INVESTIGATIONS INTO THE OVIPOSITION BEHAVIOUR OF THE MOSQUITO ANOPHELES GAMBIAE

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DECLARATION

I certify that this work has not been accepted in substance for any degree, and it is not currently being submitted for any degree other than that of Doctor of Philosophy being studied at the University of Greenwich. I also declare that this work is the result of my own investigations except where otherwise identified by references and that I have not plagiarised the work of others.

Student's signature _____ Date _____

Supervisor's signature _____ Date _____

ACKNOWLEDGMENTS

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ABSTRACT

The *Anopheles gambiae* species complex includes the most predominant malaria vectors in sub-Saharan Africa. How they locate oviposition sites is not fully known, but a greater understanding of this subject may lead to more effective monitoring and trapping of gravid females. This project investigated potential oviposition semiochemicals and the oviposition behaviour of *An. gambiae sensu stricto* Giles.

The volatile profiles of seven bacteria were analysed by gas-chromatography linked mass-spectroscopy (GC-MS) to determine their volatile profiles and behavioural assays to determine their effect on *An. gambiae*. Cage assays could not confirm attraction to bacterial solutions, but demonstrated repellence to 4-methylphenol at 1mg/ml. GC-EAG demonstrated a strong electrophysiological response to 4-methylphenol and 8 of the 9 bacterial chemicals tested gave at least 50% of the 4-methylphenol response. Direct observations of oviposition in a large arena showed that, compared to a control dish of 0.9% saline, 1mg/ml 4-methylphenol reduced the number of visits, proportion landing and visit duration, but did not completely deter oviposition. A choice between dishes of saline or 1mg/ml 4-methylphenol, showed the latter was highly repellent; a majority of females oviposited in the control, the number, proportion and duration of visits to treatment dishes were reduced and fewer eggs were laid per female.

When 4-methylphenol was presented separately from water in porous sachets, the repellency was shown to be largely volatile based, having an effect on the direction of approach to dishes, but no deterrence of egg-laying in dishes near the sachets. Short range cues, possibly involving substrate contact/sampling, appear to mediate the final stages of oviposition site selection. These findings are discussed in the context of utilising a more holistic approach than previously used to study mosquito oviposition; oviposition is clearly not a single behaviour, but a complex chain of sensory inputs and responses by the gravid insect.

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1 BACKGROUND AND LITERATURE REVIEW

1.1 Introduction

Malaria is an infectious disease, the causative agent of which is the parasite *Plasmodium* spp. The parasite cannot pass directly from human to human and requires a vector (the organism that transmits the pathogen). In the late 1890s Giovanni Grassi and Ronald Ross independently discovered that mosquitoes of the genus *Anopheles* are the vectors of malaria parasites (Capanna, 2006), the latter receiving the 1902 Nobel Prize in Medicine for this work (DFID, 2010; Nobel Media AB, 2013).

Of the total world population in 2013, 3.2 billion people were at risk of malaria and there were approximately 200 million malaria cases, resulting in nearly 630,000 deaths (WHO, 2013a). The majority of cases (~85%) and of deaths (~90%) occur in sub-Saharan Africa, predominantly killing children aged under five years old (WHO, 2009; UNICEF, 2012).

Between 2000 and 2012 ~3 million deaths were prevented in sub-Saharan Africa by malaria interventions and since 2012 there has been a massive scale-up of control programmes based on indoor residual insecticide spraying (IRS, used to target indoor resting females) and the use of bed nets treated with long lasting insecticides, (LLIN, to target female mosquitoes that enter human dwellings overnight seeking bloodmeals). The initiatives reduced malaria-related deaths annually from a high nearly 1 million in 2009 to about 600,000 in sub-Saharan Africa in 2013 (WHO, 2013a), but we clearly still have a long way to go to alleviate the burden of this disease (UNICEF, 2012).

