (Figure 3.1).

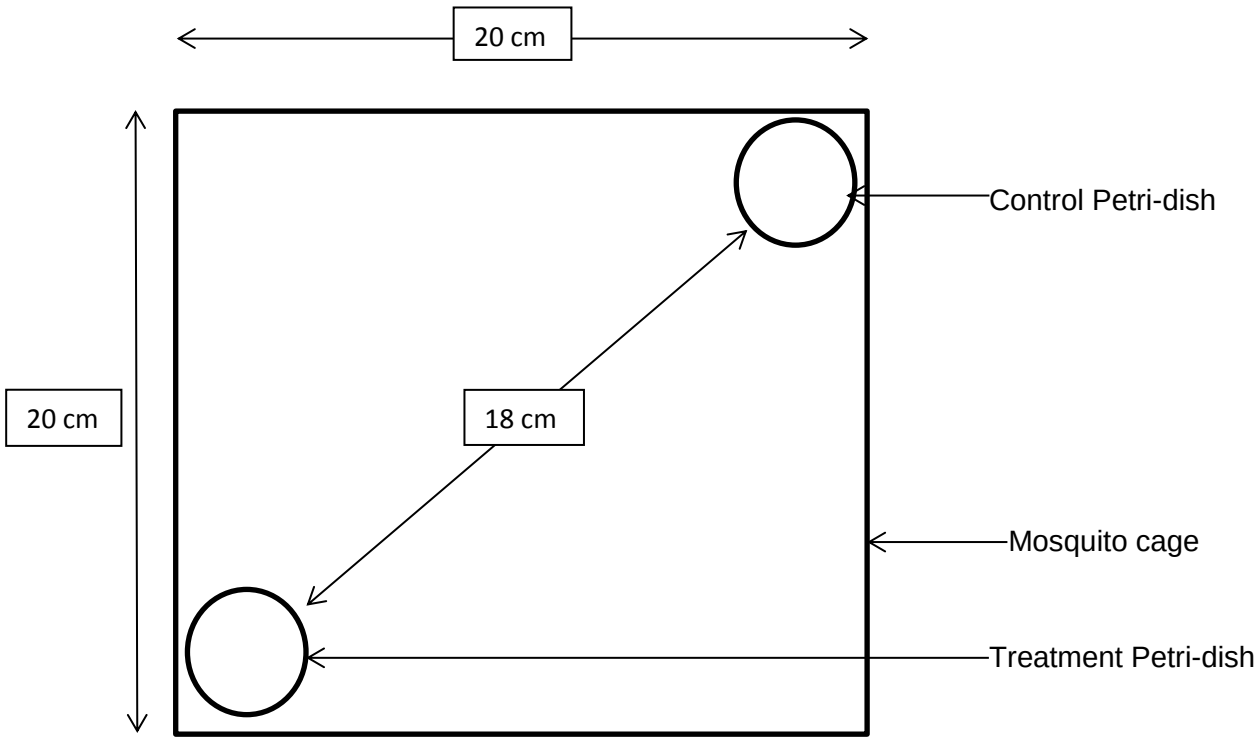

Figure 3.1 – Two dimensional representation of the set-up for the two-choice experiment: Diameter of both Petri-dishes was 5cm. The control and treatment Petri-dishes were placed at opposite corners of the cage and their positions were rotated per replicate to neutralise any potential positional bias.

Five gravid females were placed in each cage which was then enclosed within a partially transparent polythene bag (Wilkinson Ltd, UK) to prevent odours passing from one cage to another. Cages were left for 24 hours under a 12:12 hour light cycle in the bioassay room which was maintained under conditions described previously (Chapter 2; General materials and methods). After 24 hours, Petri-dishes were removed and the number of eggs counted by examination under a dissecting microscope. The number of eggs laid within a 24 hour time period

was calculated by subtracting the numbers of any pre-existing eggs from the final total.

3.3.3 Experiment 1 – Influence of conspecific eggs on oviposition site choice

This experiment was set up as shown in Figure 3.1. Two glass Petri-dishes (treatment and control) containing 10ml distilled water were placed at opposite corners of the cage. The treatment Petri-dish contained either 20, 100 or 200 eggs while the control contained no eggs. Cages were sealed in polythene bags as described and after 24 hours the numbers of eggs laid in each Petri-dish was counted by examination under a dissecting microscope.

3.3.4 Experiment 2 – Influence of pre-existing conspecific eggs on subsequent oviposition

Since it was not possible to carry out all these experiments simultaneously a separate control was run alongside each treatment. First the effect of 20 pre-existing eggs on subsequent oviposition activity was tested. A Petri-dish containing 20 eggs in 10ml distilled water was placed in the centre of the cage and the cage was sealed in a polythene bag. A control was also run simultaneously in a separate cage which consisted of a Petri-dish containing 10ml distilled water, but no eggs.

Subsequently, the same experimental set-up was used to test the effect of 100 and 200 pre-existing eggs. As before a control with zero eggs was run simultaneously.

3.3.5 Experiment 3 – To determine if methanol extract of eggs influences oviposition behaviour

200 eggs (3-4 hours old) were removed from oviposition filter paper and placed into glass vials. 1µl per egg methanol (Analytical Reagent Grade; Fisher Scientific Ltd, UK) was added using a 100µl syringe (Hamilton Microliter; Sigma-Aldrich Company Ltd, UK). The glass vial was then sealed and left overnight at room temperature. The next day, the methanol extract was decanted to a fresh vial (leaving behind the eggs) and this was sealed until required for experiments.

20µl of the egg extract (i.e. the equivalent of 20 eggs) was added using a 25µl syringe (Hamilton Microliter; Sigma-Aldrich Company Ltd, UK) instead of eggs to Petri-dishes containing 10ml distilled water and both two-choice and no-choice style bioassays were then carried out as described (section 3.3.2) with female *Ae. aegypti* 3 days after blood feeding. The control for these experiments consisted of 20µl methanol added to Petri-dishes containing 10ml distilled water to control for any effect of methanol on oviposition behavior. Additionally, mosquitoes were also given the opportunity to oviposit 4 days after blood-feeding by placing fresh Petri-dishes containing the appropriate treatment into each cage and re-sealing in polythene bags.

3.3.6 To determine whether mosquitoes were gravid

A sample of test mosquitoes from cages where no eggs were laid was kept to check retrospectively whether this was due to the mosquitoes retaining their eggs or due to the mosquitoes not being gravid in the first place. The ovaries of these mosquitoes were dissected out and examined to confirm whether they contained eggs.

3.3.7 Experimental design and data analysis

3.3.7.1 Position of petri-dish

For choice experiments, each experimental cage contained a control Petri-dish and a treatment Petri-dish. The control Petri-dish contained 10ml distilled water while the treatment Petri-dish contained 10ml distilled water plus the relevant treatment. There were four possible positions for the placement of Petri-dishes and in each cage the positions were different so that equal numbers of replicates had Petri-dishes in each configuration.

For no choice experiments, each cage contained either a control or treatment Petri-dish which was placed in the centre of the cage. The positions of the control and test cages were swapped each day.

3.3.7.2 Data analysis

All statistical tests were carried out using Minitab v15.0 (Minitab Inc., USA). Data were tested for normality using the Anderson Darling Test and if data significantly deviated from the normal distribution then they were transformed using the formula $\log_{10}$ (result + 0.5). However, since even after transformation, the data were still not normally distributed, an appropriate non-parametric test was used. For no choice experiments, data were analysed by the Mann Whitney Test (using α=0.05) to determine if there was a significant difference in the number of eggs laid between the control and test samples. For two-choice experiments, the difference between the control and test sites was calculated for each cage and the Wilcoxon Signed Rank Test (using α=0.05) was used to determine whether the median of the differences between the test and control significantly differed from zero. All statistical tests carried out were two tailed since no directional predictions

were made as to whether any influence on oviposition would be negative or positive.

3.4 Results

3.4.1 Experiment 1 – Influence of conspecific eggs on oviposition site choice (choice bioassays)

In this experiment gravid females were able to choose between a Petri-dish containing pre-existing eggs and a Petri-dish containing no pre-existing eggs (control). There was no significant difference in the numbers of eggs laid between 20 pre-existing eggs and the control (2-tailed Wilcoxon Signed Rank Test, P=0.53), 100 pre-existing eggs and the control (2-tailed Wilcoxon Signed Rank Test, P=0.53) or 200 pre-existing eggs and the control (2-tailed Wilcoxon Signed Rank Test, P=0.88). Results are shown in Figure 3.2.

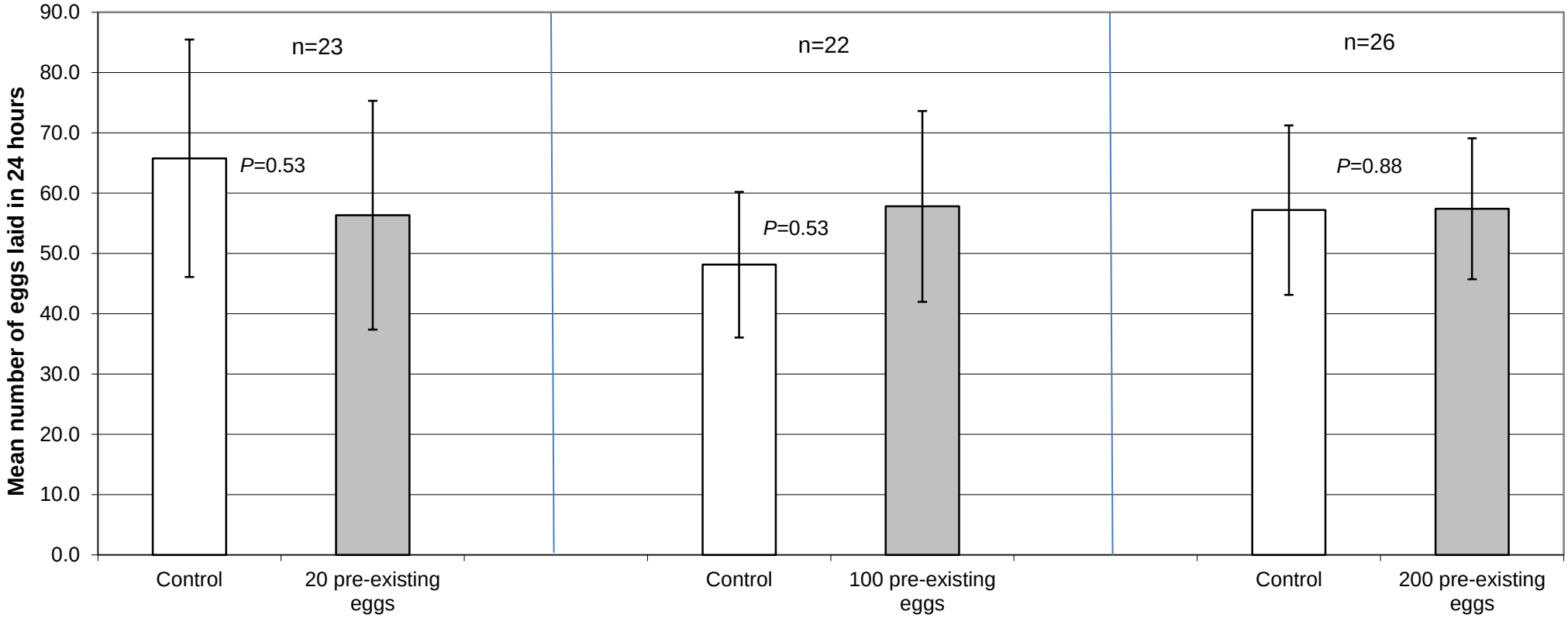

Figure 3.2 – Results of choice bioassays: Graph depicting the mean number of eggs laid within a 24 hour period in the Petri-dish containing pre-existing eggs and the Petri-dish containing no pre-existing eggs (control). Error bars depict the SEM. *P*-values were obtained by carrying out a 2-tailed Wilcoxon Signed Rank Test on the differences in the median egg counts between the control and treatment sites. "n" represents the number of replicates.

3.4.2 Experiment 2 – Influence of pre-existing conspecific eggs on subsequent oviposition (no-choice bioassays)

Neither 20, 100 nor 200 pre-existing eggs significantly affected the numbers of eggs laid compared with their corresponding controls (Figure 3.3). However, the number of eggs laid in response to 200 pre-existing eggs was significantly greater than the numbers of eggs laid in response to 100 pre-existing eggs (2-tailed Mann Whitney Test, $P = 0.046$) and 20 pre-existing eggs (2-tailed Mann Whitney Test, $P = 0.015$). There was no significant difference between the number of eggs laid in response to 20 and 100 pre-existing eggs (2-tailed Mann Whitney Test, $P = 0.36$).

However, it should be noted that there was significant variation between the controls possibly due to the fact that the no choice experiments with 200 eggs were carried out several months after the other experiments. The corresponding control for 200 pre-existing eggs was significantly higher than that for 20 pre-existing eggs (2-tailed Mann Whitney Test, $P = 0.006$), but there was no significant difference between the controls for 200 and 100 pre-existing eggs (2-tailed Mann Whitney Test, $P = 0.22$) or between the controls for 20 and 100 pre-existing eggs (2-tailed Mann Whitney Test, $P = 0.13$).

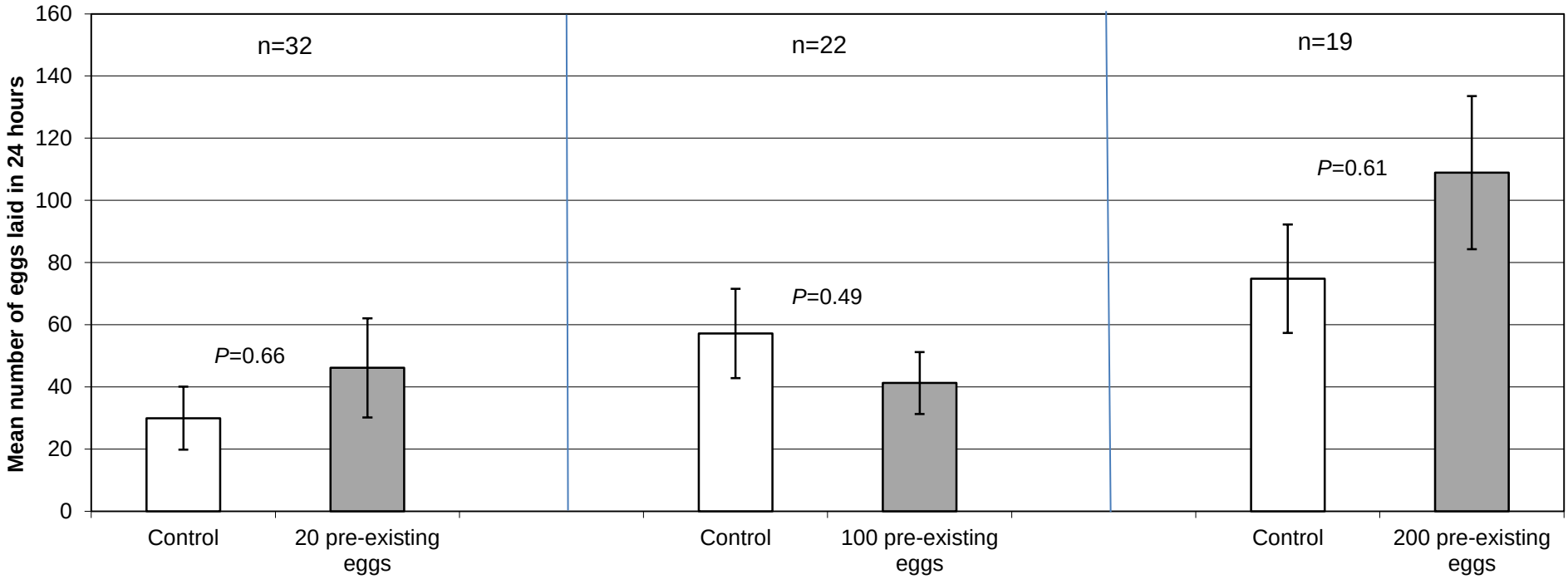

Figure 3.3 – Results of no-choice bioassays: Graph depicting the mean number of eggs laid within a 24 hour period in response to 20, 100 and 200 pre-existing eggs compared with no pre-existing eggs (control). Error bars indicate the SEM. *P*-values were obtained by carrying out 2-tailed Mann Whitney Tests. "n" represents the number of replicates.

3.4.3 Experiment 3 - To determine if methanol extract of eggs influences oviposition behaviour

Methanol extract of 20 eggs did not influence oviposition activity in either the choice or no choice experiments. 3 days after blood feeding there was no significant difference in the numbers of eggs laid in response to egg extract compared with no egg extract (control) for the choice bioassay (2-tailed Wilcoxon Signed Rank Test, *P*=0.85) or the no choice bioassay (2-tailed Mann Whitney Test, *P*=0.37). There was also no significant difference between egg extract and no egg extract 4 days after blood feeding for either the choice bioassay (2-tailed Wilcoxon Signed Rank Test, *P*=0.60) or the no choice bioassay (2-tailed Mann Whitney Test, *P*=0.52). The results of this experiment comparing the number of eggs laid in response to egg extract compared with no egg extract are shown in Table 3.3.

Table 3.3 - Number of eggs laid in response to methanol extract of 20 egg equivalents. Comparison of the number of eggs laid by gravid females in response to methanol extract of 20 eggs compared with a control using choice and no choice bioassays both 3 and 4 days after blood feeding

Number of days after blood feeding	**Type of bioassay**	**n**	**Mean number of eggs laid in 24 hours with egg extract (±SEM)**	**Mean number of eggs laid in 24 hours in absence of egg extract (±SEM)**	***P*-value***
3	Choice	14	31.29 ±13.1	22.64 ±8.4	0.85
	No choice	25	14.76 ± 6.9	27.85 ±10.5	0.37
4	Choice	14	32.92 ±15.4	51.86 ±17.9	0.60
	No choice	25	44.44 ±10.4	73.59 ±18.5	0.52

**P*-values were obtained using a 2-tailed Wilcoxon Signed Rank Test for choice bioassays and 2-tailed Mann-Whitney Test for no choice bioassays. *P*-values compare the median number of eggs laid in response to egg extract with the median number laid in the absence of egg extract.

Response of gravid females to methanol extract was tested both 3 days and 4 days after blood feeding and Table 3.4 compares the numbers of eggs laid 4 days after blood feeding with the number laid 3 days after blood feeding. Significantly more eggs were laid in the no-choice bioassays 4 days after blood feeding compared with 3 days after blood feeding both in the presence of egg

extract (2-tailed Wilcoxon Signed Rank Test, *P*=0.012) and the absence of egg extract (2-tailed Wilcoxon Signed Rank Test, *P*=0.003). In the choice bioassays, the difference in the numbers of eggs laid between 3 days and 4 days post blood feeding was less marked and interestingly for the test site in the choice bioassay, there was no significant difference in numbers of eggs laid between 3 and 4 days post blood feeding (2-tailed Wilcoxon Signed Rank Test; *P*=0.625). However, there was a significant difference between 3 and 4 days post blood feeding for the control (no egg extract) in the choice bioassay (2-tailed Wilcoxon Signed Rank Test; *P*=0.043).

Table 3.4 – Comparison of the number of eggs laid 4 days post-blood-feeding compared to 3 days post-blood-feeding. Comparison between the number of eggs laid 3 days after blood feeding compared with the number of eggs laid 4 days after blood feeding[1]

Treatment	Type of bioassay	Mean number of eggs laid 3 days after blood feeding (±SEM)	Mean number of eggs laid 4 days after blood feeding (±SEM)	*P*-value[2]
Egg extract	Choice	31.29 ±13.1	32.92 ±15.4	0.625
	No choice	14.76 ± 6.9	44.44 ±10.4	0.012
No egg extract	Choice	22.64 ±8.4	51.86 ±17.9	0.043
	No choice	27.85 ±10.5	73.59 ±18.5	0.003

[1] This table contains the same data as Table 3.3, but *P*-values are for comparisons between 3 and 4 days after blood feeding whereas in Table 3.2 comparisons are between presence and absence of egg extract.

[2] All *P*-values were obtained using a 2-tailed Wilcoxon Signed Rank Test and compare the median numbers of eggs laid in response to egg extract with the median number laid in the absence of egg extract.

3.4.4 To determine whether mosquitoes were gravid

A sample of 10 females from different replicates in which no eggs were laid during the 24 hour period were dissected to check whether they were gravid. All 10 female mosquitoes were found to be gravid as the ovaries contained eggs.

3.5 Discussion

In this chapter both choice and no-choice bioassays were carried out to determine the effect of pre-existing conspecific eggs and egg extract on oviposition. Choice bioassays were carried out so that gravid females would be able to choose between two oviposition sites. For, no-choice bioassays it was postulated that the presence of pre-existing eggs or egg extract might influence the level of egg retention and therefore the number of eggs laid within the 24-hour time period. Both choice and no choice bioassays were carried out, because Williams *et al.* (2008) had found that the effect of pre-existing eggs was dependent on an alternative oviposition site being available. Results from choice and no choice bioassays did not suggest that pre-existing conspecific eggs influence oviposition site choice and thus do not support the existence of an egg associated oviposition pheromone. Although in the no choice experiment significantly more eggs were laid in response to 200 eggs compared to 20 or 100 eggs these experiments were not run simultaneously and so are not comparable since there were also significant differences between the controls.

Methanol extract of 20 eggs was not found to influence oviposition activity in either choice or no choice experiments suggesting that the extract itself might not be biologically active even though specific compounds previously identified from methanol extracts of eggs were shown to influence oviposition activity (Ganesan *et al.* 2006). Significantly more eggs were laid 4 days after blood feeding compared with 3 days after blood feeding and this is discussed as a possible outcome of forced egg retention.

3.5.1 Effect of pre-existing eggs

The results for the choice bioassay differ from those of Williams *et al.* (2008) who found gravid females laid more eggs at sites containing an intermediate number of pre-existing eggs. However, the finding that presence of pre-existing eggs did not affect subsequent oviposition when no choice was available agrees with the finding by Williams *et al.* (2008) who found that in the absence of alternative oviposition sites the presence of pre-existing eggs had no effect.

It should be noted that in these experiments the age of the eggs used were less than four hours old. In previous studies the age of eggs may have been different to this. Among previous studies, only Williams *et al.* (2008) gave any indication as to the age of eggs used and their eggs were substantially older than the eggs used for these experiments. This raises the possibility that any egg-associated pheromone might only be present on older eggs. This contradicts the prediction by Chadee (2009) that as *Ae. aegypti* eggs age, the concentrations of any associated pheromones may be reduced, presumably due to chemical breakdown. Alternatively the oviposition response to older eggs reported in previous studies might be due to the products of microbial metabolism present on the eggs since such compounds might be present in higher concentrations on older eggs rather than the younger eggs used in these experiments. Benzon and Apperson (1988) have previously shown that results of many of the studies suggesting an oviposition aggregation pheromone of larval origin were due to bacterial contamination of the larvae rather than the result of any pheromone. Ganesan *et al.* (2006) did not report the age of the eggs used, but it is interesting that tetradecanoic acid, one of the compounds they identified has also been

reported by Ponnusamy *et al.* (2008) as present in extracts of bacteria found in leaf infusions.

Another difference in the methods used is the oviposition substrate available to gravid females. The substrate at oviposition sites can affect their allure to gravid females of *Ae. aegypti* (Wong *et al.* 2011). In this study, glass Petri-dishes containing 10ml distilled water were available to gravid females for egg laying as it was easier to be sure such sites were free from contamination. However, all published studies have used water saturated absorbent materials as oviposition substrates; Allan and Kline (1998) for example used seed germination paper. It is possible that any oviposition pheromone might be released by ovipositing females at the oviposition site rather than present on the eggs or alternatively any pheromone on the eggs might dissolve in water or be adsorbed by filter paper. In *Culex* mosquitoes the oviposition pheromone is present in droplets on the egg's apex (Bruno and Lawrence 1979) but has a low solubility in water (Lawrence and Pickett 1989). However, as noted by Hilker and Meiners (2008) oviposition pheromones in some insects can be deposited by ovipositing females near the site of oviposition rather than being directly associated with the egg. Furthermore, given that competition for resources and larval densities are a product of the size of an oviposition site in addition to the numbers of eggs laid this may also have contributed to the different results obtained compared to previous studies.

Glass Petri-dishes are highly reflective, unrepresentative of most natural oviposition substrates used by *Ae. aegypti* and it has been noted by Snow (1971) that selection of oviposition sites by *Ae. aegypti* is inversely related to the background reflectance of the site. It is possible that this may be responsible for

the large number of replicates in both control and test sites where no eggs were laid. It is therefore possible that the unfavourable egg laying environment promoted forced egg retention regardless of the presence of pre-existing eggs. It is also the case that glass Petri-dishes containing distilled water may represent a sterile environment compared with water saturated paper where microbial growth might be easier. Although bacterial effects were not directly controlled for in this study they were not controlled for in previous studies either. This therefore raises the question as to whether previous reports suggesting an *Ae. aegypti* oviposition pheromone were in fact reporting the effect of microbial metabolites associated with the eggs of this mosquito.

Another important difference in the methods used in this study compared with previous studies is the distance between the control and test sites in the choice bioassays. This was very small (about 18cm) due to the size of the cages used (20cm x 20cm x 20cm). Previous studies used larger cages. For example, Allan and Kline (1998) used cages 38cm x 37cm x 46cm, while Williams *et al.* (2008) used even larger cages of 51cm x 51cm x 51cm. Smaller cages were used so that a greater number of replicates could be carried out each day, but this may have prevented detection of any difference between the control and test sites since it is not known whether any egg-associated pheromone if it exists is non-volatile or volatile. If it is volatile, the range over which it might act is not known. For example, results from a study by Lawrence (1985) on the oviposition pheromone of *Cu. quinquefasciatus* suggest gravid females respond to molecules carried by air currents rather than molecules in the water. If any egg-associated oviposition pheromone exists in *Ae. aegypti,* and if it acts in a similar way then the

choice bioassays used in this study may have failed to detect its effect due to lack of distance between the control and test sites.

Since the focus of this chapter was on the role of egg associated chemicals the role of visual stimuli was not considered. However, it should be noted that the light source used for these experiments was fluorescent tubes rather than natural light and thus the spectral composition of the light would have been different from that experienced by *Ae. aegypti* in the field. Given the fact that the oviposition sites should have been free from all odour cues other than any emanating from the eggs it is possible that visual cues would have been utilized to a greater extent than under field conditions since Bernáth *et al.* (2012) showed that polarized light (e.g. from the glass Petri-dishes) only affects gravid females in the absence of chemical cues. Since previous studies investigating the effects of pre-existing conspecific eggs on oviposition behaviour did not distinguish between chemical and visual cues it is not clear whether any effect they have on oviposition behaviour is mediated by visual or chemical cues or an interaction between visual and chemical cues.

3.5.2 Methanol extract of 20 egg equivalents

Methanol extract of 20 egg equivalents did not affect oviposition behaviour in either choice or no choice experiments. Therefore, although individual components of this extract have earlier been shown to modulate oviposition site choice in *Ae. aegypti* (Ganesan *et al.* 2006), the results of experiments presented here show that at 20 egg equivalents, the methanol extract of eggs does not affect oviposition behaviour either 3 days or 4 days after blood feeding. Since the egg extract did not influence oviposition activity either 3 or 4 days after blood feeding it

is not possible to comment on whether differences in the age of mosquitoes or the number of days post blood feeding they were used might have contributed to seemingly contradictory results in previous studies examining the effect of pre-existing eggs. For these experiments, extract of 20 egg equivalents was tested, because Williams *et al.* (2008) had found that 11-38 pre-existing eggs resulted in significantly more eggs laid. For the purpose of this experiment it was assumed that a methanol extract of 20 egg equivalents was equivalent to 20 eggs in terms of the concentrations of volatile compounds. However, the rate of evaporation of volatile compounds associated with eggs may be different from their rate of evaporation when they are dissolved in methanol and thus there is a need to test methanol extract of eggs over a range of concentrations to confirm whether the extract itself is biologically active.

3.5.3 Effect of time since blood feeding

For most treatments where gravid females were used four days after blood feeding in addition to three days after blood feeding, significantly more eggs were laid four days after blood feeding compared to three days after blood feeding. It is likely that this was a result of forced egg retention. The oviposition sites provided in these experiments lacked any source of kairomones and so (assuming no effect of the methanol) will not have been attractive to gravid females since it has been shown for example that gravid *Ae. aegypti* females prefer water containing oak leaf infusions to plain water (Ponnusamy *et al.* 2008). It could therefore be speculated that 3 days after blood feeding very few eggs were laid as the mosquitoes retained most of their eggs in the “hope” of finding more suitable oviposition sites. However, 4 days after blood feeding (with the same oviposition sites available) the need to lay their eggs might begin to overcome the need to find

suitable sites. This would support the finding by Siju *et al.* (2010) that ORNs in female *Ae. aegypti* sensitive to compounds known to emanate from oviposition sites (such as indole and phenolic compounds) are less sensitive 72 hours after blood feeding compared to 24 hours after blood feeding. This would suggest that 72 hours after blood feeding gravid females are less responsive to oviposition related odour stimuli and will therefore be more likely to oviposit without assessing the suitability of an oviposition site.

Interestingly, there was no significant difference between the numbers of eggs laid 3 days after blood feeding and 4 days after blood feeding for the treatment site in the choice bioassay. It is also interesting to note that 3 days after blood feeding a greater number of eggs were laid in the Petri-dish containing extract of 20 eggs (even though this was not significant) whereas 4 days after blood feeding more eggs were laid in the control Petri-dish (although this was also not significant). This would suggest that the egg extract shifted the proportion of eggs laid to the first 24 hours (i.e. 3 days after blood feeding) and thereby negated the difference between 3 and 4 days after blood feeding. However, if this is the case it is not clear why in the no choice bioassay significantly more eggs were still laid 4 days after blood feeding in those cages containing methanol extract of 20 eggs and thus this finding may merit further investigation. This finding nevertheless suggests that the age of gravid females and/or the time since their last blood meal may affect their response to oviposition cues.

3.5.4 Conclusions

The results of these experiments do not show any evidence for an egg associated oviposition pheromone, but do show that the rate of oviposition is

significantly higher 4 days after blood feeding compared with 3 days after blood feeding. As has been discussed it is possible that any egg associated pheromone if it is volatile may act over longer distances than the size of the cages used in these experiments and this would explain why its effect on oviposition activity was not detected in these experiments. Furthermore, if any egg associated pheromone acts solely as an attractant or repellent rather than a stimulant or deterrent then in an enclosed cage it might not have any effect on the numbers of eggs laid. For example, if gravid females respond anemotactically to the pheromone then the behavioural bioassays described in this chapter might not be able to detect such a behavioural response.

It is also possible that kairomones rather than any pheromone(s) represent the main chemical cue used by *Ae. aegypti* in oviposition site selection. In mosquito species that lay their eggs singly rather than in rafts the need for an egg associated pheromone might be assumed to be less pressing although a pheromone that inhibits further oviposition at a site would help to prevent overcrowding. Larval overcrowding has been shown to result in fewer and smaller eggs being laid by the females that emerge (Mitchell-Foster *et al.* 2012) and thus a mechanism that reduced the chances of overcrowding at breeding sites would be evolutionarily advantageous. Nevertheless, it is also possible that if an egg-associated oviposition pheromone(s) does/do exist they are only effective in the presence of certain kairomones indicating sufficient nutrients for larval development. For example, synergistic interactions between oviposition pheromones and kairomones have been documented in *Lu. longipalpis* (Dougherty *et al.* 1993). There is thus a need to examine the effect of preexisting eggs on the response of gravid females to kairomones.

Chapter 4 - Development of an olfactometer based bioassay to investigate mating behaviour

4.1 Introduction

This chapter describes attempts to develop an olfactometer to investigate the role of semiochemicals in *Ae. aegypti* mate attraction.

4.1.1 Role of semiochemicals in mating behaviour

It is likely that in *Ae. aegypti* host odour has a role in facilitating mating, because both male and female *Ae. aegypti* are attracted to vertebrate hosts even though it is only the females that blood feed (Hartberg 1971; Nelson *et al.* 1978). Indeed, *Ae. aegypti* males have been shown to respond electro-physiologically to carbon dioxide (Grant *et al.* 1995), which is a component of host odour and Cabrera and Jaffe (2007) observed that the presence of a host triggers swarming behaviour in male *Ae. aegypti* as well as increasing the frequency of copulations between males and females.

Some studies have suggested that chemical cues in the form of contact sex pheromones (Nijhout and Craig 1971) or volatile male produced pheromones (Cabrera and Jaffe 2007) might facilitate mating in *Ae. aegypti* in addition to host odour. Nevertheless, no sex pheromones have yet been identified from any species of mosquitoes.

4.1.2 Evidence for volatile sex pheromones in *Aedes aegypti*

Cabrera and Jaffe (2007) carried out behavioural assays on *Ae. aegypti* which suggested that a volatile pheromone secreted by males attracted males and females. Cabrera and Jaffe (2007) found that air blown over male mosquitoes significantly increased the numbers of females flying for at least 60 seconds. Additionally, females also responded in the same manner to air blown over females suggesting that females also emit a pheromone.

The apparatus used by Cabrera and Jaffe (2007) was not a "closed system" as it consisted of air being blown through a cage containing males and into a cage containing females 10 cm away. It is also unclear what material the cages used by Cabrera and Jaffe (2007) were constructed from or how they were cleaned in between experiments. There is therefore a possibility that their experimental design was not able to adequately exclude any effect of odour contamination on the results. It would therefore be useful to develop a "closed system" olfactometer to re-examine the claims by Cabrera and Jaffe (2007) that would aim to more thoroughly exclude other potential sources of odour that might impact the results.

Furthermore Cabrera and Jaffe (2007) measured the response of a group of females to male odour. Baker (1985) suggested that using groups of insects rather than individuals in behavioural experiments may result in "group effects". As noted by Bray and Hamilton (2007), testing the behavioural response of groups of insects simultaneously means that even if only a small proportion of insects respond to the odour being tested, the response can be magnified by interaction between individual insects. Testing the response of virgin females individually rather than collectively would give a better indication of whether the chemical cue

could be successfully used to bait traps and may also better mimic the situation that would exist under field conditions. If upwind anemotaxis in response to male odour could also be measured rather than simply the flight response as measured by Cabrera and Jaffe (2007) then this would confirm the potential for the use of compounds in male odour as attractants in traps as well as demonstrating the likely function of any male produced pheromone in attracting females.

4.1.3 Olfactometer based bioassays

Behavioural bioassays can be used to measure the response of living insects to odours that may contain biologically active components. Where the aim is to examine behavioural evidence for the existence of a pheromone the bioassay needs to be able to measure a response to the odour that may contain the putative pheromone. The objective of a well-designed olfactometer study is to reduce the response to a single key behaviour that can be reproduced and thus measured many times (Baker 1985).

In the case of a sex or aggregation pheromone one of the measurable responses would be upwind anemotaxis. Therefore an olfactometer that is appropriate in scale to the size of the insect can be used to determine whether the insect responds to any putative pheromone. The olfactometer design can take many forms depending on the insect under study and the nature of the odour to be tested. One type of olfactometer widely used to study mosquito response to host odours consists of a flight chamber (wind tunnel) into which protrudes two odour baited traps as used to test *Ae. aegypti* response to volatile plant odours by Jepson and Healy (1988) or as used more recently by Spitzen *et al.* (2008) to test the response of *An. gambiae* to human odours. In this olfactometer, mosquitoes

are released into the wind tunnel and are attracted to either the control or test trap with the test traps baited with the odour cue that is to be tested. Another approach is to use a Y-tube olfactometer design such as those described for *Ae. aegypti* by Mukwaya (1976), Geier and Boeckh (1999) and many others. In a Y-tube olfactometer there are two arms (the test and the control arm) and insects are able to respond by moving upwind and entering either of the two arms.

In those olfactometers which rely on the odour being present in a moving column of air, flow can be maintained either by incorporation of a fan or by use of compressed air. Not all olfactometers use air flow; for example, the olfactometer used by Eiras and Jepson (1994) to investigate *Ae. aegypti* response to host odours relied on passive diffusion of the odour. However, olfactometers that rely on airflow are useful where the insect is expected to respond anemotactically to the odour source.

Various materials can be used to construct olfactometers although as noted by Birch and Haynes (1982) flight chambers with glass walls unlike those made from Perspex can be cleaned with solvent.

4.1.4 Use of host odour mimics in bioassays

In order to check that apparatus is capable of detecting response to odours it is useful to first test the response to a known attractant such as host odour. Furthermore, since *Ae. aegypti* mate in the vicinity of a host their response to a potential source of pheromone and/or release of any pheromone may be affected by the presence of host odour and thus it may be useful to test potential sources of pheromone in both the presence and absence of host odour. Although host visual cues may also be important in attracting males to hosts and in stimulating release

of any pheromone and/or the response of females to any pheromone this chapter focuses solely on olfactory cues.

Worn socks are a source of host odour that has been successfully shown to attract both *Ae. aegypti* (Kline 1998) and *An. gambiae* (Smallegange *et al.* 2010; Olanga *et al.* 2010) in olfactometers. Kline (1998) also found that worn socks attracted *Ae. aegypti* to traps in the field and that addition of carbon dioxide at 500ml min^{-1} synergised increased attraction of *Ae. aegypti* to worn sock odour. Nevertheless, the usefulness of worn socks as a host odour mimic in the laboratory can be compromised by the presence of non-human contaminants from the materials used in the manufacture of the socks making subsequent GC-MS analysis difficult as noted by Smallegange *et al* (2010).

There has been much endeavour to recreate host odour through the development of synthetic host odours although synthetic blends developed to date have not been as attractive as natural skin odours (Williams *et al.* 2006). An example of a commercially available synthetic blend is the BG-Lure which contains lactic acid, ammonia and various unspecified fatty acids (Biogents 2012).

4.2 Aims and objectives

4.2.1 Aims

The main aim of the work presented in this chapter was to develop an olfactometer based bioassay to test behavioural evidence for pheromone mediated mating behaviour in *Ae. aegypti.* The aim was to optimise the design of a "closed system" olfactometer and to check that it was capable of demonstrating behavioural response to odours by testing it using a known attractant (worn socks). Additional aims were to examine evidence for synergistic or additive

interactions between host odour and any pheromone as well as to test a synthetic alternative to worn socks for use as a host odour mimic in bioassays.

4.2.2 Objectives

1. To develop an olfactometer to test the behavioural response of female Ae. aegypti to odour from males.
2. To evaluate olfactometer designs by testing the response of mosquitoes to a known attractant (worn socks).
3. To determine whether air blown over virgin males is attractive to virgin females.
4. To determine whether odour from virgin males enhances attraction of virgin females to odour from worn socks.
5. To determine whether odour from virgin males enhances attraction of virgin females to odour from worn socks using an elevated level of carbon dioxide.
6. To determine whether a synthetic host odour mimic (BG Lure) can be used to replace worn socks as a source of host odour in bioassays.

4.3 Materials and methods

4.3.1 Mosquitoes

Unmated male and female mosquitoes were used 4 days post-emergence for all experiments outlined in this chapter. Sucrose solution was removed from female cages at least 12 hours prior to commencement of experiments to increase their responsiveness to host odour.

4.3.2 Bioassay room

Conditions in the room used for carrying out bioassays were as described in Chapter 2. The compressed air supply to the olfactometer contained moisture of 50ppm by volume (BOC Industrial Gases, UK).

4.3.3 Olfactometers

The same basic design was used for all olfactometers, but the material and dimensions of some components varied. Table 4.1 summarises the main differences between the four olfactometer designs. All olfactometers were designed to measure the upwind flight response of virgin females to an odour source and consisted of two chambers. A positive response was recorded when mosquitoes moved from the chamber into which they were introduced to the chamber upwind of this (i.e. from component “F” to component “E” in Figure 4.1).

Table 4.1 Summary of differences between four olfactometer designs

Olfactometer design	Material	Internal diameter of component F	Length of component F	Other details	Rationale
1	Perspex	6.0 cm	12.0 cm	Previously used as an olfactometer for *An. gambiae*	Readily available at Keele and previously used for *Anopheles* mosquitoes
2	Glass	1.1 – 2.2 cm	17.0 cm	Originally designed for western flower thrips	Readily available at Keele; all glass made washing easier
3	Glass	2.3 cm	35.0 cm	Same as design 2 but a glass distillation column used for component "F"	Wider entry tube thought to be more suitable for insect the size of *Ae. aegypti*; glass distillation column readily available. However, not of uniform diameter.
4	Glass	2.1 cm	13.2 cm	Same as design 2 but a glass cylinder used for component "F"	Wider entry tube than in design 3 and uniform diameter.

4.3.3.1 Basic design

The basic design is shown in Figure 4.1. Air flowed from a cylinder of compressed air (component “A”; BOC Industrial Gases, UK) via FEP tubing (Chemfluor; 0.25 inch internal diameter; 0.625 inch outer diameter; Sigma-Aldrich Company Ltd, UK) through a carbon trap to remove any impurities (component “B”; Alltech, USA) followed by a Supelco rotameter (component “C”; PMRI-011426 Rotameter, Sigma Aldrich, UK) and continued to flow via FEP tubing through a 5L glass jar (component “D”; FV5L, Quickfit; Fisher Scientific, UK) containing the odour source. The 5L glass jar was covered with a glass lid (MAF 2/2; 19/26, Quickfit, Fisher Scientific, UK) secured using four Quickfit clamps (JC35, Quickfit, Fisher Scientific, UK). A Drechsel bottle head (Quickfit; joint B/L 19/26, Fisher Scientific, UK) inserted into the central port (joint BL 19/26) of the lid was used to pass air through the glass jar while the other ports were blocked with ground glass stoppers (Quickfit BL joint 19/26; Fisher Scientific, UK). The inlet side of the Drechsel head was connected via FEP tubing to the rotameter while the outlet side was connected via FEP tubing to the olfactometer (component “E”). Four different olfactometer designs described in the following sections were tested.

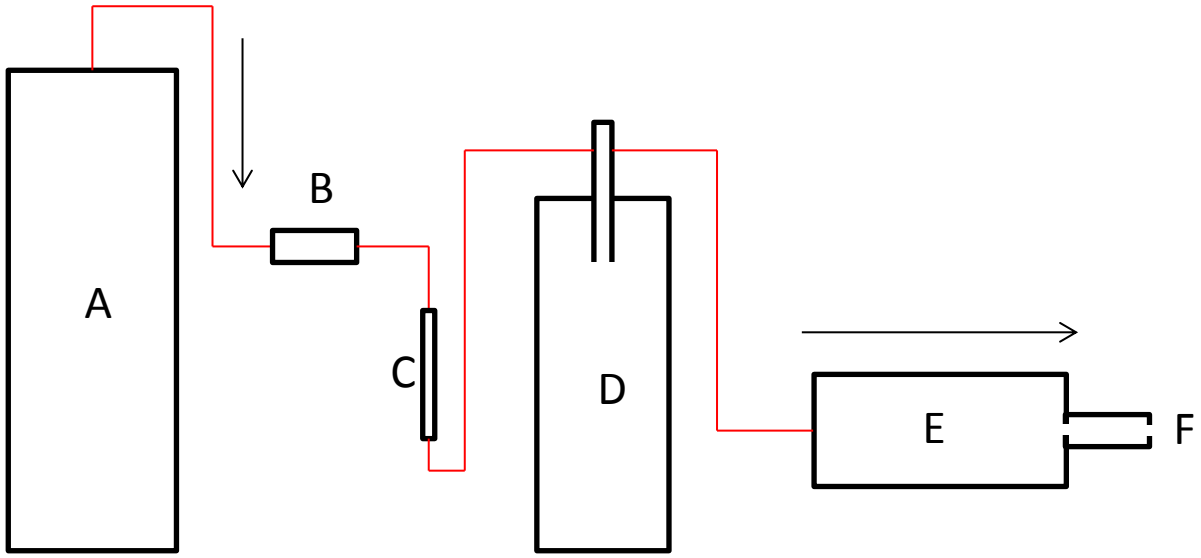

Figure 4.1 Schematic design of basic olfactometer layout: The overall design of the olfactometer is shown systematically. Red Lines indicate connections consisting of FEP tubing and arrows indicate direction of air flow. **A:** air cylinder; **B**: filter; **C**: rotameter for adjusting air flow; **D**: Flask containing odour source through which air stream flows; **E**: A positive response is recorded if the mosquito enters compartment "E"; **F**: Enclosure into which each test mosquito was introduced. See text for a more detailed description of components.

4.3.3.2 Design 1

The main body of the olfactometer consisted of a Perspex tube (17cm diameter x 40cm long); Figure 4.2. This apparatus had previously been used to study the behaviour of *An. gambiae* mosquitoes (Abu Hasan 2008). One end of the Perspex tube had a permanently fixed end plate in the centre of which was an opening that enabled the introduction of the air supply via FEP tubing. The other end of the tube was enclosed by a lid that could be attached with the aid of metal clips. In the centre of this lid was an opening (2cm diameter) to enable mosquitoes to move from compartment “F” to compartment “E” in response to attractive odours. On the outer face of the detachable lid, four Velcro strips were attached around the opening and this was used to attach a second Perspex tube (6cm diameter; 12cm long) (part F). The end of this second tube that abutted the first tube contained an opening (3cm diameter) that allowed a connection between the two tubes. The other end of this tube was covered with Nylon mesh. A 1cm hole (blocked with cotton wool) in the centre of the nylon mesh allowed the introduction of mosquitoes.