In spite of highly effective control programmes, there have been reports of increasing insecticide resistance (Roberts and Andre, 1994; N'Guessan *et al*., 2007, Ranson *et al*., 2011, Maxman, 2012), and higher incidence of disease transmission outdoors, away from insecticide treated walls and bed nets (Reddy *et al*., 2011; Russell *et al*., 2011). This has led to a widespread interest in measures to reduce mosquito breeding populations, which was highly successful in eradicating malaria in the southern USA and southern Europe during the 20th Century (Williams, 1963; de Zulueta, 1998; Tognotti, 2009; Majori, 2012).

Ecologically safe ways of controlling mosquito breeding are urgently needed (Gatton *et al.,* 2013). Larviciding can be effective (Fillinger *et al.*, 2004; Samnotra & Kumar, 1980) but financially expensive, labour intensive and ecologically unsound (Tusting *et al*., 2013).

The control of ovipositing ('egg-laying') mosquitoes is currently not widely used. Such an approach would likely be based on exploiting the natural behaviour patterns of ovipositing females, e.g., either luring them to traps baited with oviposition attractants (Dugassa *et al*., 2012; Dugassa *et al*., 2014; Okal *et al*., 2015a), or creating barriers of repellent-treated material, or repellent plants (Hill *et al*., 2007, Achee *et al*., 2012). However, while previous studies have focused on other genera (e.g. *Culex*) there is insufficient background knowledge of the semiochemicals that affect *Anopheles* mosquito oviposition (Fillinger & Lindsay, 2011; Wilson *et al.*, 2015), and the aim of this research project was to contribute to filling the knowledge gaps.

Malaria therefore, refers to the infectious disease, the causative agent of which is the parasite *Plasmodium* spp. The parasite cannot pass directly from human to human and requires a vector (the organism that transmits the pathogen). In the late 1890's Giovanni Grassi and Ronald Ross independently discovered that mosquitoes of the genus *Anopheles* are the vectors of malaria parasites (Capanna, 2006), the latter receiving the 1902 Nobel Prize in Medicine for this work (DFID, 2010; Nobel Media AB, 2013).

Mosquitoes vector many pathogens that are the causative agents of disease, and while it has been suggested that malaria may be over diagnosed in some low-resource areas (Crump *et al.*, 2013), through malaria and other vector-borne diseases, mosquitoes of the genus *Anopheles* account for over 1 million deaths per annum globally and contribute to the bulk of human mortality in tropical regions (WHO, 2013b).

Knowledge of both the ecology (the animal's relationship with its surrounding) and the ethology (the animal's behaviour) of mosquitoes are important in helping fight against disease vectored by these insects. The features determining the location of oviposition sites clearly depend on mosquito ecology and behaviour, neither of which are well understood in many mosquito species, and because of this control can be difficult as the sites may be difficult, or at the very least, time consuming (Anderson *et al*., 1980; Dongus *et al*., 2007).

Greater knowledge would also allow us to target interventions and avoid detrimental effects on non-pest species and non-vector mosquitoes, many of which play important roles in the ecosystems they inhabit. While arguably the ecological damage from removing all mosquitoes would not be unassailable,

there would still be a great ecological impact, if only in the short term (Fang, 2010).

Furthermore, the cost of controlling mosquito-borne disease is high, with a projected expenditure of US$5.1bn every year between 2011 and 2020 for controlling malaria globally (RBM, 2011). A better understanding of the vector and how to control it will save money, which in turn will save lives.

The primary vectors of malaria parasites in sub-Saharan Africa include some of the sibling species within the *Anopheles gambiae* Giles species complex. Their lifecycle is largely known, but many details of their behaviour are not fully understood. Most importantly, it is not fully known how females locate suitable breeding sites, or why a female lays her eggs in one potential breeding site and not others. Until recently much research has focused on how females locate a host for blood-feeding (Gibson and Torr, 1999). This research has led to a greater understanding of the vector and its human–host relationship, and has helped lead to successful disease interventions (Raghavendra *et al*., 2011). But in spite of these successes the burden of these tropical diseases is yet to be fully brought under control.