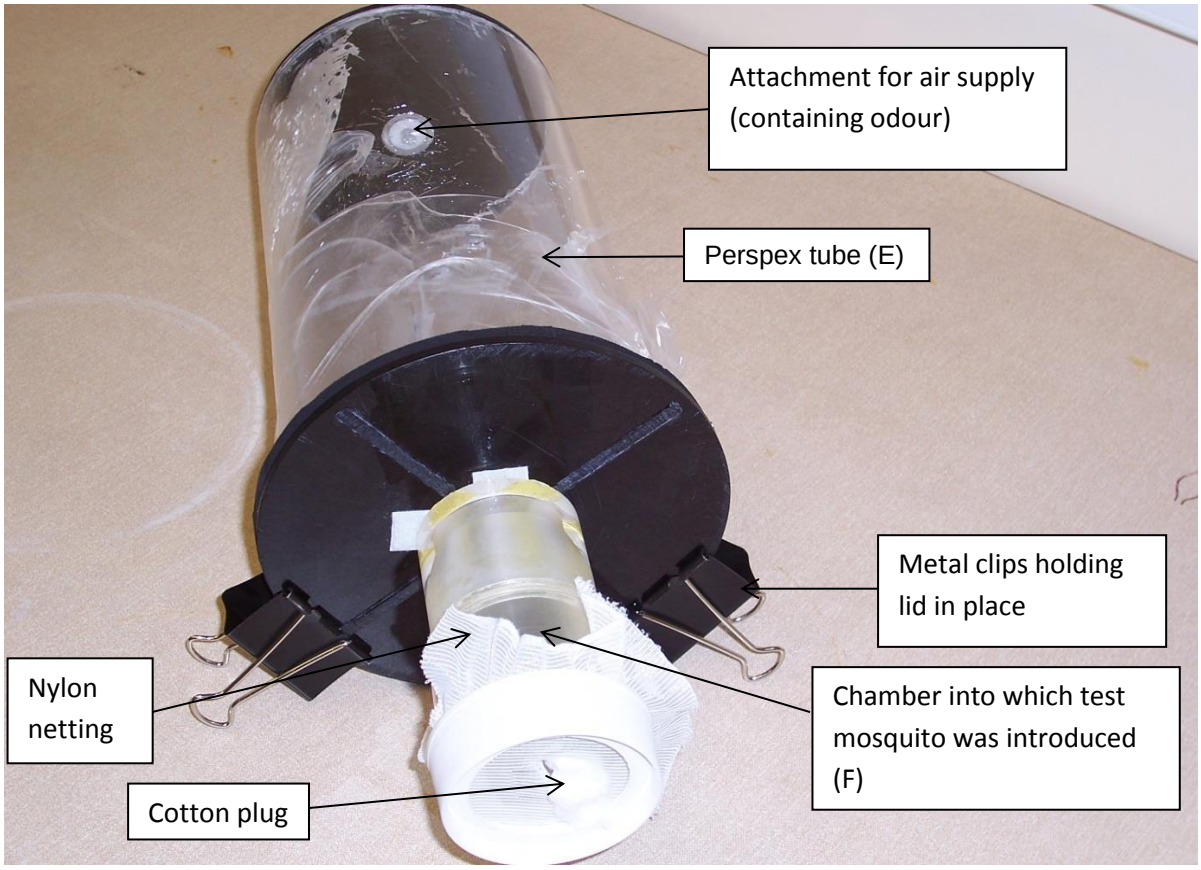

Figure 4.2 - Olfactometer Design 1: Photo shows components "E" and "F" in olfactometer Design 1. Other components which were the same in all designs are not shown.

4.3.3.3 Design 2

This all-glass apparatus was set up as has previously been described by Dublon (2009) for bioassays on western flower thrips, *Frankliniella occidentalis* (Thysanoptera: Thripidae). This apparatus is shown in Figure 4.3. Component “E” in this design consisted of a 2L flat bottomed glass jar (Quickfit FV2L; Scientific Glass Laboratories Ltd, UK) which had been modified by the insertion of a ground glass BL24/29 port at its base (Scientific Glass Laboratories Ltd, UK; design as per Dublon 2009). A BL24/29 ground glass insert was inserted into this port which narrowed at its outermost side to enable attachment of the external air supply via FEP tubing. The modified 2L jar was sealed with a Quickfit multi-port lid (MAF 2/32, Quickfit, Fisher Scientific, UK).

Instead of using a Perspex enclosure for component “F”, a glass insert in the upper-most port of the lid described by Dublon, 2009 (Scientific Glassware Laboratories Ltd, UK) was used for introducing the mosquito. All other ports were blocked with Quickfit BL 19/26 ground glass stoppers. A small piece of cotton wool was inserted at the end of the insert following introduction of the mosquito to prevent escape.

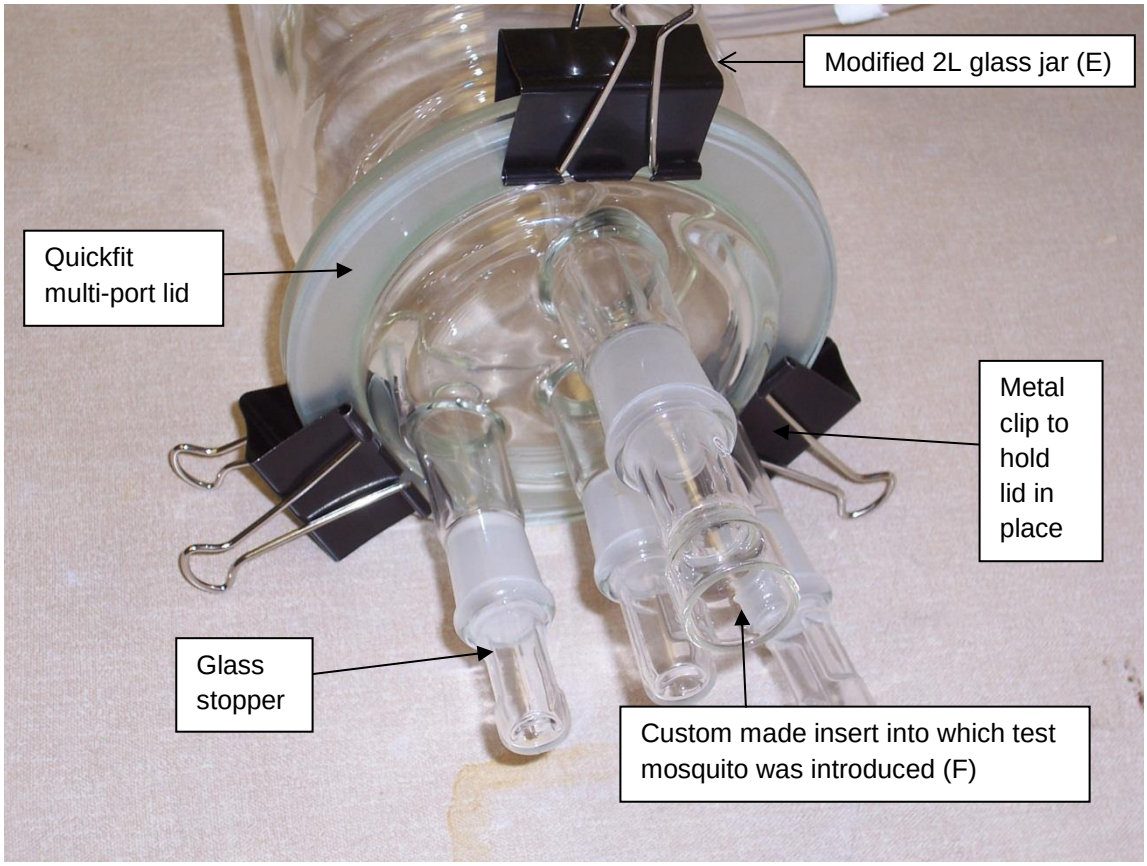

Figure 4.3 - Olfactometer design 2: Photo shows components “E” and “F” of olfactometer Design 2. Components “E” and “F” consisted of all-glass apparatus originally designed for bioassays on western flower thrips. Other components which were the same in all designs are not shown.

4.3.3.4 Design 3

This apparatus differed from Design 2 in that the custom made insert used for component “F” was replaced with a glass distillation column (35 cm long; approximately 2.3 cm maximum internal diameter; FC7/23 Quickfit, Fisher Scientific, UK) shown in Figure 4.4. As with Design 2, a small piece of cotton wool was inserted at the end of the insert following introduction of the mosquito to prevent escape.

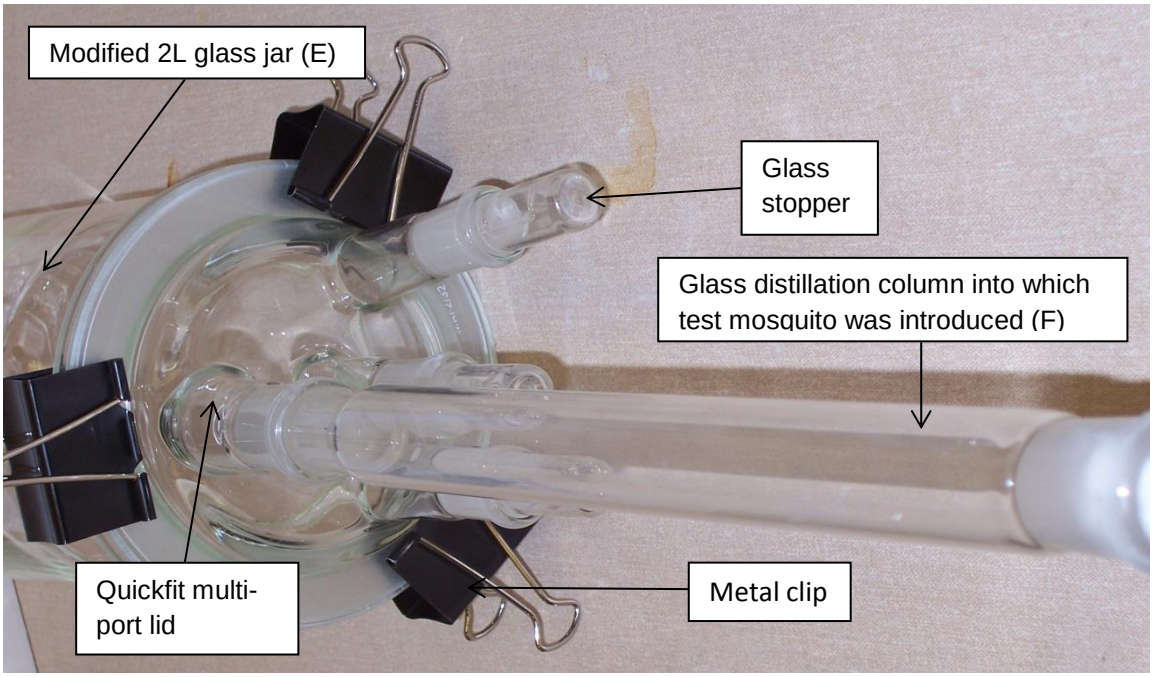

Figure 4.4 - Olfactometer Design 3: Photo shows components "E" and "F" in olfactometer Design 3. Other components are not shown. A glass distillation column replaced the custom made insert used in Design 2

4.3.3.5 Design 4

Design 4 differed from Design 3 in the entry port (component “F”) used to introduce the mosquitoes. The glass distillation column used in Design 3 was replaced with a glass cylinder (13.2cm long; 2.1cm internal diameter) as shown in Figure 4.5. The glass lid affixed to component “E” in Design 3 was replaced with a glass lid containing one BL 34/35 port (Quickfit MAF 2/52). The four remaining BL 19/26 ports were blocked with Quickfit BL 19/26 ground glass stoppers. Nylon netting was secured to the end of component “F” with the polyethylene cap of a 30ml universal container (Fisher Scientific Ltd, UK). A slit in the nylon netting of a few mm was made to enable introduction of the test mosquito. A small (5 x 5 mm) piece of nylon netting was used to cover the slit following introduction of the mosquito.

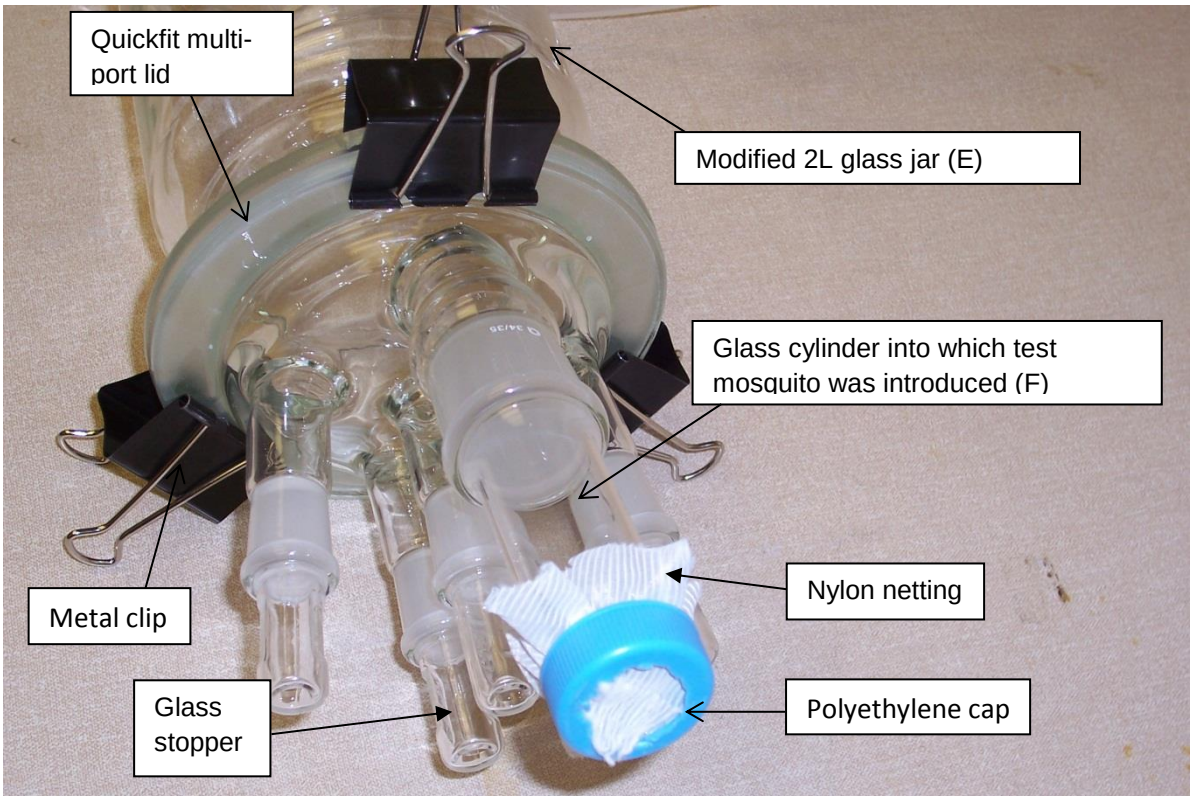

Figure 4.5 - Olfactometer Design 4: Photo shows components "E" and "F" in olfactometer Design 4. Other components are not shown. See text for details.

4.3.4 Bioassay protocol

All experiments were carried out 0 – 3 hours prior to the end of photophase since previous studies have found that maximum flight activity in *Ae. aegypti* occurred at both the beginning and end of the photophase (Jones, 1981).

Mosquitoes were placed in the bioassay room at least one hour prior to being used for experimentation to enable acclimatisation to the conditions in the bioassay room. Mosquitoes were introduced one at a time into the small chamber labelled “F” in Figure 4.1. The mosquito was left for 3 minutes and after that time it was noted whether or not it had entered component “E”. If the mosquito had entered component “E” within this time it was recorded as a positive response. In all cases any mosquito that had entered part “E” or that remained in part “F” of the apparatus was removed prior to the introduction of the next mosquito. Each test mosquito was used once.

4.3.5 Different host odours

Cotton socks that had not been treated with an anti-microbial treatment were worn by the same person for 3 consecutive days for 12 hours each day and were placed into sealed polythene bags prior to use in experiments. In Experiment 6, BG Lure (Biogents AG, Germany) was used in place of worn socks.

4.3.6 Preliminary experiments

Preliminary experiments were carried out using Olfactometer Design 1. The response of virgin females and virgin males to worn sock odour was tested to determine whether the olfactometer was capable of measuring behavioural responses of *Ae. aegypti* to known attractants. Worn socks were placed in the

glass vessel (Figure 4.1 component “D”). A control consisting of no odour i.e. with no socks in component “D” was also run.

4.3.7 Details of main experiments

4.3.7.1 Experiment 1 – Attraction of virgin females to 100 virgin males in presence and absence of worn sock odour

In this experiment the response of 3 day old virgin females to worn socks alone was compared to that of worn socks with 100 3-day old virgin males. For comparison; response of virgin females to no odour, worn socks alone and 100 virgin males alone was also tested. 20 virgin females were tested per treatment (no odour, 100 males, worn socks and worn socks plus 100 males) each day over eight different days. Two treatments were carried out per day; either i) no odour followed by 100 males or ii) worn socks followed by worn socks and 100 males.

In all cases where 100 males were used, the 100 males were given 20 minutes to acclimatise after being introduced into component “D” before commencement of the experiment.

4.3.7.2 Experiment 2 – Response of virgin females to virgin males with 0.1% CO_2 in presence and absence of worn sock odour

Experiment 1 with worn socks was repeated, using an air cylinder containing 0.1% CO_2. The concentration of CO_2 in ambient air is 0.038%. It was thought that odour from worn socks by itself might not be sufficient to mimic host odour without the presence of an elevated concentration of CO_2 and this might limit the responsiveness of virgin females to odour from virgin males. As with

experiment 1, two treatments were carried out per day; either i) no odour followed by 100 males or ii) worn socks followed by worn socks and 100 males.

4.3.7.3 Experiment 3 – Response to different numbers of males with 0.1% CO_2

The response of virgin females to 25, 100 and 200 males with worn socks was tested as well as their response to no odour and worn socks alone. Treatments were fully randomised using the random number generator in Excel and two treatments were carried out each day. In order to facilitate an adequate supply of worn socks for these experiments socks were only worn for 24 hours.

4.3.7.4 Experiment 4 – Response to different numbers of males with 0.3% CO_2

This experiment was carried out in the same manner as for experiment 3 except that compressed air containing 0.3% CO_2 was used instead of 0.1%.

4.3.7.5 Experiment 5 – Use of an alternative entry tube (13.2 cm long x 2.1 cm internal diameter)

These experiments were carried out using olfactometer design 4 using the same procedure as described in section 4.3.7.1.

4.3.7.6 Experiment 6 – Trial of a synthetic host odour mimic as an alternative to worn socks

These experiments were carried out with olfactometer design 4 using the BG Lure instead of worn socks. The BG Lure was wrapped in aluminium foil and

stored at -80°C in between experiments. The experimental procedure was the same as described in section 4.3.7.1.

4.3.8 Data analysis

These experiments were no choice and each mosquito tested either responded or did not respond to the odour it was exposed to. Therefore, for each experiment, data from each of the days over which the experiment was run were pooled and analysed using the 2-sided Pearson χ^2 test (SPSS v18.0, SPSS Inc., USA). The *P*-values reported were those computed by SPSS using the Exact method. This test was used in order to determine whether the proportion of mosquitoes responding to the test odour (observed) differed significantly from the proportion responding to the control (expected) at α=0.05. χ^2 test was also used to check whether there was any significant difference in mosquito response to each odour treatment between the different days over which the experiment was run.

4.4 Results

4.4.1 Preliminary experiment: male and female response to worn socks using olfactometer design 1

Results are shown in Figure 4.6.1. Significantly more male (χ^2 = 23.8, 1 d.f., *P* = <0.001) and female (χ^2 = 3.34, 1 d.f., *P* = 0.04) mosquitoes responded to worn sock odour compared with no odour indicating that the bioassay was capable of measuring the response of mosquitoes to different odours. Interestingly, 14% more males than females responded to sock odour although this was not significant (χ^2 = 2.37, 1 d.f., *P* = 0.08).

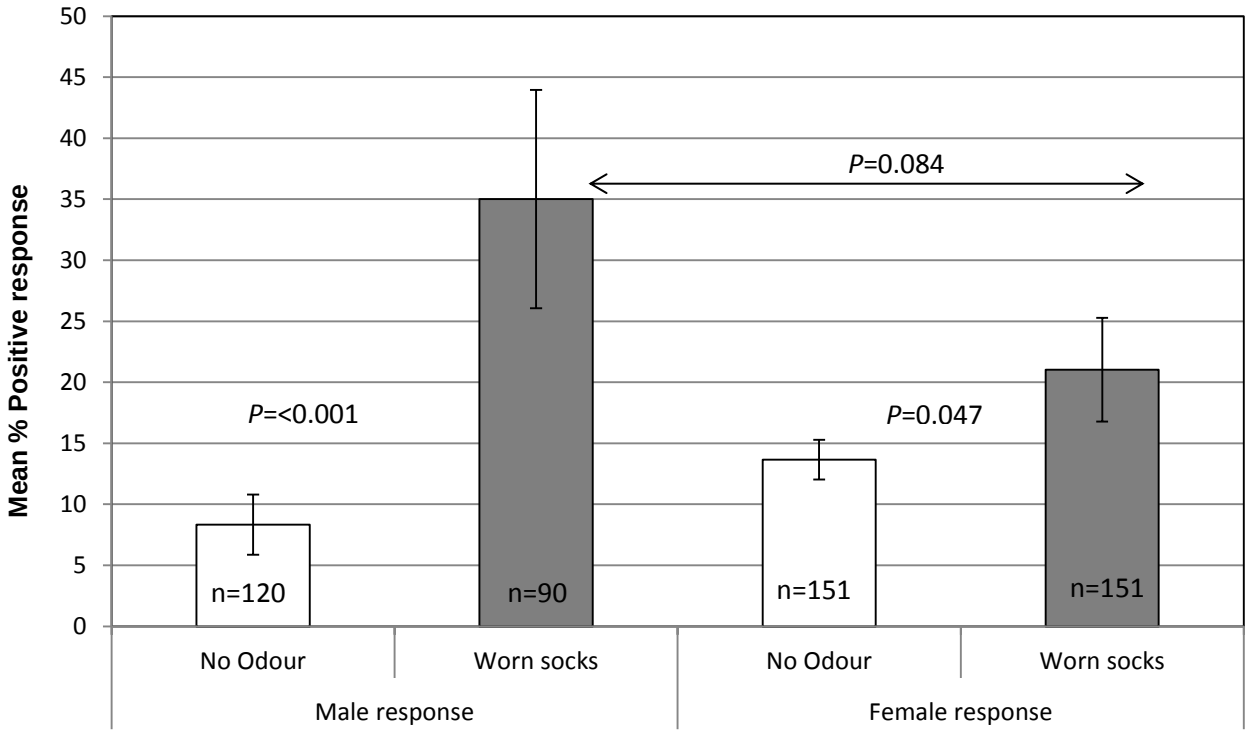

Figure 4.6.1 – Response of males and females to worn sock odour in the olfactometer: The mean daily positive response as a percentage of virgin males (left) and virgin females (right) to worn sock odour is shown compared to the response to a control consisting of no odour. Error bars depict the SEM. *n* is the number of mosquitoes tested to each treatment; each treatment was carried out over 5 – 7 days. *P*-values were calculated using the Pearson χ^2 test.

4.4.2 Experiment 1: Virgin female response to male odour in the absence or presence of worn sock odour using olfactometer design 1

Results of Experiment 1 are summarised in Figure 4.6.2.1. Odour from 100 males alone was not significantly attractive to females (χ^2 = 0.56, 1 d.f., *P* = 0.57). The percentage of virgin females responding positively to socks plus 100 males was actually lower than in response to worn socks alone. Significantly less virgin females entered chamber E when worn socks plus 100 males was present compared to host odour alone (χ^2 = 4.80, 1 d.f., *P* = 0.04). Female *Ae. aegypti* were significantly more attracted (χ^2 = 7.47, 1 d.f., *P* = 0.009) to worn sock odour (21.25 % mean daily positive response $\pm$ 3.23 SEM) compared to no odour (8.75 % mean daily positive response $\pm$ 2.23 SEM) indicating that mosquitoes were able to respond to a known attractant in this olfactometer.

However, there was a significant difference in response of virgin females between days for the treatment worn socks plus 100 males (χ^2 = 18.36, 7 d.f., *P* = <0.001), but no significant difference between days for the other treatments suggesting this was the result of variation in the males.

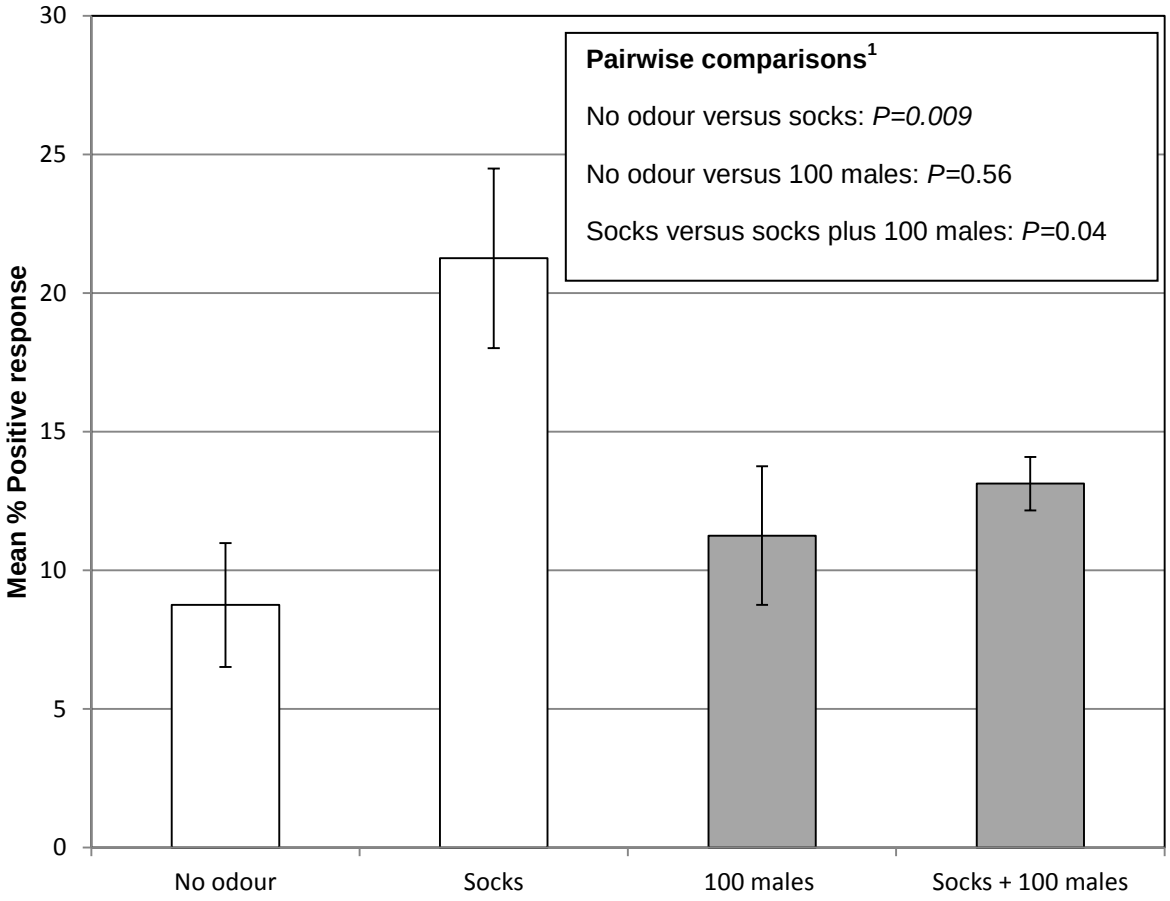

Figure 4.6.2.1 - Response to worn socks and/or 100 Males: The mean daily percentage of mosquitoes responding to no odour, worn socks, 100 males and 100 males with worn socks is shown. n=160 virgin females tested over 8 days per treatment. Error bars indicate the SEM.

[1]*P*-values were obtained using Pearson χ^2 test on the pooled data

4.4.3 Experiment 2: Response to male odour and worn sock odour with an elevated level of CO_2 (0.1%) using olfactometer design 2

Results of this experiment are summarised in Figure 4.6.2.2. Significantly more (χ^2 =9.29, 1 d.f., *P* = 0.003) *Ae. aegypti* females were attracted to worn socks with 100 males (56.7% mean daily positive response ± 15.9 SEM) than were attracted to worn socks alone (35.0% mean daily positive response ± 8.6 SEM). There was no significant difference in attraction of virgin females to air blown over 100 virgin males compared to no odour (χ^2 =3.07, 1 d.f., *P* = 0.116). There was significant variation between days for the treatment socks plus males (χ^2 = 29.74, 7 d.f., *P* = <0.001), but not for either males alone or socks alone.

Significantly more (χ^2 = 11.93, 1 d.f., *P* = 0.001) *Ae. aegypti* females responded positively to sock odour (35.0% mean daily positive response ± 8.6 SEM) than no odour (12.5% mean daily positive response ± 3.2 SEM) indicating that mosquitoes were able to respond to a known attractant in this olfactometer.. However, it was also observed that in these bioassays test mosquitoes struggled to fly and often walked towards the stimulus probably due to the small size of the entry tube (component “A”) used in Olfactometer Design 2.

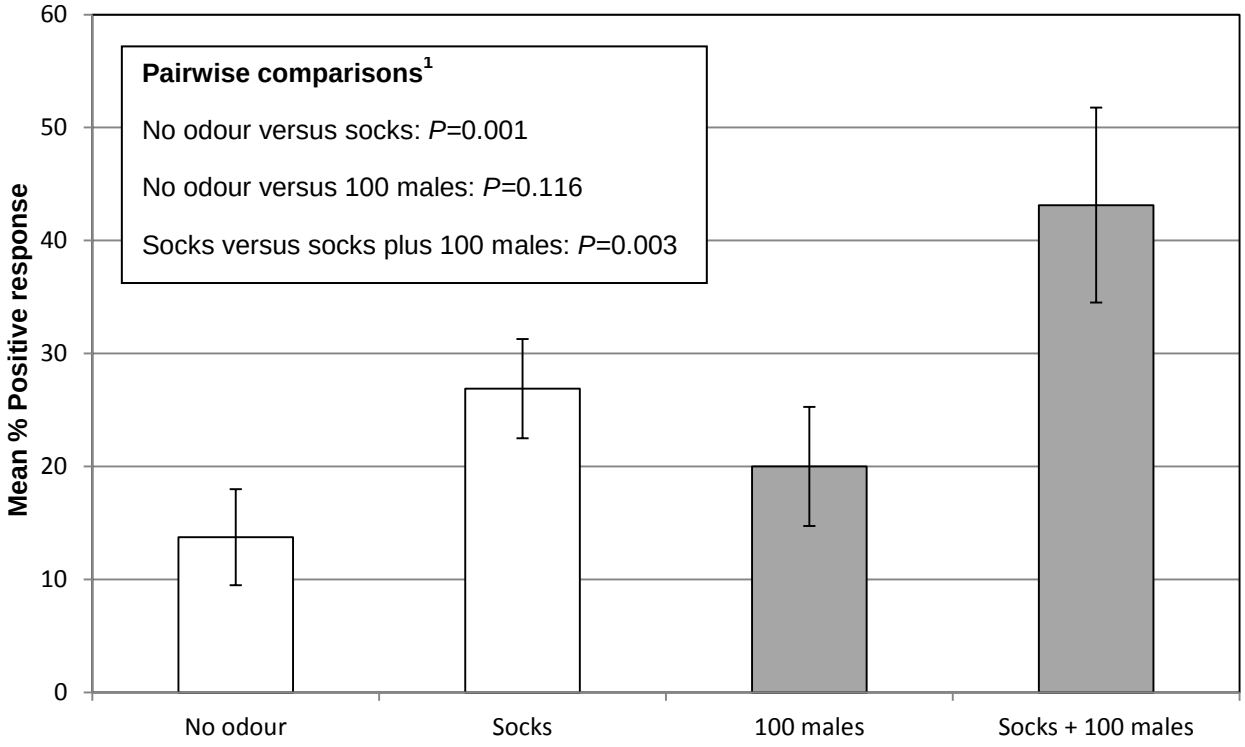

Figure 4.6.2.2 - Mean daily percentage of virgin females responding to worn socks and/or 100 males when using 0.1% CO_2: The mean daily percentage of mosquitoes responding positively to no odour, worn socks, 100 males and 100 males with worn socks is shown. n=160 virgin females tested over 8 days per treatment; except for 100 males where n=80 virgin females tested over 4 days per treatment. Error bars indicate the SEM.

[1]*P*-values were obtained using the Pearson χ^2 test on the pooled data

4.4.4 Experiment 3: Response to different numbers of virgin males using olfactometer design 3 with 0.1% CO_2

In this experiment the response of virgin females to different numbers of males in the presence of worn socks and 0.1% carbon dioxide was tested using olfactometer design 3. Results of this experiment are summarised in Figure 4.6.2.3. A 4 x 2 χ^2 analysis found a significant difference in response to different numbers of males (χ^2 = 4.97, 3 d.f., *P* = 0.029). However as can be seen from Figure 4.6.2.3 there was no obvious trend in response to increasing numbers of males.

Significantly more *Ae. aegypti* females (χ^2 = 19.78, 1 d.f., *P* = <0.001) responded positively to sock odour (25.0% mean daily positive response ± 4.8 SEM) than no odour (1.3% mean daily positive response ± 1.1 SEM) indicating that mosquitoes were able to respond to a known attractant in this olfactometer.

There was no significant difference in response of virgin females between different days for any of the treatments.

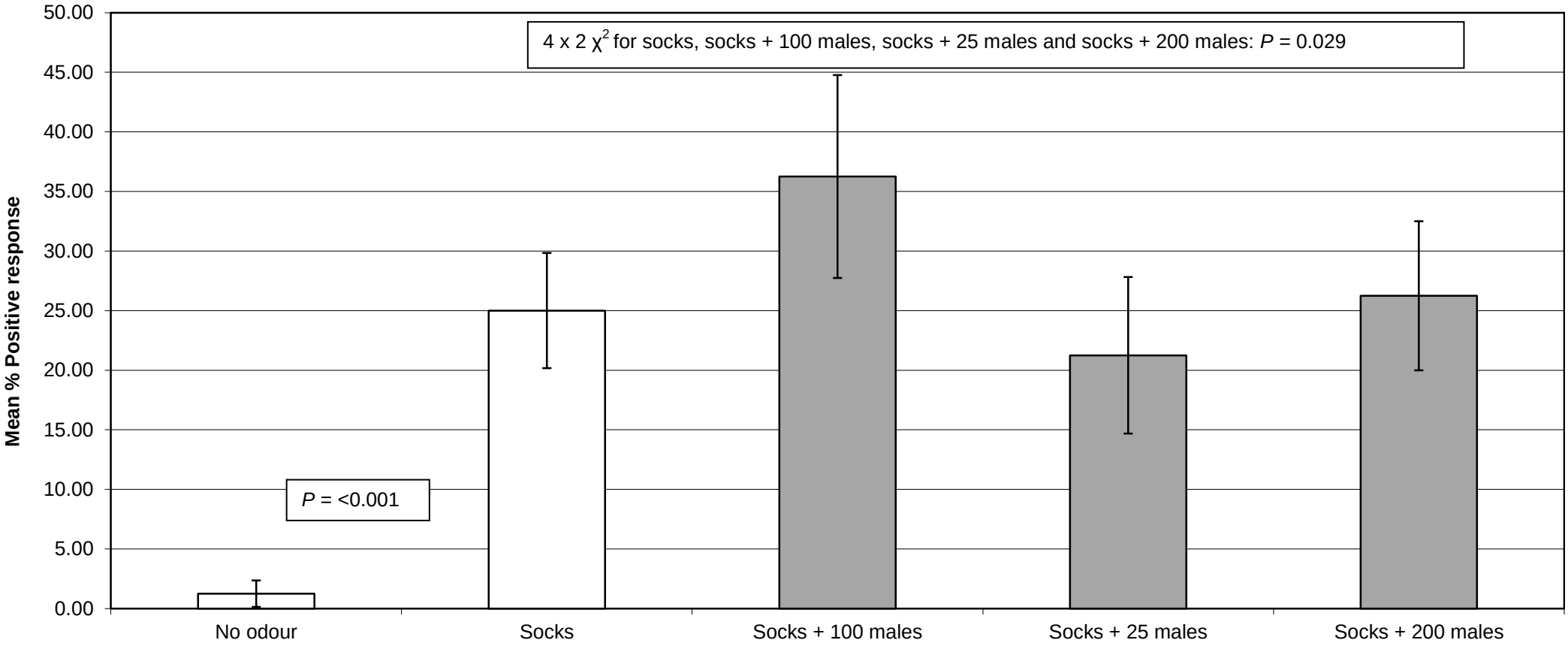

Figure 4.6.2.3 - Mean daily percentage of virgin females responding to worn socks and 100 males, 200 males or 25 males when using 0.1% CO_2: The percentage of mosquitoes responding to no odour, worn socks, 100 males with worn socks, 200 males with worn socks and 25 males with worn socks is shown. n = 80 virgin females tested over 4 days for each treatment. Error bars indicate the SEM. [1]*P*-values were obtained using the Pearson χ^2 test on the pooled data

4.4.5 Experiment 4: Response to male odour and worn sock odour with 0.3% CO_2 using olfactometer design 3

In this experiment the response of virgin females to different numbers of males in the presence of worn socks and 0.3% carbon dioxide was tested using olfactometer design 3. Results of this experiment are summarised in Figure 4.6.2.4. A 4 x 2 χ^2 analysis found no significant difference in the response of virgin females to different numbers of males (χ^2 = 1.893, 3 d.f., *P* = 0.151). Significantly more *Ae. aegypti* females (χ^2 = 7.83, 1 d.f., *P* = 0.009) responded positively to sock odour (15.00% mean daily positive response ± 3.65 SEM) than no odour (2.50% mean daily positive response ± 1.29 SEM) indicating that mosquitoes were able to respond to a known attractant in this olfactometer. There was no significant difference in the response of virgin females between different days for any of the treatments.

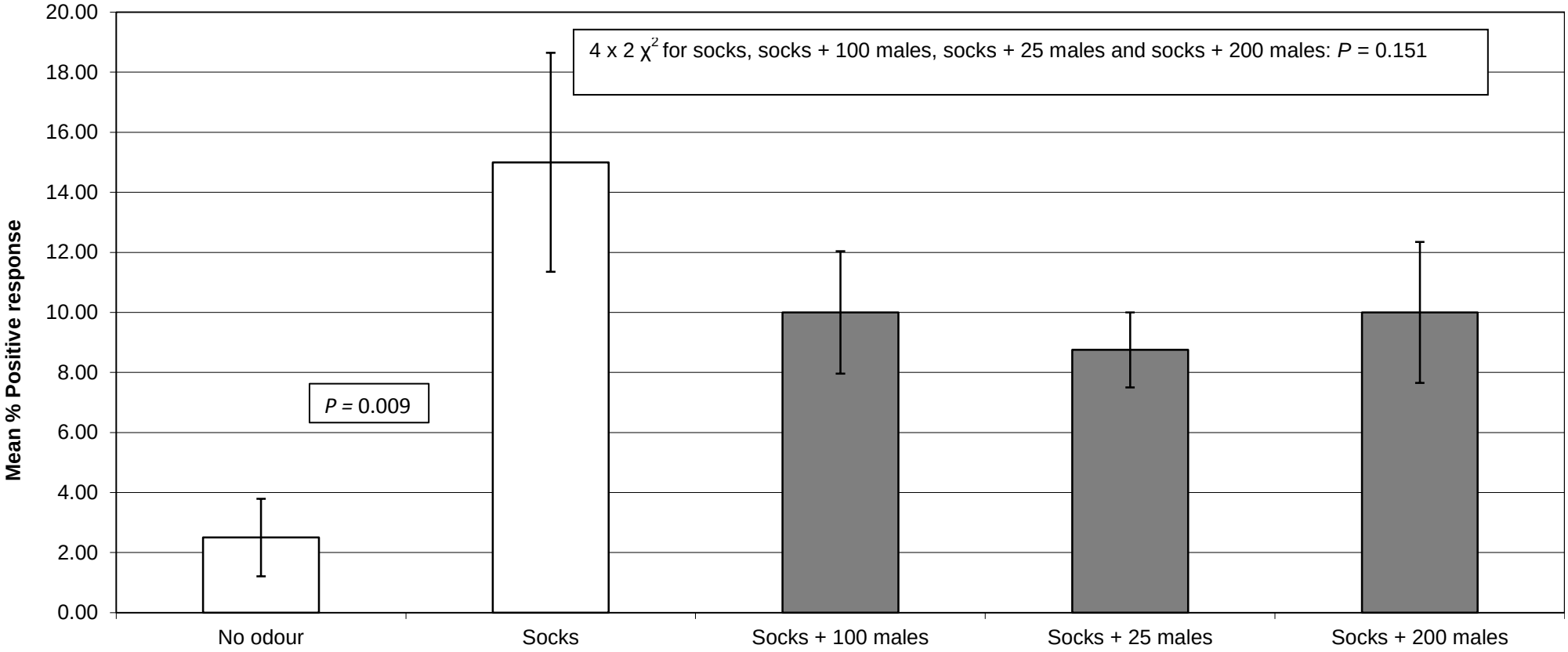

Figure 4.6.2.4 - Mean daily percentage of virgin females responding to worn socks and 100 males or 25 males when using 0.3% CO_2: The percentage of mosquitoes entering the tunnel in response to no odour, worn socks, 100 males with worn socks, 200 males with worn socks and 25 males with worn socks is shown. n = 80 virgin females tested over 4 days for each treatment. Error bars indicate the SEM. [1]*P*-values were obtained using the Pearson χ^2 test on the pooled data

4.4.6 Experiment 5: To determine the response to male odour plus worn sock odour with 0.1% CO_2 using olfactometer design 4.

There was no significant difference in the number of females responding to odour from 100 males plus worn socks compared to odour from worn socks alone (χ^2 =<0.001, 1 d.f., *P* = >0.99). Significantly more (χ^2 = 6.26,1 d.f., *P* = 0.02) *Ae. aegypti* females responded positively to sock odour (17.5% mean daily positive response ± 3.2) than no odour (5.0% mean daily positive response ± 4.3) indicating the olfactometer apparatus was working. There was no significant difference in the response of virgin females between different days for any of the treatments. Results are summarised in Figure 4.6.2.5.

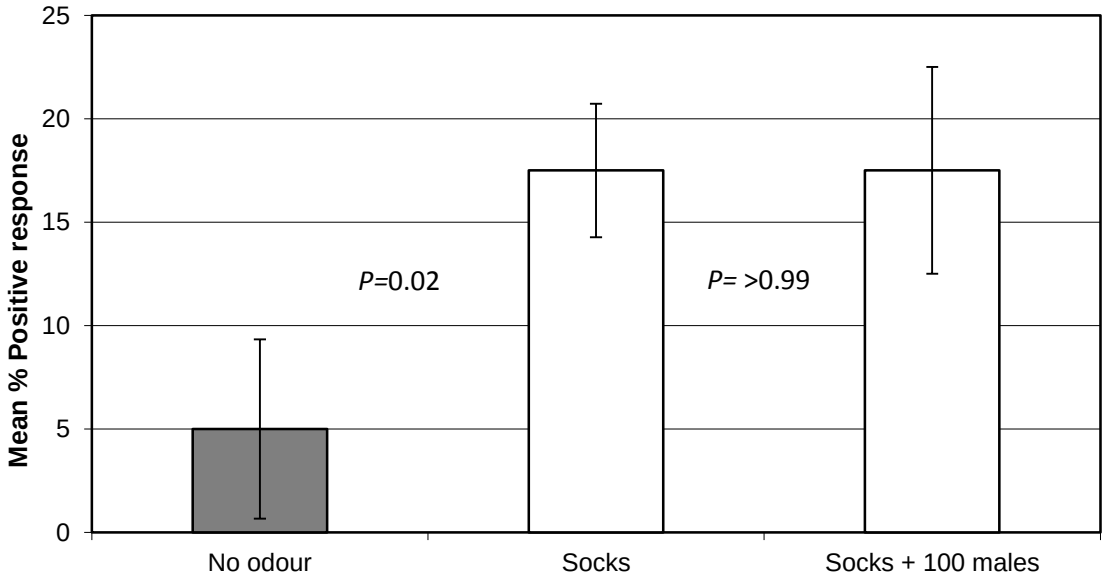

Figure 4.6.2.5 - Mean daily percentage of virgin females responding to worn socks and 100 males using 0.1% CO_2 and a shorter wider entry tube (13.2cm long; 2.1cm internal diameter): The percentage of mosquitoes responding to no odour, worn socks and 100 males with worn socks is shown. *n* = 80 virgin females tested over 4 days for each treatment. *P*-values were obtained using the Pearson χ^2 test on the pooled data. Error bars indicate the SEM.

4.4.7 Testing BG Lure as a substitute for worn sock odour

Although more females were attracted to BG Lure than to no odour this was not significant (χ^2 = 0.06, 1 d.f., *P* = >0.99). More females were also attracted to BG Lure plus male odour than BG Lure alone, but this was also not significant (χ^2 = 0.46, 1 d.f., *P* = 0.65).There was also no significant difference (χ^2 = 2.06, *P* = 0.23) between no odour and lure plus 100 males. There was no significant difference in the response of virgin females between days for any of the treatments. Results are summarised in Figure 4.6.2.6

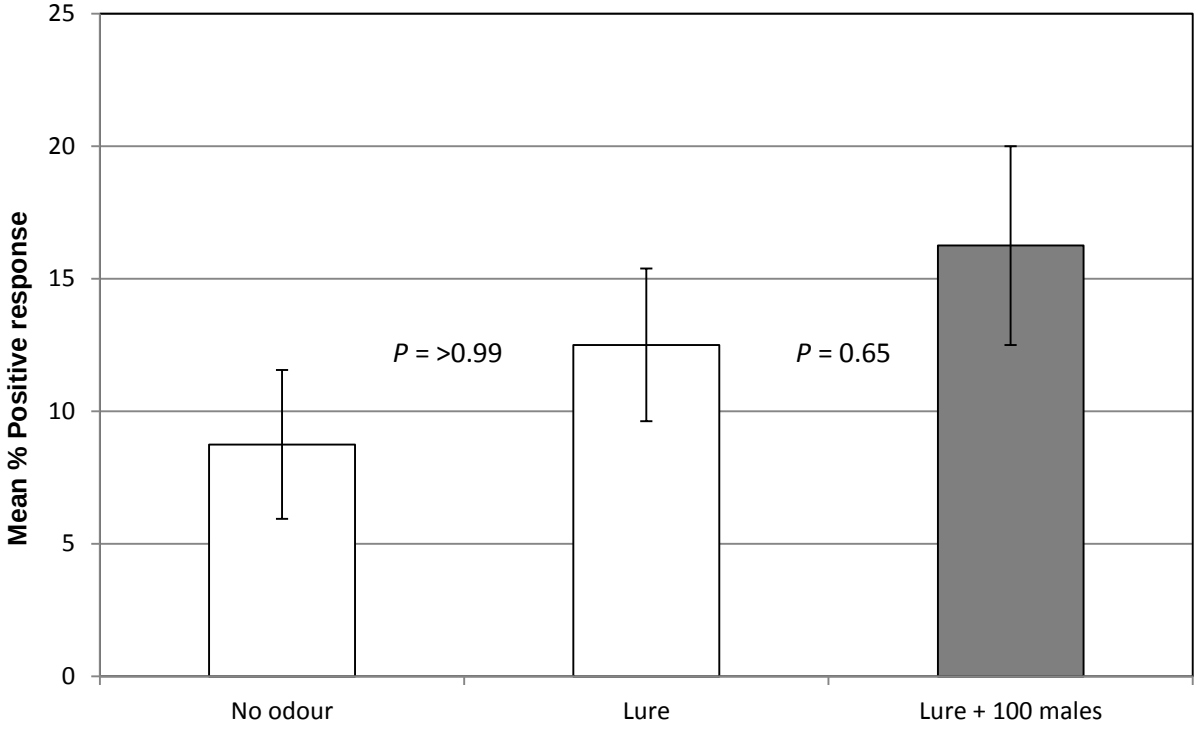

Figure 4.6.2.6 – Use of the synthetic host odour mimic BG Lure as an alternative to worn socks: The mean daily percentage of mosquitoes responding to no odour, BG Lure and 100 males plus BG Lure is shown. n = 80 virgin females tested over 4 days for each treatment. *P*-values were obtained using the Pearson χ^2 test on the pooled data. Error bars indicate the SEM.

4.5 Discussion

In this chapter a number of different olfactometer designs have successfully detected the attraction of *Ae. aegypti* to a known attractant (worn sock odour). It was important to test potential olfactometer based bioassays with a known attractant since if a bioassay is unable to detect attraction to a known attractant then it is unlikely to be capable of detecting responses to unknown attractants including potential pheromones. No evidence was found for the attraction of virgin females to male odour alone in these experiments. In Experiment 1, male odour appeared to reduce the attraction of worn socks to virgin females. However, in Experiment 2 evidence suggested that males plus worn sock odour with 0.1% carbon dioxide were more attractive than worn sock odour with 0.1% carbon dioxide alone. Interestingly it was also noted that there was significant variation between days in the response to worn sock odour plus 100 males, but not in the response to worn sock odour alone.

4.5.1 Evaluation of olfactometer designs

All four olfactometer designs used in these experiments were able to detect the response of *Ae. aegypti* to a known attractant (worn sock odour). This suggests that all four designs had the potential to detect behavioural responses to any potential source of pheromone assuming that the males were emitting any pheromone when in the confines of a 5L glass jar.

When conducting experiments using olfactometer design 2 it was observed that mosquitoes were unable to respond to odour stimuli with full flight due to the narrowness of the entry tube in which they were placed. In olfactometer designs 1

and 4 the flight response of mosquitoes did not appear to be impeded suggesting that these designs are more appropriate for assessing anemotactic response.

4.5.2 Response to host odour

In addition to confirming the response of mosquitoes to a known attractant (worn sock odour), results from the preliminary experiments also showed that male *Ae. aegypti* are also attracted to worn sock odour and thus the presence of males in the vicinity of hosts reported by earlier field studies (Hartberg 1971; Gubler and Bhattacharya 1972) likely results from males responding to host odour in a similar manner to females.

While research into host location in mosquitoes has almost exclusively focused on the response of females de Ázara *et al.* (2013) found that addition of carbon dioxide to traps increased the numbers of males caught as well as females although the increased catch rate was only 9% for males compared with 23% for females. McCall *et al.* (1996) have previously tested the response of males to sticky traps baited with mouse odour in the laboratory and found no significant difference in the number of males caught by mouse odour baited traps compared with control traps not baited with mouse odour whereas significantly more females were caught in the mouse odour baited trap. However, since males do not themselves blood feed it could be speculated that unlike females they might fly around hosts (or sources of host odour) rather than landing and furthermore the bioassay used by McCall *et al.* (1996) used still air and thus prevented mosquitoes responding with upwind anemotaxis. The finding that male *Ae. aegypti* are attracted to worn sock odour confirms the results of previous field studies (Hartberg 1971; Nelson *et al.* 1978; Corbet and Smith 1974; Trpis *et al.* 1973)

which suggested male *Ae. aegypti* are attracted to vertebrate hosts. These results suggest male attraction to vertebrate hosts is mediated by host odour volatiles, although whether males are attracted to the same host odour components as females remains to be determined. Since mating in *Ae. aegypti* takes place in the vicinity of a host, the attraction of males to host odour must be an adaptation enabling males to access females and this assertion is further supported by the observation that unmated blood-fed *Ae. aegypti* continue host-seeking behaviour until they have mated successfully (Fernandez and Klowden 1995). When investigating other potential semiochemicals that might modulate mating behaviour it is therefore important to consider potential interactions with host odour.

A greater proportion of males responded to worn sock odour than females although this was not significant ($P = 0.08$). Field observations of *Ae. albopictus* by Gubler and Bhattacharya (1972) claimed that male *Ae. albopictus* formed swarms around a host prior to the arrival of females i.e. suggesting a faster or stronger response of males to host odour compared to females. Field experiments by Hartberg (1971) found that most *Ae. aegypti* females that alighted on a host were inseminated and this might indicate that females were intercepted by males before reaching the host which would support the theory that males tend to arrive before females. However, *Ae. aegypti* landing catches by Hartberg (1971) contained only 12-40% males suggesting male response to host odour might be weaker than that of females although landing catches may under-estimate the numbers of males attracted to the host since presumably males once in the vicinity of a host would tend to swarm around the host in order to intercept females rather than alight on the host. Landing catches carried out by Nelson *et al.* (1978) in Indonesia caught similar numbers of males and females although as with Hartberg (1971) use of

landing catches may under-estimate the total numbers of males that were attracted to the host. However more recently behavioural observations by Cator *et al.* (2011) suggest that males may form swarms around vertebrate hosts prior to the arrival of females and thus there is a need to further investigate the response of males to host odour volatiles to determine whether this is primarily mediated by chemical cues or visual cues.

An elevated level of carbon dioxide (0.1%) enhanced the response of female mosquitoes to worn sock odour. The ambient concentration of carbon dioxide in air (not in the vicinity of a host or other source of carbon dioxide) and that used in Experiment 1 is about 0.038%. Elevated carbon dioxide concentrations have been shown to act by sensitising *Ae. aegypti* to other host odour components (Dekker *et al.* 2005) and this would explain why response to worn sock odour was higher when using air containing 0.1% carbon dioxide compared with ambient levels.

BG lure was not significantly attractive to mosquitoes when used in the olfactometer as an alternative to worn sock odour despite its successful widespread use in traps. This contrasts with Williams *et al.* (2006) who tested BG Lure in a Y-tube olfactometer and found it to be significantly more attractive than a water control although it was significantly less attractive than odour from a human finger. However, it should be noted that the bioassay and apparatus described by Williams *et al.* (2006) was quite different from that used in this chapter. For example, Williams *et al.* (2006) pre-selected host-seeking mosquitoes by luring them from their cages using human odour and thus only mosquitoes that had already been shown to respond to host odour were tested. This would have made their bioassay more sensitive than those used in this chapter where mosquitoes

were not pre-selected and this would suggest that while the bioassay used for this experiment was sensitive enough to detect response to worn sock odour; it was not sensitive enough to detect response to BG Lure.

4.5.3 Response of virgin females to odour from virgin males

Odour from virgin males in the absence of host odour was not attractive to virgin females in any of the olfactometer designs used in these experiments. Worn sock odour plus odour from 100 males was significantly less attractive (P=0.04) than worn sock odour alone when using olfactometer design 1 and there was also significant variation between days for socks plus 100 males, but not for worn sock odour alone or for 100 males alone. It is possible that the variation was a reflection of the varying interaction between males and worn sock odour and this could be interpreted as indirect evidence that the males were producing something that was interacting with the worn sock odour.

When olfactometer design 2 was used, worn sock odour plus odour from 100 males with 0.1% carbon dioxide attracted significantly more females than worn sock odour with 0.1% carbon dioxide alone. It may be that either virgin females only responded positively to pheromone in the presence of both worn sock odour and 0.1% carbon dioxide or alternatively that virgin males only released pheromone in the presence of both worn sock odour and an elevated level of carbon dioxide. It is important to note that in olfactometer design 2 the entry tube was too narrow for the females to respond with flight.

When using olfactometer design 3 with 0.1% carbon dioxide, there was a significant difference in the response of virgin females to different numbers of males but no obvious directional trend with numbers of males. When 0.3% carbon

dioxide was used there was no significant difference in the response of virgin females to worn socks with different numbers of males.

However, an attempt to replicate Experiment 2 with a wider entry tube (i.e. design 4) that could facilitate mosquito flight was unsuccessful suggesting that any compound(s) emitted by the males might not act as simple attractants. *Ae. aegypti* are flying insects and have been observed to mate in flight and thus the response of females observed when using olfactometer design 2 is unlikely to take place in the field. Nevertheless, as noted by Birch and Haynes (1982) walking assays have been useful in investigating the response of bark beetles to pheromone sources even though the beetles would fly to the pheromone source in the field.

4.5.4 Conclusions

This chapter has described the successful construction and testing of four olfactometer designs all of which were capable of detecting attraction to worn sock odour. Interestingly males as well as females were attracted to worn sock odour. The response of virgin females to odour from virgin males in the presence and absence of worn sock odour and/or elevated levels of carbon dioxide was also tested. Overall, there was no consistent evidence for male associated attractants, but results also suggested that any response to male odour is dependent on host odour including carbon dioxide concentration. An attempt to replace worn socks with a synthetic odour blend was not successful. The observed interaction between male odour and host odour is further explored in Chapter 5 using olfactometer design 4 since this design enabled test mosquitoes to respond with flight to odour stimuli.

Chapter 5 –Lab and field based studies of virgin female response to male odour

5.1 Introduction

While the results of Chapter 4 failed to provide any consistent evidence for a male produced pheromone, there was some evidence suggesting a possible interaction between male odour and host odour. This chapter describes attempts to further investigate the response of virgin females to male odour through laboratory and field based experiments.

5.1.1 Kairomone-Pheromone interactions

In many diptera where pheromones have been identified, synergistic or additive interactions with kairomones have been identified. In *Lu. longipalpis*, for example, a male produced pheromone increases the attraction of host odour to both virgin females and virgin males (Kelly and Dye 1997; Bray and Hamilton 2007).

Kairomone-pheromone interactions are especially common in phytophagous insects including major agricultural pests and have long been acknowledged in the literature. Bedard *et al.* (1969) observed that the response of male and female western pine beetles, *Dendroctonus brevicomis* (Coleoptera: Curculionidae) to a female produced pheromone was enhanced by a host derived compound that was not an attractant by itself. Since then Dickens *et al* (1993), for

example, have noted that certain kairomones (green leaf volatiles) enhance the response of male tobacco budworms, *Heliothis virescens* (Lepidoptera: Noctuidae) to a female produced sex pheromone. More recently Saïd *et al.* (2011) have shown that the interaction between aggregation pheromone and plant odour in the American palm weevil, *Rhynchophorus palmarum* (Coleoptera: Curculionidae) may be synergistic as the pheromone is only weakly active alone.

In addition to synergising response to pheromones, host odour may also stimulate release or production of pheromones. While Ruiz-Montiel *et al.* (2009) did not find any evidence for host odour stimulating pheromone release in *Scyphophorus acupunctatus* (Coleoptera: Curculionidae), other authors have reported stimulation of pheromone release by host odour. For example, Landolt *et al.* (1994) found evidence that plant host odour stimulated pheromone release in female cabbage looper moths, *Trichoplusia ni* (Lepidoptera: Noctuidae). When Landolt *et al.* (1994) passed air over cotton plants before passing it over females (i.e. the females were exposed to plant odour) significantly more males were attracted compared with when air was passed first over females and then over cotton plants (i.e. the females were not exposed to plant odour). If *Ae. aegypti* males only release pheromone in response to host odour, but females respond to pheromone in the absence of host odour then traps baited with synthetic pheromone would be attractive without the need to additionally bait them with host odour and thus it is important to further investigate the nature of the interaction between male odour and host odour that has been described in chapter 4.

5.1.2 Source of host odour

Experiments presented in Chapter 4 used worn sock odour and/or elevated levels of carbon dioxide to mimic host odour. Worn sock odour and carbon dioxide will not fully mimic mammalian host odour from live hosts since other compounds from mammalian breath (other than carbon dioxide) have been shown to play a role in host location by mosquitoes. For example, some compounds that are known to play a role in host location by mosquitoes such as 1-octen-3-ol (Takken and Kline 1989) and ammonia (Geier *et al.* 1999) are present in both mammalian sweat and mammalian breath, but other compounds such as acetone (Bernier *et al.* 2003) are only present in mammalian breath. Furthermore, the rate of release of volatile odours from previously worn socks may also differ from when they are being worn.

Varela *et al.* (2011) have investigated the role of host plant odour components in synergising attraction of male *Grapholita molesta* (Lepidoptera: Tortricidae) to synthetic sex pheromone and found that one component of plant odour, benzaldehyde, did not synergize attraction to sex pheromone on its own, but that when this was removed from a blend containing several plant host odour compounds it resulted in a reduction in the synergistic response to sex pheromone. As far as haematophagous insects are concerned a previous study that successfully demonstrated the synergistic and additive effects of host odour on attraction to sex pheromone in *Lu. Longipalpis* used live hamsters as a source of host odour (Bray and Hamilton 2007). It is not clear if an alternative source of host odour (other than a live host) would have synergised attraction of sandflies to sex pheromone to the same extent as this was not tested. The specific components of host odour that might stimulate release of the postulated

pheromone by male *Ae. aegypti* and/or synergise response of virgin females to male odour are not known and so there is therefore a need to repeat experiments described in chapter 4 with a live host as this would better replicate what takes place under natural conditions.

Although, *Ae. aegypti* is a highly anthropophilic species, it has been shown to respond to odour from rodents in the laboratory (e.g. McCall *et al.* 1996; Cabrera and Jaffe 2007). Ponlawat and Harrington (2005) found a low frequency of blood-fed *Ae. aegypti* caught in Thailand contained blood meals from non-human hosts which included rats suggesting that *Ae. aegypti* in the field are also attracted to rodent odour. The relatively small size of rodents makes them more amenable for use in both field and laboratory experiments compared to other hosts such as humans and thus rodents have recently been used in olfactometer studies to investigate the behaviour of both *Ae. aegypti* (Cabrera and Jaffe 2007) and *Lu. longipalpis* (Bray and Hamilton 2007).

5.1.3 Importance of confirming results in the field

While laboratory based studies enable greater control of environmental conditions which can help to reduce variation, behaviour of insects in the laboratory cannot completely mimic behaviour that occurs in the field. Field based experiments can therefore sometimes yield different results from those obtained in the laboratory. For example, Verhulst *et al.* (2011) found a synthetic blend of compounds identified from skin microbiota were significantly attractive to *An. gambiae* in laboratory based experiments, but not in semi-field experiments. It can therefore be useful to replicate behavioural bioassays under field conditions to ensure that behavioural responses observed under laboratory conditions are

representative of what occurs in the natural habitat of the insect. Furthermore, where the eventual aim is to exploit the behavioural response of the insect to semiochemicals for use in monitoring and control, it is useful to determine whether the semiochemicals being tested can be used as attractive bait in a trap. For example, Bray *et al.* (2009) found that *Lu. longipalpis* was attracted to traps baited with synthetic sex pheromone in the field.

5.2 Aims and objectives

5.2.1 Aims

The main aims of work presented in this chapter were to: i) confirm results of chapter 4 using a live host rather than worn socks as a source of host odour to determine whether males emit and pheromone and females respond to this when exposed to a natural source of host odour, ii) determine whether male release of the postulated pheromone is dependent on exposure to host odour and iii) determine whether male odour can be used to enhance the attraction of *Ae. aegypti* to traps in the field.

5.2.2 Objectives

1) To determine whether odour from 100 virgin males enhances the attraction of mouse odour to virgin females using olfactometer based bioassays in the laboratory.

2) To determine whether virgin males need to be exposed to mouse odour in order for their odour to affect behaviour of virgin females.

3) To compare the attractiveness of different numbers of males to virgin females in the presence of mouse odour.

4) To test whether BG Sentinel traps in the field baited with hamster odour plus odour from 100 males catch more *Ae. aegypti* mosquitoes than traps baited with hamster odour alone.

5.3 Materials and Methods

5.3.1 Laboratory based experiments

5.3.1.1 Olfactometer apparatus set-up and bioassay protocol

The apparatus used for laboratory based bioassays is shown diagrammatically in Figure 5.1. Laboratory based bioassays were carried out as described previously (Chapter 4; section 4.2.4) using olfactometer design 4 (described in Chapter 4; section 4.2.3). However, the basic olfactometer design was modified by connecting an extra 5L jar (Quickfit FV5L; Fisher Scientific UK Ltd.) labelled as "D" in Figure 5.1 so that the males and mouse were in separate glass jars. Thus air flowing at 300 ml min^{-1} passed through two 5L glass jars labelled "D" and "E" which were connected in series by FEP tubing (Chemfluor tubing; 0.25 inch internal diameter, Sigma-Aldrich Company Ltd, UK) into an olfactometer ("F" and "G"). Odour sources were placed in jars "D" and "E" according to the experiment. In all experiments except Experiment 3, the mouse was placed in jar "D" while males were placed in jar "E" so that the males were themselves exposed to host odour. In Experiment 3, this was reversed and thus the males were placed in jar "D" while the mouse was placed in jar "E" so that the males were not exposed to host odour.

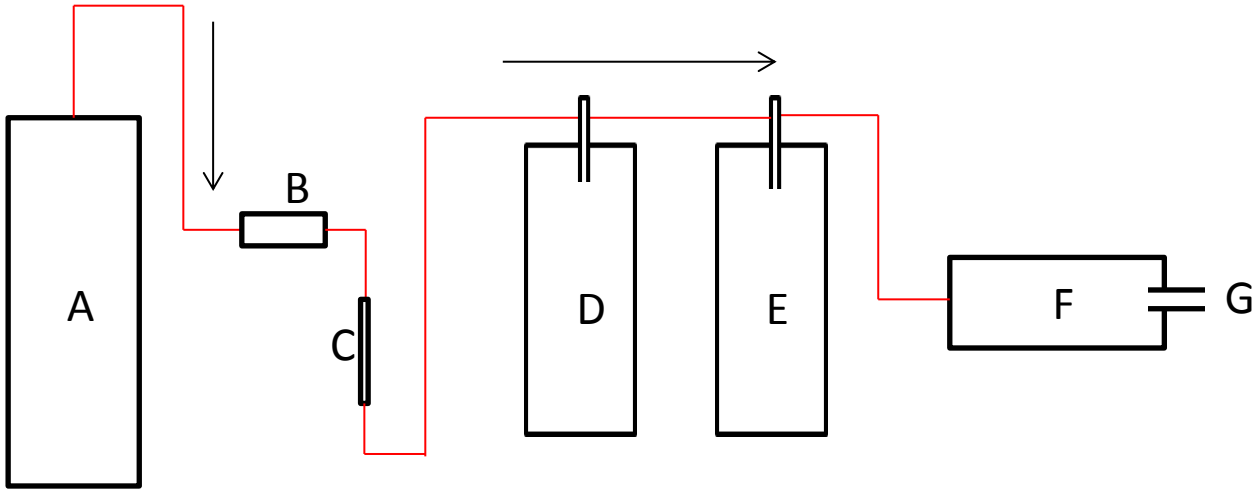

Figure 5.1 – Diagrammatic representation of olfactometer: A: Compressed air cylinder **B**: Charcoal filter **C**: Rotameter **D**: 5L glass jar **E**: 5L glass jar **F**: Horizontally laid 2L flat bottomed glass vessel with Quickfit lid at outer end **G**: Glass entry tube into which test mosquito was introduced. Arrows indicate direction of air flow.

5.3.1.2 Animal maintenance

Male laboratory mice were used as sources of host odour for all laboratory based experiments described in this chapter. No regulated procedures were carried out on these mice since they were solely used as a source of host odour. All mice were maintained in accordance with UK Home Office regulations. Mice were kept in RB3 cages (North Kent Plastic Cages, UK) containing bedding material and provided with rodent food (Special Diet Services Ltd, UK) and water *ad libitum*. Each day a different mouse was used for experiments and afterwards its tail was marked with a marker pen before the mouse was returned to its cage.

5.3.1.3 Bioassay protocol

The response of 140 virgin females (3-5 days old) was tested to each treatment. Each experiment was run over 7 days and for each day the control was run first followed by the test treatment (n = 20 for each). All experiments in the laboratory were carried out within 3 hours before photophase. Between experiments the olfactometer was dismantled and cleaned as described in Chapter 2. The response of virgin females was tested 30 minutes after placing the relevant odour sources (male mosquitoes or mouse) into jars “D” and “E” to allow odour to percolate through the olfactometer.

5.3.1.4 Experiment 1 – Response to virgin male odour in absence of mouse odour

This experiment is summarised in Table 5.1. In the first part of the experiment air passed first through jar “D” (empty) and then jar “E” (containing 100 males) and the response of females was measured. In the second part of the experiment air passed through jar “D” (containing a mouse) and then through jar

"E" (containing 100 males) and the response of females was measured. The response of females to a control where both jar "D" and "E" were empty was also carried out.

Table 5.1 – Summary of Experiment 1; the response of virgin females to 100 males, mouse plus 100 males and no odour. The mouse was placed in jar "D" while the males were placed in jar "E" so that the males were exposed to mouse odour

Treatment	Description of Test	Jar "D"	Jar "E"
1	100 males	empty	100 males
2	mouse + males	mouse	100 males
3	no odour	empty	empty

5.3.1.5 Experiment 2 – Does odour from males exposed to mouse odour enhance attraction of virgin females to mouse odour?

This experiment is summarised in Table 5.2. In the first part of the experiment air passed first through jar "D" (containing a mouse) and then jar "E" (empty) and the response of females was measured. In the second part of the experiment air passed through jar "D" (containing a mouse) and then through jar "E" (containing 100 males) and the response of females was measured. The response of females to a control in which jars "D" and "E" were both empty was also carried out.

Table 5.2 – Summary of Experiment 2; the response of virgin females to mouse, mouse plus 100 males and no odour. The mouse was placed in jar “D” and the males were placed in jar “E” so that the males were exposed to mouse odour

Treatment	Description	Contents of jar “D”	Contents of jar “E”
1	mouse	mouse	empty
2	mouse + 100 males	mouse	100 males
3	no odour	empty	empty

5.3.1.6 Experiment 3 – Does odour from males that have not been exposed to mouse odour enhance attraction of virgin females to mouse odour?

This experiment is summarised in Table 5.3. Experiment 3 was arranged so that air passed first through the jar containing the males and then through the jar containing the mouse so that the males were not exposed to host odour, but the females were exposed to both male odour and mouse odour.

In the first part of the experiment air passed first through jar “D” (100 males) and then jar “E” (empty) and the response of females was measured. In the second part of the experiment air passed through jar “D” (100 males) and then through jar “E” (mouse) and the response of females was measured.

Table 5.3 – Summary of Experiment 3; response of virgin females to mouse plus 100 males (that have not been exposed to the mouse odour) and mouse odour alone. In this experiment the males were placed in jar “D” and the mouse was placed in jar “E” (downstream of the males) so that the males were not exposed to the mouse odour

Treatment	Description of test	Jar “D”	Jar “E”
1	mouse	empty	mouse
2	mouse + 100 males	100 males	mouse

5.3.1.7 Experiment 4 – To determine whether mouse odour plus 200 males is more attractive than host odour plus 100 males

This experiment is summarised in Table 5.4. In the first part of the experiment air passed first through jar “D” (mouse) and then jar “E” (100 males) and the response of females was measured. In the second part of the experiment air passed through jar “D” (mouse) and then through jar “E” (200 males) and the response of females was measured.

Table 5.4 – Summary of Experiment 4; the response of virgin females to mouse plus 100 males and mouse plus 200 males. The mouse was placed in jar “D” and the males were placed in jar “E” so that the males were exposed to mouse odour.

Treatment	Description of test	Jar “D”	Jar “E”
1	mouse plus 100 males	mouse	100 males
2	mouse plus 200 males	mouse	200 males

5.3.1.8 Experiment 5 - To determine whether mouse odour plus 20 males is more or less attractive than host odour plus 100 males

This experiment is summarised in Table 5.5. In the first part of the experiment air passed first through jar “D” (mouse) and then jar “E” (20 males) and the response of females was measured. In the second part of the experiment air passed through jar “D” (mouse) and then through jar “E” (100 males) and the response of females was measured.

Table 5.5 – Summary of Experiment 5; the response of virgin females to mouse plus 20 males and mouse plus 100 males. The mouse was placed in jar “D” and the males were placed in jar “E” so that the males were exposed to mouse odour.

Treatment	Description of test	Jar “D”	Jar “E”
1	mouse plus 20 males	Mouse	20 males
2	mouse plus 100 males	Mouse	100 males

5.3.1.9 Data analysis for laboratory experiments

For each experiment, data from each of the 7 days were pooled and the difference in response to different odour treatments was analysed by the 2-sided Pearson χ^2 test in SPSS 18.0 (SPSS Inc., USA) as used to analyse data in Chapter 4. This test was used to compare the test (observed) with the control (expected) to determine whether the proportion of mosquitoes responding positively to the test significantly differed from the proportion of mosquitoes responding to the control (α=0.05).

5.3.2 Field based experiments

Field experiments were carried out in the grounds of the Mosquito Research and Control Unit (MRCU) in Grand Cayman between 23rd August 2011 and 16th September 2011. *Ae. aegypti* (Liverpool strain) eggs were brought to Grand Cayman from Keele. Polystyrene cups containing male pupae were placed in 30 x 30 x 30 cm BugDorm cages (Megaview Science Co. Ltd, Taiwan) with access to 10% w/v sucrose solution and 3-5 day old virgin males were used for these experiments.

5.3.2.1 Apparatus set-up

A modified version of the apparatus used in laboratory experiments at Keele was used in Grand Cayman using glassware that was available at the Mosquito Research and Control Unit. In this case the apparatus was set up as a 2 arm choice experiment shown diagrammatically in Figure 5.2 and photographically in Figure 5.3. Air from a compressed air cylinder (P.M. Industrial Gas Ltd., Grand Cayman) was passed through a carbon trap (Alltech, USA) to remove any impurities followed by a Supelco rotameter (PMRI-011426 Rotameter, Sigma-Aldrich Ltd., UK) before passing through a 1L conical flask (Pyrex; baffled Erlenmeyer flask; CLS44501L; Sigma-Aldrich Co. LLC, USA) containing a hamster. This flask was sealed with a rubber stopper (Size 11; laboratory grade; The Science Company, Colorado, USA) which had been cut to fit inside the neck of the flask. Two holes were drilled into the rubber stopper (0.625 inch each) for inserting two FEP tubes (Chemfluor; 0.25 inch internal diameter; 0.625 inch outer diameter; Sigma-Aldrich Ltd., UK). Each FEP tube was connected to one of two 2L conical flasks (CLS44442L; Sigma-Aldrich Co. LLC, USA) via rubber stoppers into which two holes (0.625 inch) had been drilled. One flask contained males

(treatment flask) while the other flask was empty (control flask). FEP tubing (0.25 inch internal diameter) from both conical flasks then led to two traps so that one trap was baited with hamster odour alone (control) while the other trap was baited with hamster odour plus male odour (treatment) as shown in Figure 5.2. The FEP tubing leading to the control and test traps were of equal length to ensure an equal flow rate; air flow was measured using a bubble air-flow meter and adjusted to give initial air flow of 300 ml min^{-1} at both traps. All joints were sealed using PTFE sealing tape (A. L. Thompson Building Supplies Ltd, Grand Cayman).

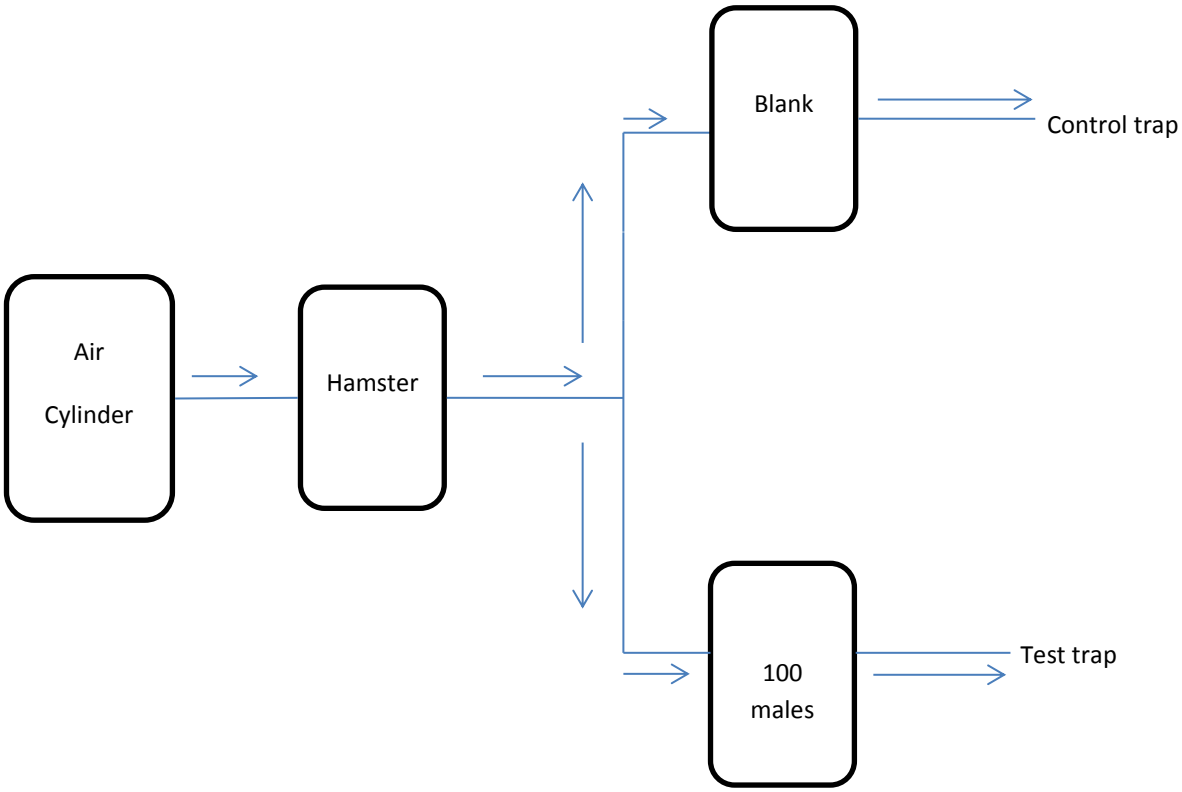

Figure 5.2 - Diagrammatic representation of apparatus used for field experiments: Air passed through a conical glass flask containing a hamster following which it branched off to flow through an empty conical glass flask to the control trap or through a conical glass flask containing 100 males to the test trap. Arrows indicate direction of air flow.

Figure 5.3 - Apparatus Set-up for field experiments: From the jar containing the hamster there were two arms; one going to the empty flask and one to the flask containing 100 males. Air was then directed to the test and control traps via FEP tubing (not shown in photograph).

Before each experiment, all glass apparatus was washed as described in Chapter 2. FEP tubing was washed with odour free detergent (Teepol L; BDH Laboratory Supplies Ltd, UK) and water before being rinsed with acetone (Sigma Aldrich Co. LLC, USA) and then left in an area receiving direct sunlight to dry. Other components including the traps were washed with odour free detergent and water and left in a place receiving direct sunlight to dry.

5.3.2.2 Animal maintenance

Four male hamsters were used as a source of host odour and one hamster was used for each replicate selected alternately. No regulated procedures were carried out on these mice since they were solely used as a source of host odour. All mice were maintained in accordance with UK Home Office regulations. The hamsters were checked at regular intervals during the course of experiments to ensure they were not distressed and on all occasions they were either sleeping or engaging in grooming behaviour. Hamsters were kept in a plastic hamster cage (Animal House Ltd, Grand Cayman) containing bedding material. Hamsters were provided with mixed hamster food (Animal House Ltd, Grand Cayman) and water *ad libitum.*

5.3.2.3 Traps

BG Sentinel traps (Biogents AG, Germany) were used which were connected to lead-acid batteries to power their motor for the duration of trapping. Traps were used without the BG Lure so that the only attractant was that delivered by the FEP tubing. A distance was maintained between control and test traps of 6.7m and as far as possible control and test traps were located in locations that were as similar as possible to minimise positional effects. Since some positional effects were still likely to occur this was controlled for by rotating the positions of the control and test traps each day.

A total of six BG Sentinel traps were used for experiments which were organized into three pairs of control and test traps. The pair of traps used for each replicate was randomised using the random number feature in Microsoft Excel.

5.3.2.4 Location of experiments

Due to localised fluctuations in abundance of *Ae. aegypti* (that led to none being caught in either trap on some days), three different locations were used for experiments to try to minimise the number of days when no mosquitoes were caught in either trap. The locations used were:

1) Next to the Mosquito Research and Control Unit main building. Both traps were set against the wall and both were an equal distance from the window.
2) Adjacent to a hangar at the airport. Both traps were placed next to two disused shipping containers.
3) Behind the operations building of the Mosquito Research and Control Unit. Both traps were placed next to the fence under the shade of a tree.

5.3.2.5 Protocol

The apparatus was set up as shown in Figure 3. Experiments were run for 3 hours from 4pm to 7pm. Sunset began around 7pm in Grand Cayman during the period of study. After each day's experiment (replicate) the collection bags in the traps were transferred to appropriately labelled polythene freezer bags which were placed in the freezer to kill any trapped mosquitoes. The contents were then analysed and any *Ae. aegypti* from both the control and test traps were counted and recorded. 18 replicates were carried out over 18 days.

5.3.2.6 Data analysis for field based experiments

Since the data were assumed to be paired (i.e. for each replicate mosquitoes could "choose" either the test or control trap with both test and control traps baited with odour from the same hamster) and were also non-parametric, the

Wilcoxon signed rank test (Minitab v15.0, Minitab Inc. USA) was used to determine whether traps baited with hamster plus male odour (test) trapped higher numbers of females than traps baited with hamster odour alone (α=0.05). All tests were one tailed since based on the results of laboratory experiments it was predicted that more females would be caught in traps baited with hamster odour plus male odour compared with hamster odour alone. Additionally a χ^2 Exact Test was carried out (SPSS v18.0, SPSS Inc., USA) to determine whether *Ae. aegypti* females were more likely to be present in test traps compared to control traps (α=0.05).

5.4 Results

5.4.1 Experiment 1 – Response to virgin male odour in absence of mouse odour

Odour from 100 males was not significantly more attractive than no-odour (χ^2 = 0.97, 1d.f., *P* = 0.36), but mouse plus 100 males (31.4% mean daily positive response ± 3.3 SEM) was significantly more attractive than either male odour alone (19.3% mean daily positive response ± 3.2 SEM; χ^2 =5.45, 1 d.f., *P* = 0.028) or no odour (17.1% mean daily positive response ± 3.9 SEM; χ^2 = 11.49, 1d.f., *P* = 0.001). These results are depicted in Figure 5.4.1. This experiment confirms results of experiments in chapter 4 which showed that male odour alone is not attractive to virgin females.

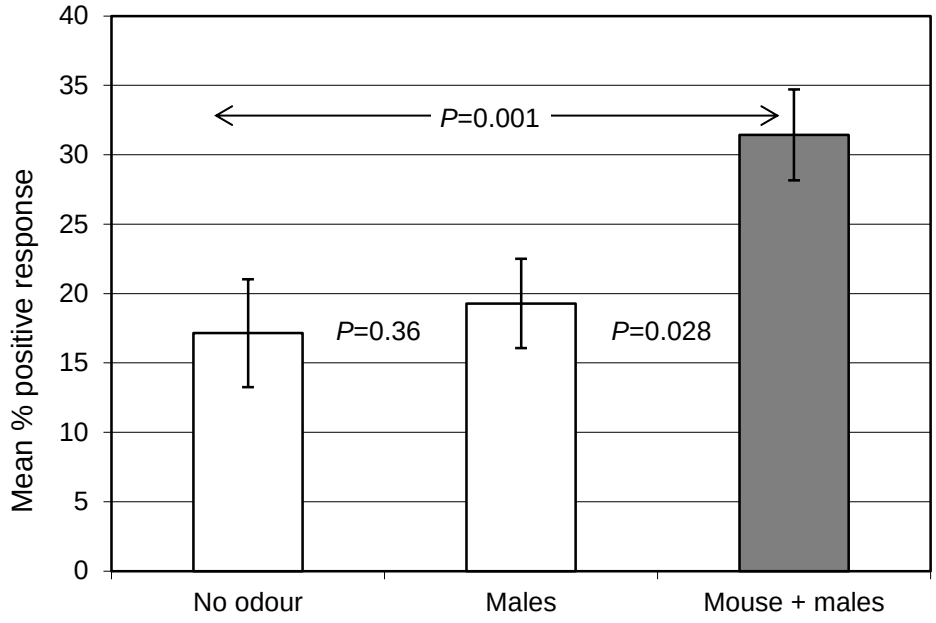

Figure 5.4.1 - Graph showing mean daily percentage of mosquitoes responding to i) no odour, ii) odour from 100 males and iii) mouse odour plus odour from 100 males: n=140 females tested over 7 days. *P*-values were obtained using the Pearson χ^2 test on the pooled data. Error bars indicate the SEM. Significantly more virgin females responded to mouse plus males compared with males alone, but there was no significant difference between the numbers of virgin females responding to males alone compared with no odour.

5.4.2 Experiment 2 – Does odour from males exposed to mouse odour enhance attraction of virgin females to mouse odour?

Odour from mouse plus 100 males (30.7% mean daily positive response ± 2.5 SEM) attracted significantly more virgin females (χ^2 = 14.34, 1 d.f., *P* = <0.001) than odour from mouse alone (17.9% mean daily positive response ± 1.9 SEM) as depicted in Figure 5.4.2. More virgin females responded to mouse odour than no-odour, but this was not significant (χ^2 = 1.79, 1 d.f., *P* = 0.24).

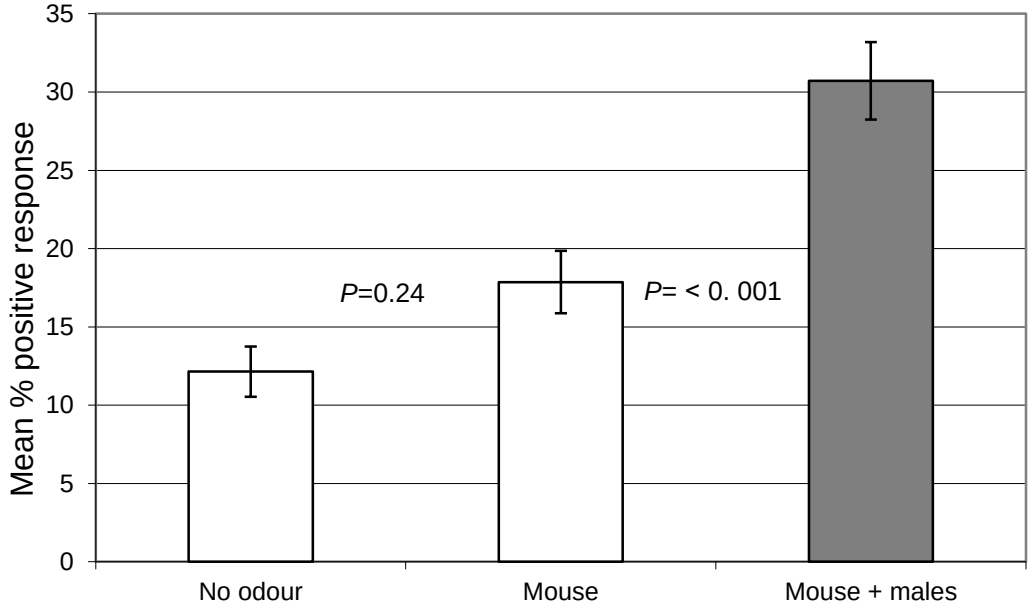

Figure 5.4.2 - Graph showing mean daily percentage of mosquitoes responding to i) no odour, ii) mouse odour and iii) odour from mouse and 100 males: n=140 females tested over 7 days. *P*-values were obtained using the Pearson χ^2 test on the pooled data. Error bars indicate the SEM. Significantly more virgin females responded to mouse plus males compared with mouse odour alone, but there was no significant difference between the numbers of virgin females responding to mouse alone compared with no odour

5.4.3 Experiment 3 – Does odour from males that have not been exposed to mouse odour enhance attraction of virgin females to mouse odour?

For this experiment, air passed through the virgin males first before passing through the flask containing the mouse meaning that the males were not exposed to the host odour. Results are shown in Figure 5.4.3. Although more virgin females responded to 100 males plus mouse odour (31.4% mean daily positive response ± 4.4 SEM) than mouse odour alone (22.9% mean daily positive response ± 3.4 SEM) this was not significant (χ^2 = 2.17, 1 d.f., *P* = 0.18).

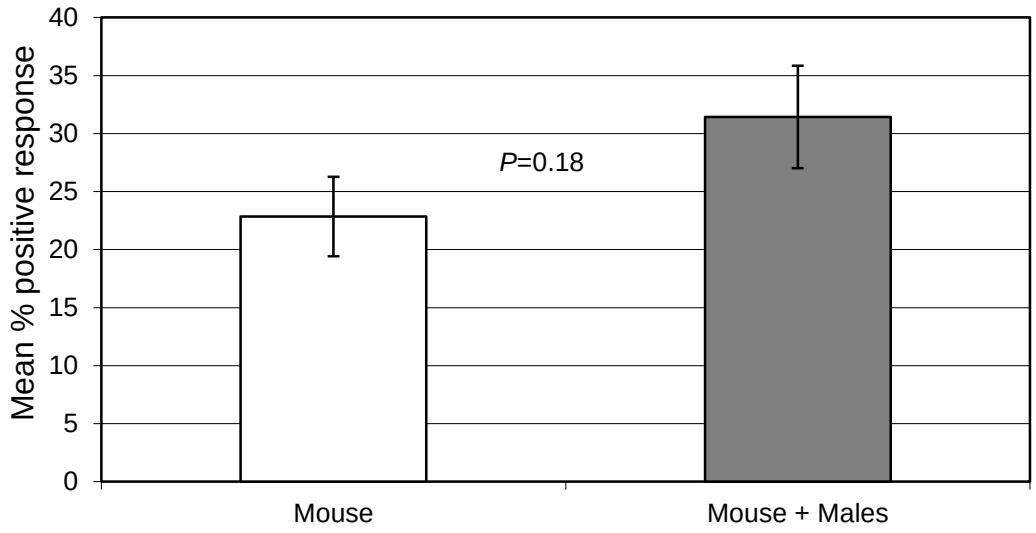

Figure 5.4.3 - Graph showing mean daily percentage of mosquitoes responding to i) mouse, ii) mouse plus odour from 100 males that have not themselves been exposed to mouse odour: n=140 females tested over 7 days. *P*-value was obtained using the Pearson χ^2 test on the pooled data. Error bars indicate the SEM. There was no significant difference between the number of virgin females responding to mouse plus males compared with mouse odour alone

5.4.4 Experiment 4 – To determine whether mouse odour plus 200 males is more attractive than host odour plus 100 males

The attraction of 200 males plus mouse odour was compared with that of 100 males plus mouse odour. As depicted in Figure 5.4.4, odour from 200 males plus mouse odour was not significantly more attractive to virgin females than odour from 100 males plus mouse odour (χ^2 = <0.001, 1 d.f., *P* = 0.99).

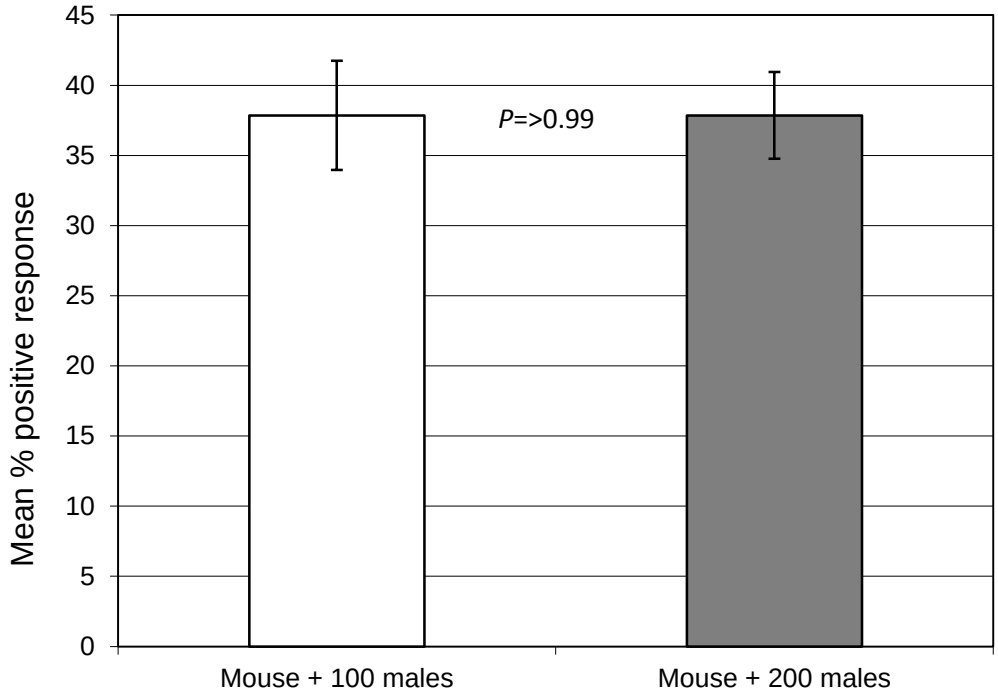

Figure 5.4.4 - Graph showing mean daily percentage of mosquitoes responding to i) mouse odour plus odour from 100 males and ii) mouse odour plus odour from 200 males: n = 140 females tested over 7 days. *P*-value was obtained using the Pearson χ^2 test on the pooled data. Error bars indicate the SEM. There was no significant difference between the number of virgin females responding to mouse plus 200 males compared with mouse plus 100 males.

5.4.5 Experiment 5 - To determine whether mouse odour plus 20 males is more or less attractive than host odour plus 100 males

As shown in Figure 5.4.5 there was no significant difference in the response of virgin females to 20 males plus mouse odour compared to 100 males plus mouse odour ($\chi^2 = 2.22$, 1 d.f., $P = 0.17$).

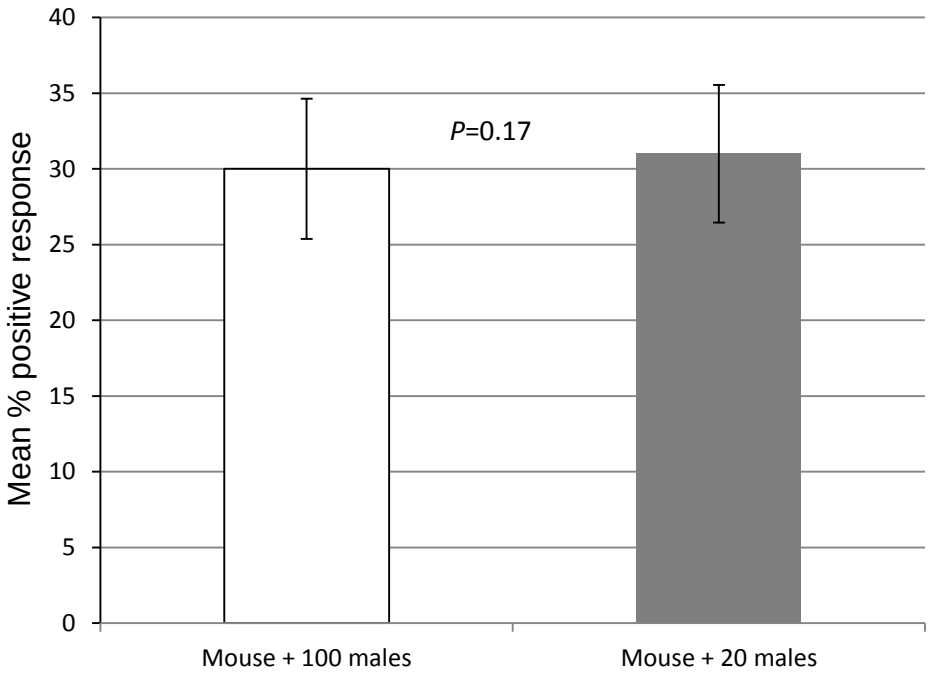

Figure 5.4.5 - Graph showing mean daily percentage of mosquitoes responding to i) mouse odour plus odour from 100 males and ii) mouse odour plus odour from 20 males. n = 140 females tested over 7 days. *P*-value was obtained using the Pearson χ^2 test on the pooled data. Error bars indicate the SEM. There was no significant difference between the number of virgin females responding to mouse plus 20 males compared with mouse plus 100 males.

5.4.6 Field based experiments

5.4.6.1 Numbers of females caught in traps

Experiments were carried out over 23 days of trapping but there were 5 days on which no *Ae. aegypti* were caught in either control or test traps. Male and/or female *Ae. aegypti* were caught on 18 days of trapping and the numbers caught in the trap baited with hamster odour alone were compared with the numbers caught in the trap baited with odour from a hamster plus 100 males. During the course of these experiments a total of 62 females and 8 males were caught in the control traps while 73 females and 13 males were caught in test traps.

Days when no *Ae. aegypti* were caught in either the test or control traps were excluded from the data. Significantly more females (1-tailed Wilcoxon Signed Rank Test, $P = 0.042$) were caught in the traps baited with hamster odour plus male odour (test traps) than in traps baited with hamster odour alone (control traps). As can be seen from Figure 5.4.6 there were a number of days when much higher numbers were caught which corresponded to those days when experiments were conducted adjacent to an airport hangar. When data from days when experiments were conducted adjacent to an airport hangar were excluded from results the result was even more significant (1-tailed Wilcoxon Signed Rank Test, $P = 0.013$) and a daily mean average of 2.08 (± 0.29 SEM) female *Ae. aegypti* were caught in test traps compared with a daily mean average of 1.08 (± 0.31 SEM) in control traps as shown in Figure 5.4.7. Test traps were also more likely to contain *Ae. aegypti* females than control traps ($\chi^2 = 6.19$, 1 d.f., $P = 0.039$).

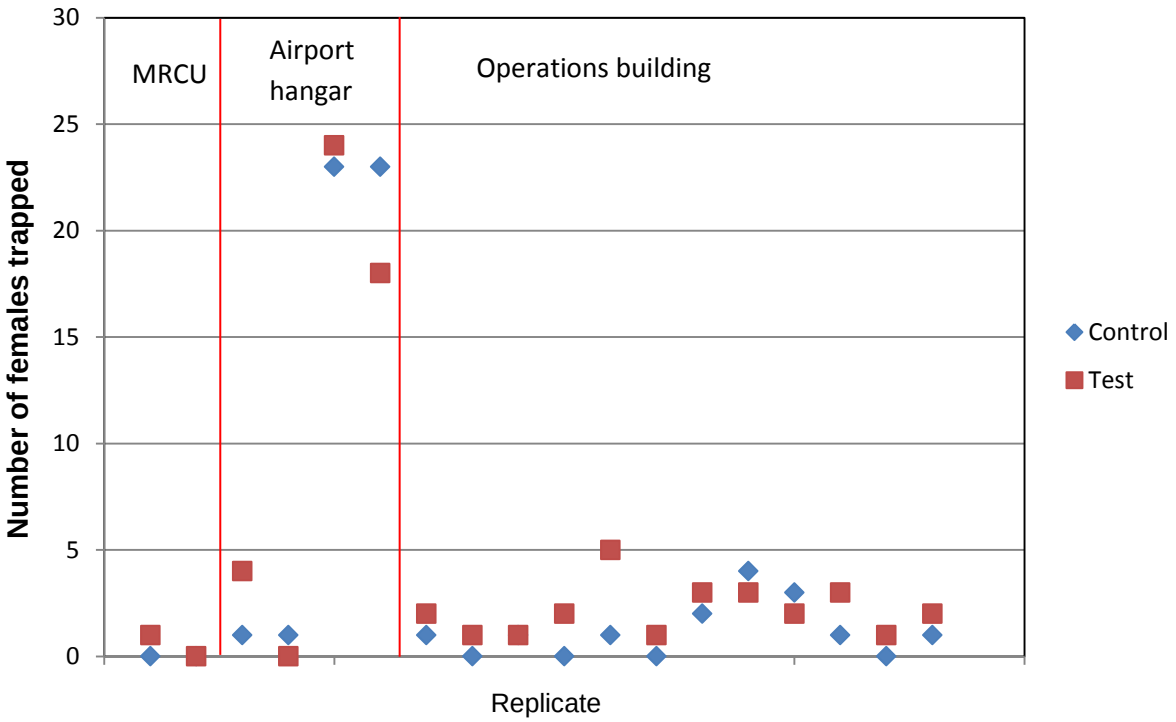

Figure 5.4.6 - Scatterplot of trap catches of females for both the control and test traps: There were only four days when the control traps caught more than the test traps. Numbers caught ranged between 0 and 5 except for 2 days when carrying out experiments near the airport hangar. Text labels at the top of the graph indicate the trapping locations.

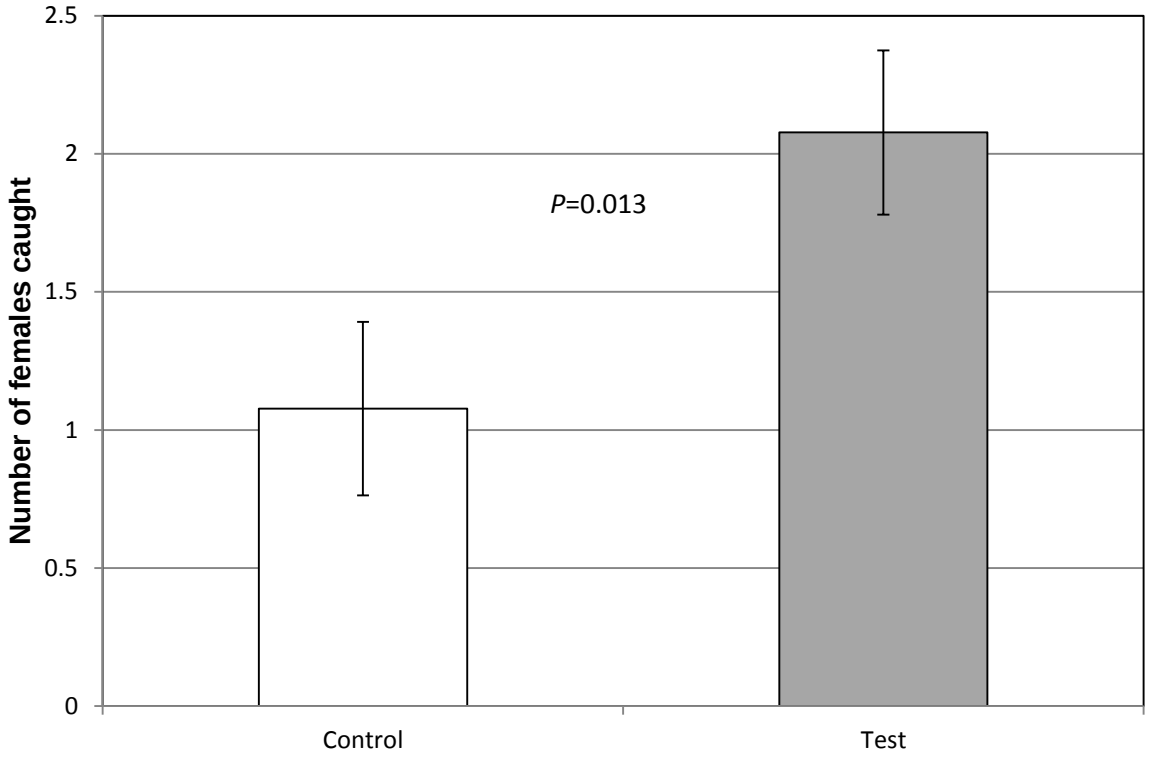

Figure 5.4.7 - Mean number of females caught daily in the test and control traps: n = 13 days of trapping. Error bars indicate SEM. The *P-value* was calculated using the Wilcoxon Signed Rank Test.

5.4.6.2 Numbers of males caught in traps

Significantly fewer males than females were caught in both the test traps (1-tailed Wilcoxon Signed Rank Test, *P* = 0.002) and control traps (1-tailed Wilcoxon Signed Rank Test, *P* = 0.025). There were only 10 days when male *Ae. aegypti* were found in either the control or test traps. There was no significant difference between the number of males in the test and control traps (1-tailed Wilcoxon Signed Rank Test, *P* = 0.187). Figure 5.4.8 shows graphically the average number of males caught in the control and test traps.

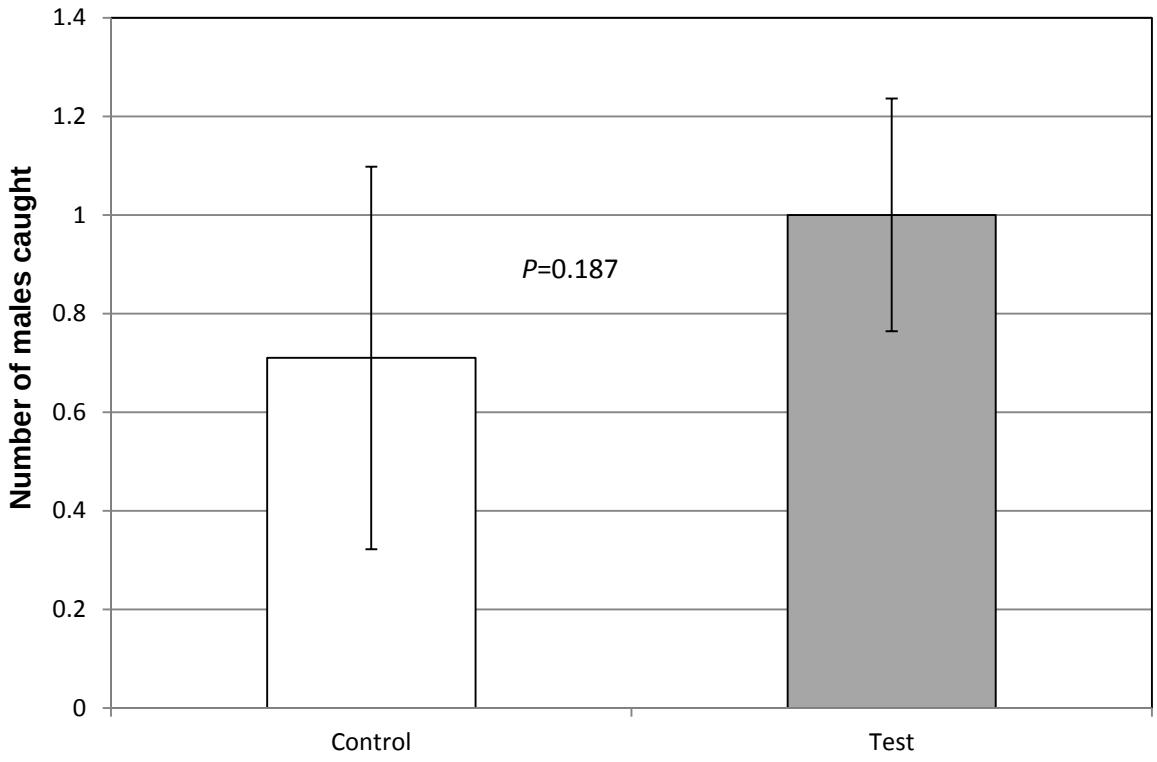

Figure 5.4.8 - Mean number of males caught daily in the test and control traps: n = 7 days of trapping. Error bars indicate SEM. The *P-value* was calculated using the Wilcoxon Signed Rank Test.

5.5 Discussion

The results of experiments described in this chapter suggest that virgin male odour enhances the attraction of host odour to virgin females. It was not possible to relate this effect to the numbers of males since there was no significant difference in the response of females to 20, 100 or 200 males plus host odour. One explanation for the increased attraction to rodent odour might be that males respond to host odour by increasing release of the postulated pheromone since the enhanced attraction of females was not significant when males were not themselves exposed to host odour. Results from field experiments support the results of laboratory based bioassays with significantly more females being caught

in traps baited with male odour plus hamster odour compared with traps baited with hamster odour alone.

5.5.1 Laboratory based bioassays

Significantly more females responded to mouse odour plus male odour than mouse odour alone when the males were themselves exposed to mouse odour (Experiment 2). This confirms results from some experiments in Chapter 4 using worn socks and carbon dioxide as a host odour mimic that suggested the males were releasing a pheromone that enhanced the attraction of host odour to females. This also confirms the results of behavioural bioassays by Cabrera and Jaffe (2007) suggesting the existence of a male produced pheromone. The fact that in Experiment 2, response to mouse odour (a known attractant) was not significantly higher than that to no odour suggests that either the bioassay was not very sensitive or that attraction of *Ae. aegypti* females to mouse odour is relatively weak. In fact, *Ae. aegypti* is known to have a preference for human blood and it is thought that differences in host odour composition, especially carboxylic acids such as lactic acid are the main factors responsible for host specificity in anthropophilic mosquitoes such as *Ae. aegypti* (Smallegange *et al.* 2011). It is therefore possible that this may have resulted in a low response rate to mouse odour in the olfactometer.

Male odour in the absence of host odour was not attractive to virgin females (Experiment 1) indicating that the interaction between host kairomones and the postulated pheromone is synergistic rather than additive or that any response of females to pheromone is secondary to their response to host kairomones. Experiments described in Chapter 4 using olfactometer design 1 and olfactometer

design 2 also failed to find any attraction to male odour in the absence of host odour. Cabrera and Jaffe (2007) who measured the flight response of virgin females did find that male odour alone elicited a behavioural response in virgin females with a doubling in the number of females flying for at least 60 seconds. However, the authors did not directly measure attraction and thus it should be noted that the behavioural response they measured differs from what was measured in these experiments. Cabrera and Jaffe (2007) measured the number of females flying in response to male odour whereas in these experiments the number of females that moved past a fixed point (i.e. from part “G” to part “F” in Figure 5.1) was measured. It is therefore possible that the postulated male produced pheromone primarily serves to promote flight in virgin females which these experiments failed to detect since the flight response of responding females was not directly measured. This may help to explain the mechanism by which the postulated pheromone might enhance the attraction of host odour to virgin females since mosquitoes which are already in flight mode may be better able to orient towards sources of host odour. This should result in females responding faster to vertebrate hosts around which males are available compared to vertebrate hosts around which no potential mates are available. Theoretically this should increase the chance of a female mating and blood feeding in short succession without the need to expend energy reserves a second time.

When males were not themselves exposed to host odour, more females responded to mouse odour plus male odour than mouse odour alone, but this was not significant. This suggests that the males respond to mouse odour by increasing release of the pheromone. It is possible that even in the absence of host odour males were releasing a pheromone, but at a level which was too low

for the response of responding virgin females to be detected given the limitations of the apparatus. It is likely that it is release of pheromone rather than pheromone production that responds to the presence of host odour, because release of pheromone is likely to be under direct neurological control and thus would enable a more rapid response to the presence of host odour than up-regulation of pheromone synthesis. For example, Delisle *et al.* (2000) found that it took 24 hours for mating to significantly suppress pheromone production in the spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae) and such a long response time would not enable response to fast changing stimuli such as the presence of a vertebrate host.

These experiments did not directly address the issue of whether virgin females only respond to the postulated pheromone when presented in combination with host odour. Host odour may be necessary both to stimulate pheromone release in males and to sensitise virgin females to pheromone. This could be investigated by testing the response of virgin females to males that have been pre-exposed to host odour. Alternatively once the pheromone has been identified the response of virgin females to different concentrations of pheromone could be determined in both the presence and absence of host odour.

These experiments were unable to establish any difference in the response of females to different numbers of males plus mouse odour. This may be due to the limitations of the olfactometer based bioassay used since any differences in female response to different numbers of males are likely to be much smaller than the difference between no males and males. However, it is also not clear what advantages to virgin females there would be to seek male aggregations of specific

sizes although larger male aggregations might be expected to be easier to detect and so it would seem logical for there to be a minimum number of males required to elicit the observed response, and these results suggest that if so, this number will be less than 20. Since 200 males do not significantly increase the response of females compared with 100 males it seems that once the threshold number of males has been reached female response remains constant. These results therefore failed to find any evidence for a dose dependent response as described by Bengtsson (2008). Interestingly, Bray and Hamilton (2007) were also unable to detect an increased response to pheromone in *Lu. longipalpis* with a 10-fold increase in pheromone concentration in Y-tube olfactometer experiments in the laboratory, but Bray *et al.* (2010) were able to detect an almost 3-fold increase in recruitment of female sandflies to pheromone baited chicken sheds in the field in response to ten times the number of pheromone dispensers (i.e. equivalent to a 10-fold increase in pheromone concentration). As noted by Bray *et al.* (2010) it is possible that greater recruitment of insects in response to increased release of pheromone may partly result simply from larger quantities of pheromone being easier to detect and thus it is possible that pheromone concentration/release rate is more important under field conditions than in an olfactometer.

5.5.2 Field based experiment

Data from experiments conducted adjacent to the airport hangar where the numbers caught were extremely high were excluded from the results prior to analysis for two reasons: i) very high densities of *Ae. aegypti* within the vicinity of the traps would have increased the chance that mosquitoes were trapped which did not specifically respond to the odour plume; ii) higher densities of male *Ae. aegypti* in the vicinity would have increased the competition from natural sources

of the postulated pheromone. The exclusion of these two data points was therefore thought to be justified.

Significantly more females were caught in traps baited with hamster odour and male odour compared to traps baited with hamster odour alone. Although more males were also caught in the test trap compared to the control this was not significant. However, it should be noted that males were only caught in 10 days of trapping and thus the number of replicates for males may be too small to determine whether males respond to the postulated male produced pheromone since power analysis using Minitab 15.0 showed that to achieve a power of 0.8 for males would require 33 days of trapping. The fact that the response of females to the trap baited with both male odour and hamster odour is significantly higher confirms the results of the laboratory based bioassays. The higher number of males caught in the test traps although not significant merits further investigation since behavioural experiments by Cabrera and Jaffe (2007) suggested the postulated male produced pheromone in *Ae. aegypti* acts as an aggregation pheromone by attracting both males and females

The field based experiments were set up as a two arm choice experiment assuming mosquitoes in the vicinity could choose between a trap baited with male odour plus hamster odour and a trap baited with hamster odour alone. This contrasts with laboratory experiments which were no-choice. However, since it is not known over what range the postulated pheromone acts it is not clear whether the field based experiments were in reality the equivalent of a two-choice bioassay.

5.5.3 Conclusions

These results have shown strong behavioural evidence for a male produced pheromone that enhances attraction of rodent odour to females. The chemical nature of this pheromone remains to be determined. This pheromone may comprise a single compound or a blend of several compounds. It is also not clear from these experiments over what range these volatiles act or which elements of host odour are required in order for females to respond to these volatiles. Repetition of laboratory based experiments using mated females would help to confirm whether the pheromone primarily acts to facilitate mating (i.e. as a sex pheromone) since if this is the case then mated females would not be expected to respond to it.

Chapter 6 – General Discussion

Pheromones are known to modulate key behaviours in many insects and as discussed in Chapter 1 several studies have suggested a role for volatile pheromones in *Ae. aegypti* oviposition and mating behaviour. This thesis aimed to re-examine behavioural evidence for the role of pheromones in oviposition and mating behaviour. Oviposition experiments described in this thesis (Chapter 3) differed in methodology from previous studies in that specific numbers of eggs less than one day old were tested whereas previous studies had used much older eggs and had not tested specific numbers of eggs. With regards to mating behaviour, the aim was to develop an olfactometer based bioassay that could measure the anemotactic attraction of individual virgin females to odour from virgin males rather than simply the flight response of females as measured by Cabrera and Jaffe (2007). Various olfactometers were designed and assembled using readily available components to test the response of virgin females to male odour (Chapter 4) and laboratory and field based experiments were carried out to investigate the interaction between male odour and host odour (Chapter 5). No behavioural evidence was found for an egg-associated oviposition pheromone, but behavioural evidence was found that was consistent with a male produced pheromone. Results suggested that males might release this pheromone in response to host odour. This chapter discusses these results in the wider context of *Ae. aegypti* behaviour.

6.1 Oviposition behaviour

6.1.1 Search for an egg-associated oviposition pheromone

As described in Chapter 3, choice and no-choice oviposition bioassays were carried out with the aim of investigating behavioural evidence for egg aggregation. Results obtained did not support the existence of an egg associated pheromone that influences oviposition behaviour. While previous studies have suggested pre-existing eggs do influence subsequent oviposition activity (Allan and Kline 1998; Williams *et al.* 2008; Chadee 1990), they did not test specific numbers of eggs and their methods differed from those described in this thesis since eggs used were older (or the age of eggs was not specified) and different oviposition substrates were used. It is therefore not possible to exclude the possibility that more mature eggs (i.e. several days old rather than several hours old) might influence subsequent oviposition activity since eggs of different ages were not tested.

Methanol extracts of *Ae. aegypti* eggs did not influence the number of eggs laid by gravid females even though compounds identified from such extracts were shown to influence gravid females at certain concentrations by Ganesan *et al.* (2006). It is thus likely that the concentrations at which these compounds were tested by Ganesan *et al.* (2006) do not represent the concentrations that would exist in an extract of 20 eggs. While extracts of different numbers of eggs were not tested it should be noted that experiments by Chadee *et al.* (2009) found significantly more ovitraps in the field contained less than 30 eggs suggesting that an extract of 20 eggs is representative of natural oviposition sites. Nevertheless, it is also not clear whether an extract of 20 eggs is equivalent to 20 actual eggs. For example, volatile compounds might evaporate at a faster rate when they are

dissolved in methanol compared to when they are contained in the egg and compounds which are not normally on the surface of eggs and so not accessible to the odour receptors of gravid females might dissolve in methanol. As discussed in Chapter 3, these results suggest that an approach to pheromone research which starts with identification of compounds as pursued by Ganesan *et al.* (2006) may lead to misleading conclusions regarding their likely biological role. Thus care also needs to be exercised in the use of "reverse chemical ecology" approaches such as described by Leal *et al.* (2008) that use odorant binding proteins as a molecular target in binding assays to identify potential pheromones. Compounds should not be labelled as pheromones until there is strong behavioural evidence for a pheromone communication system controlling behaviour in the species concerned.

It should be noted that *Ae. aegypti* eggs can remain dormant for many months and survive extended periods of desiccation (Christophers 1960; Kliewer 1961) and recently *Ae. aegypti* has been shown to exhibit greater desiccation tolerance than *Ae. albopictus* (Juliano *et al.* 2002). Indeed *Ae. aegypti* eggs may not hatch for up to one year in the absence of appropriate hatching stimuli which include both reduction of dissolved oxygen and either bacteria or bacteria secreted compounds (Ponnusamy *et al.* 2011). It could therefore be speculated that the presence of pre-existing eggs may not be a good indicator of potential competition since there is no guarantee as to when those eggs will hatch and it does not give any information on the suitability of the site in terms of food resources or disease. Presence of larvae at a site however can indicate both potential competition (Benzon and Apperson 1988), availability of food for developing larvae (Zahiri *et al.* 1997) and risk of disease to developing larvae (Lowenberger *et al.* 1994).

Furthermore, given the fact that in many mosquito species such as *Ae. aegypti,* fourth instar larvae can prey on first instar larvae (Edgerly *et al.* 1999), presence of pre-existing conspecific larvae may be of major importance in determining the survival rate of a gravid female's progeny. It is therefore possible that gravid *Ae. aegypti* females do not respond to cues from freshly laid eggs, because larvae-associated cues are a better indication of potential competition and/or the suitability of the oviposition site.

Furthermore, it is also worth noting that one of the advantages of aggregation of immature stages in some species is to ensure emerging adults have ready access to mates. For example, with the codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae), females at the pupal stage release a pheromone that acts as an arrestant on adult males (Duthie *et al.* 2003). This may also be the case with some mosquito species such as *Ae. iriomotensis* and *Culiseta inornata*, where males remain near the emergence site and mate with newly emerged females (Miyagi and Toma 1981). However, given the centrality of vertebrate host odour to the *Ae. aegypti* mating system it would seem that oviposition pheromones are unlikely to play any role in facilitating mating in *Ae. aegypti.*

6.1.2 Effect of time since blood feeding on the oviposition response of gravid females

Results from experiments where gravid females were used both 4 days and 3 days after blood feeding suggest that the response of gravid females to oviposition stimuli may be affected by the length of time since they were blood fed. Overall, significantly more eggs were laid 4 days after blood feeding than after 3 days. This suggested that when gravid females were first given the opportunity to

oviposit (3 days after blood feeding) they chose to retain most of their eggs. This may partially reflect the skip oviposition strategy of *Ae. aegypti,* but given the large number of replicates in which no eggs were laid it may also reflect the poor suitability of the oviposition substrates with which the gravid females were provided. Glass Petri-dishes filled with distilled water were used as oviposition sites for all these experiments due to the ease with which they could be cleaned, but glass is a highly reflective material and as noted by Snow (1971) higher reflectance has been shown to result in reduced oviposition by *Ae. aegypti.* It is therefore possible that four days after blood feeding the need of gravid females to lay eggs may have started to overcome any choosiness regarding the selection of appropriate sites since Xue *et al.* (2005) have shown that prolonged forced egg retention can significantly reduce hatch rates. This interpretation would suggest that oviposition site choice is dependent on the time since blood feeding occurred as well as the oviposition sites available and that where oviposition sites are limited gravid females will become increasingly less choosy in order to balance the risks of an unsuitable oviposition site with the risk of reduced hatch rates due to prolonged forced egg retention. However, it is also possible that 3 days after blood feeding, the ovaries of some of the gravid females had not fully matured which might have contributed to the significantly higher numbers of eggs laid 4 days after blood feeding compared with 3 days .

A complete investigation of the effect of time since blood feeding was not carried out in this study, but these results suggest that this issue merits further investigation since the response of gravid females to oviposition cues may change in proportion to the time since blood feeding/ovary maturation occurred. The age

of gravid females and the number of gonotrophic cycles they have undergone may also be important although this was not examined here.

6.2 Mating behaviour

6.2.1 Olfactometer based bioassays and evidence for a male produced pheromone

Responses of mosquitoes in the olfactometer designs used in Chapter 4 show that olfactometers need to be designed with adequate knowledge of the behaviour they are intended to quantify as well as the need for careful observation of the behavioural responses insects exhibit when placed in the apparatus. Olfactometers used in this study were designed on the assumption that any response to male odour would involve anemotactic attraction and thus effort was made to ensure test mosquitoes would be able to fly towards the odour source. However, in olfactometer design 2 the narrowness of part of the olfactometer meant that test mosquitoes were unable to respond with flight and even in olfactometer 3 the ability of mosquitoes to fly towards the odour source appeared to be restricted which led to a further modification being made as in olfactometer design 4 which enabled apparently unhindered flight. However, interestingly when using worn socks rather than live rodents the only olfactometer in which significantly more females responded to male odour was olfactometer design 2 in which the flight of mosquitoes was restricted by the small diameter (Chapter 4). It is possible that the main effect of the male odour was to induce flight in females and that in olfactometer design 2 this resulted in the females exiting the entry tube which was recorded as a positive result. In other olfactometer designs the greater

diameter of the entry tube may have meant that females were able to respond with flight without exiting the entry tube and thus a positive response was not recorded.

It is therefore possible that male odour primarily acts as a flight stimulant in virgin females which is why it only works in combination with host odour and thus it may be necessary to re-examine the most appropriate behavioural response to measure when conducting such experiments. This might explain why Cabrera and Jaffe (2007) who measured the flight response of *Ae. aegypti* were able to detect a significant positive response by virgin females to male odour in the absence of host odour, but in these olfactometer based experiments designed to measure attraction, male odour did not attract virgin females in the absence of host odour.

In Chapter 5, olfactometer design 4 was used successfully to detect the response of females to male odour, but live rodents rather than worn socks were used as a source of host odour. This would suggest that the quality of host odour may also be an important factor in determining the response of females to male odour.

6.2.2 Interaction between male odour and host odour

In preliminary experiments described in Chapter 4 more males responded to worn sock odour than females although this was not significant. A complete investigation of male response to host odour was not carried out here, but further investigation of male response to host odour will help determine whether males respond in the same way as females. For example, if males are attracted to host odour more strongly than females then this would suggest that in the field they are likely to reach the host prior to females in order to intercept the females that subsequently arrive. Field results described in Chapter 5 found significantly fewer

males than females in traps baited with hamster odour or hamster plus male odour, although this might be due to the BG Sentinel trap being visually more attractive to females. Greater attraction of males to host odour compared with females would be consistent with a situation whereby males release a pheromone that enhances the attraction of host odour since this would presumably require the males to reach the host first. Indeed Kelly and Dye (1997) found that male *Lu. longipalpis* (which are known to emit an aggregation pheromone which attracts both males and females) arrived at lek-like aggregations in the vicinity of hosts before females and that the rate at which males arrived was partly dependent on host abundance. Kelly and Dye (1997) suggested that by searching for a host after male aggregations have become established female *Lu. longipalpis* are able to make maximum use of male produced pheromone in locating these male aggregations and it is possible that the same might also be true for *Ae. aegypti.*

Results from some laboratory based bioassays described in Chapter 4 and Chapter 5 suggest a male produced pheromone does indeed enhance the attraction of host odour to virgin females although the results of Experiment 1 of Chapter 4 (Figure 4.6.2.1) also suggests that in the absence of an elevated concentration of carbon dioxide male odour might reduce the attractiveness of host odour volatiles. Since, it is thought that response to auditory cues only occurs within 150-300 mm in *Ae. aegypti* (Clements 1999) it is unlikely that auditory cues were responsible for the response of virgin females due to the fact that the distance between the jar containing the males and where the virgin females were introduced (>500 mm) exceeded this range. It is not clear why male odour might under certain conditions reduce the attraction of host odour and this phenomenon

was not investigated further. However, it is possible that male odour might impart information to females on the quality of a potential host.

Results described in Chapter 5 also suggest that host odour might be required to stimulate release of pheromone by males. This is similar to the female produced pheromone of cabbage looper moths, *Trichoplusia ni* (Lepidoptera: Noctuidae) where exposure of females to plant host odours appears to increase pheromone release (Landolt *et al.* 1994) and also mirrors the release of the male produced aggregation pheromone of the palm weevil, *Rhynchophorus palmarum* (Coleoptera: Curculionidae) in response to ethyl-acetate emanating from the host plant tissue (Jaffe *et al.* 1993). Regarding Diptera, it is worth noting that in *Lu. Longipalpis,* pheromone release is thought to be associated with male wing-flapping (Jones and Hamilton 1998) although it is not clear whether this behaviour itself is stimulated by host odour. Release of pheromone in response to host odour may be an adaptation that enables males to conserve energy since releasing pheromone in the absence of host odour would be energetically wasteful assuming that females only respond to pheromone in the presence of host odour.

It was not possible to distinguish between the responses of females to different numbers of males plus host odour regardless of whether worn socks and carbon dioxide (Chapter 4) or mice (Chapter 5) were used. It is possible that it is the presence or absence of males at a host rather than their number that determines the response of virgin females. For example, a greater numbers of males might only be relevant to female mating success if other females were present, although given the fact that male *Ae. aegypti* are able to mate repeatedly (Choochote *et al.* 2001) this may be of limited importance. However, it is also possible that the olfactometer apparatus is only measuring response to

pheromone over a relatively short range and in the field a larger amount of pheromone being released (i.e. a larger aggregation of males) might increase the distance from which females are able to detect the pheromone. Indeed, studies investigating the response of *Lu. longipalpis* to pheromone were unable to detect an increase in response to higher concentrations of pheromone using Y-tube olfactometer bioassays in the laboratory (Bray and Hamilton 2007), but were able to detect a significantly greater attraction of female sandflies to greater numbers of pheromone dispensers in the field (Bray *et al.* 2010). This suggests it would be worthwhile repeating experiments comparing female response to different numbers of males under field conditions.

6.2.3 Field based experiments

Significantly more *Ae. aegypti* females were caught in the trap baited with hamster plus male odour compared with hamster odour alone. There was no significant difference in the number of males caught suggesting that the male produced pheromone might only be attractive to females and thus acts as a sex pheromone rather than an aggregation pheromone. In these field experiments males were placed in 2L jars whereas 5L jars were used for laboratory experiments. Despite the smaller size of jars used (which might be expected to restrict swarming behaviour) results suggest that the males were emitting a pheromone that attracted females to traps and thus this suggests that male release of pheromone might not be dependent on any swarming/lekking behaviour of the type observed by Cabrera and Jaffe (2007).

The mating status of *Ae. aegypti* mosquitoes caught in traps was not examined. Ideally the mating status of mosquitoes should have been checked

since if the postulated pheromone acts as a sex pheromone it would be expected that traps baited with male odour would trap a greater proportion of unmated females compared with traps that were not baited with male odour.

6.2.4 Role of postulated pheromone as an indicator of male fitness

Production and release of pheromones is metabolically expensive and thus there must be a competitive advantage that justifies the cost. One such advantage could be to bring females and males into contact so that mating can take place. However, since male and female *Ae. aegypti* are both attracted to host odour (i.e. host odour brings males and females into proximity with each other) and given that species recognition in *Ae. aegypti* is known to be mediated by auditory cues (Cator *et al.* 2009), it could be argued that there must be other advantages of a male produced pheromone. One further possible explanation is that the pheromone released by male *Ae. aegypti* is used by females to identify those males which are "fitter" since the olfactory attractiveness of males to females in many insects has been correlated with desirable male phenotypic traits (Harari *et al.* 2011). It could be speculated that a volatile male produced pheromone will be more advantageous than a contact pheromone, because once a female has made contact with a male; it may be difficult to avoid copulation and anyway significant energy reserves may have already been utilised in making contact.

6.3 Strain of mosquito used

In these experiments, a laboratory strain of *Ae. aegypti* was used that was readily available at Keele. Due to time constraints it was not possible to repeat any of the experiments using a more recently colonised strain. Strains that have been maintained in the laboratory for numerous generations may differ in important

phenotypic traits from field strains and Clark *et al.* (2011) have recently shown that laboratory colonisation of *Ae. aegypti* results in a significant change in host seeking behaviour. It is thought that colonisation of mosquito strains in the laboratory can lead to fixation of alleles that affect mating behaviour and also lead to genetic bottle-necks (Howell and Knols 2009). Delisle and Vincent (2002) found reduced levels of sex pheromone production in insecticide resistant females of the oblique banded leafroller, *Choristoneura rosaceana* (Lepidoptera: Tortricidae) compared to susceptible females. If insecticide selection pressures can impact on pheromone production then laboratory colonization may also have some impact. It is therefore possible that production of the postulated male produced pheromone might be greater in more recently colonized strains of *Ae. aegypti* since it could be speculated that there will be little competitive advantage from pheromones when mosquitoes are mating in cages with limited space. It is therefore interesting that even with a highly inbred laboratory strain it was possible to obtain behavioural evidence for a male produced pheromone. This suggests that the genes encoding pheromone production in male *Ae. aegypti* and encoding the corresponding odorant binding proteins in female *Ae. aegypti* must be highly conserved and not strain specific. Nevertheless, this would need to be confirmed by repeating experiments with recently colonised strains from several different geographic origins and by testing the response of females from one strain to odour from males of a different strain.

6.4 Future directions

Results presented in this thesis demonstrate behavioural evidence for a male produced pheromone in *Ae. aegypti*. Experiments should be carried out to determine whether the postulated pheromone also affects males. Furthermore, in

order to confirm that the main function of the pheromone is to facilitate mating, the responses of mated females to male odour should also be tested in addition to blood fed unmated females. It is predicted that mated females will not respond to male odour, but that blood fed unmated females will respond to male odour. Ideally experiments should also be carried out using some form of Y-tube based choice olfactometer to confirm that unmated females “choose” host odour plus male odour when presented with an alternate choice of host odour alone. If the pheromone primarily acts as a flight stimulant rather than an attractant it is predicted that it will not be possible to replicate results in a choice olfactometer.

My efforts to identify the compound(s) were unsuccessful and thus have not been presented in this thesis. Male headspace volatiles from males were collected using Tenax columns and hexane was used to elute any adsorbed volatiles. Hexane extracts were then concentrated and analysed using GC-MS and compared with an appropriate control. No compounds were identified that were not present in the control and efforts to elute any male produced compounds using dichloromethane were also unsuccessful (unpublished data). Even when males were exposed to rodent odour in an attempt to stimulate release of any pheromone no compounds could be identified and thus the chemical nature of the postulated pheromone remains to be discovered. Unlike some other disease vectors, mosquitoes do not have any known pheromone glands from which they might secrete pheromones. Since it is not clear where this postulated pheromone is stored and from where it is released it will not be possible to make solvent extracts of pheromone glands for analysis by GC-MS as has been used for the identification of pheromones from species with known pheromone glands. An alternative to the use of porous polymer coated columns to collect male

headspace volatiles would be Solid Phase Microextraction (SPME) as used recently by Levi-Zada *et al.* (2011) to identify the sex pheromone of the lesser date moth, *Batrachedra amydraula* (Lepidoptera: Cosmopteridae). GC-MS could then be used to identify the compounds present.

In the long term, a synthetic version of the pheromone could be used to enhance the attraction of mosquito traps. Since the pheromone only appears to be attractive in combination with host odour the traps would still need to be baited with host odour, but addition of pheromone should theoretically increase trap catches. Recently Salazar *et al.* (2012) have described the development of a push-pull vector control strategy for *Ae. aegypti* in which a mosquito trap is used as the "pull" component and thus if the pheromone could be synthesised it could potentially enhance the efficacy of this method. Thus characterization and synthesis of this pheromone could potentially contribute to control in addition to improved monitoring.

An aspect of *Ae. aegypti* mating behaviour that remains unresolved by the postulated male produced pheromone is how males are able to detect the mating status of females approaching a host. Attempting to mate with previously inseminated females is unlikely to be successful due to the fact that once a female mosquito has been inseminated it becomes refractory to further mating which in *Ae. aegypti* can occur within only 10 seconds following successful insemination (Clements 1999). Studies using *An. gambiae* suggest that mating is costly (i.e. increases male mortality) for male mosquitoes (Dao *et al.* 2010) and there is no reason to suggest that this may not also be the case with *Ae.* aegypti. It therefore seems possible that some adaptation may exist that enables males to avoid

females which will not be receptive to insemination. As reviewed by Thomas (2011) there is evidence that in many insects chemical cues are used by males to discriminate between mated and unmated females and thus this raises the possibility of a female produced pheromone in *Ae. aegypti* that would signal female mating status although this was not investigated in this thesis. Experiments by Cabrera and Jaffe (2007) suggest that further investigation of any female produced pheromone may be worthwhile.

So far no volatile male produced pheromone has been identified in any species of mosquito. Behavioural evidence for such a pheromone in *Ae. aegypti* should stimulate interest in examining whether a male produced pheromone may exist in other mosquito species. Any male produced pheromone in other species of the *Stegomyia* subgenus such as *Ae. albopictus* would be likely to interact with host odour in a similar manner since *Ae. albopictus* is also thought to mate in the vicinity of a host (Gubler and Bhattacharya 1972). However, for other mosquito species which are not thought to mate in the vicinity of a host any pheromone may be released by males/attractive to females in the absence of host odour.

6.5 Conclusions

This study has demonstrated behavioural evidence for a male produced pheromone in *Ae. aegypti* that enhances the attraction of host odour to virgin females. Results also demonstrate the challenge of designing apparatus to study mosquito behaviour and the need to correctly identify the behavioural response to be measured. The challenge now is to identify the specific compounds that make up this pheromone and determine where it is stored and how it is released. Lack of evidence for an egg-associated oviposition pheromone suggests that if freshly laid

con-specific eggs are used as an indicator of site suitability by ovipositing gravid females they might only effect oviposition when in combination with other larval cues and/or kairomones.

With the increasing frequency and size of dengue epidemics over the past 40 years and the continuing acceleration of urbanisation and globalisation likely to further the spread of this disease (Gubler 2012) there is a greater need than ever for new approaches that can improve our defences against this disease. Any increased understanding of the biology of the major insect vector of this disease might play a small part in improving existing vector control programmes and may even lead to the development of novel vector control tools. Results presented in this thesis suggest that the behaviour of males, an area that has traditionally been neglected, may require further attention since their presence around hosts might increase recruitment of females and thus male presence around hosts may indirectly facilitate disease transmission even though males do not themselves blood feed. As discussed, if the postulated pheromone suggested by results presented in this study could be synthesised then it could be used to enhance the attraction of traps and this could improve monitoring of *Ae. aegypti* populations or if used as part of a mass trapping strategy could contribute to control efforts. Combined with the expected future availability of a licensed vaccine for dengue and greater deployment of existing vector control strategies, further research into the chemical ecology of *Ae. aegypti* may eventually help contribute to improved control of dengue.

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