Further knowledge of oviposition site selection by this mosquito may aid in the early identification of breeding sites, allowing us to build on the existing gains in control further helping reduce the burden of malaria and improving the lives of millions.

1.1.1 Mosquitoes

Mosquitoes are biting insects of the order Diptera. All Diptera are adapted to ingest liquids. They have visible forewings and greatly reduced hind wings, which are in the form of halteres (Figure 1.1), drumstick-like objects which aid in balance during flight.

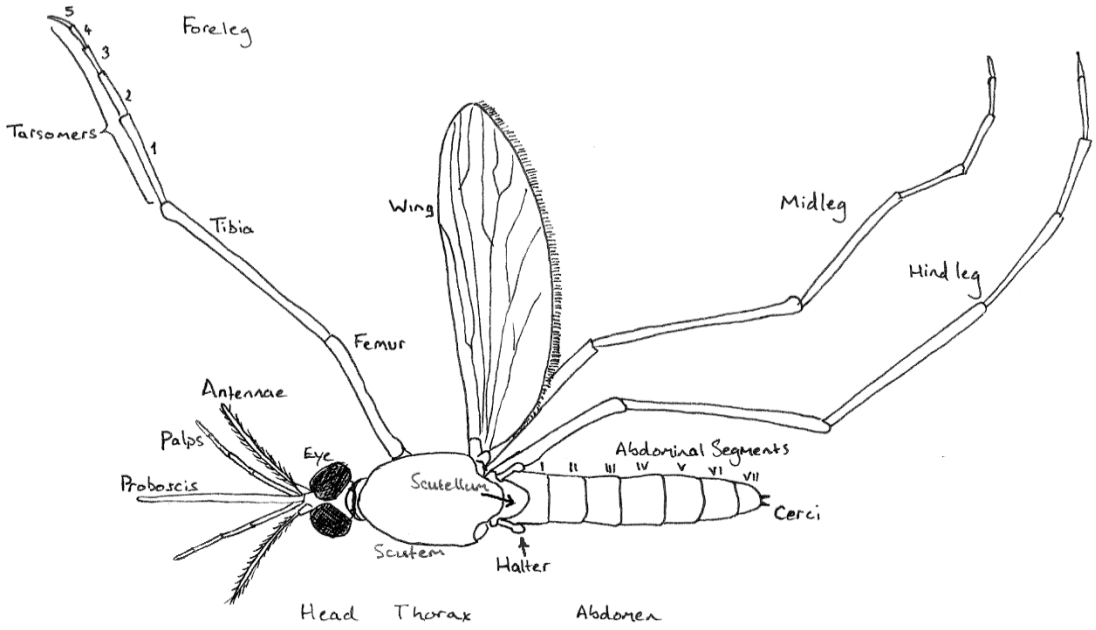


Figure 1.1 Typical external features of the Nematocera. Line drawing of the major anatomical features of the mosquito *Anopheles gambiae* Giles, a Nematoceran Diptera, showing elongated abdomen, long legs, and a single pair of forewings with hind-wings reduced to halters. Drawn by Author.

The Diptera are a large insect order, containing over 120,000 species in 170 families spread over two sub-orders: the Brachycera (e.g. flies such as *Musca domestica* L.) and the Nematocera (including crane flies and mosquitoes). There are 42 families of Nematocera, including Culicidae (mosquitoes). There are three mosquito subfamilies (Anophelinae, Culicinae and Toxorhynchitinae), with 54 genera. Genus *Anopheles* contains approximately 450 species,

including the *Anopheles gambiae* species complex, commonly referred to as *Anopheles gambiae sensu lato.*

Anopheles gambiae is a typical mosquito in form (Figure 1.2). The body is elongated with a multi segmented abdomen, terminating in cerci. The thorax is large with a prominent, humped scutum and small halters. The legs terminate in five tarsomeres. At rest the hind leg is carried high and the body posture points at an angle to the surface it is resting on, contrasting with other mosquito genera, such as *Culex* spp. that rest perpendicular to the surface they are on.

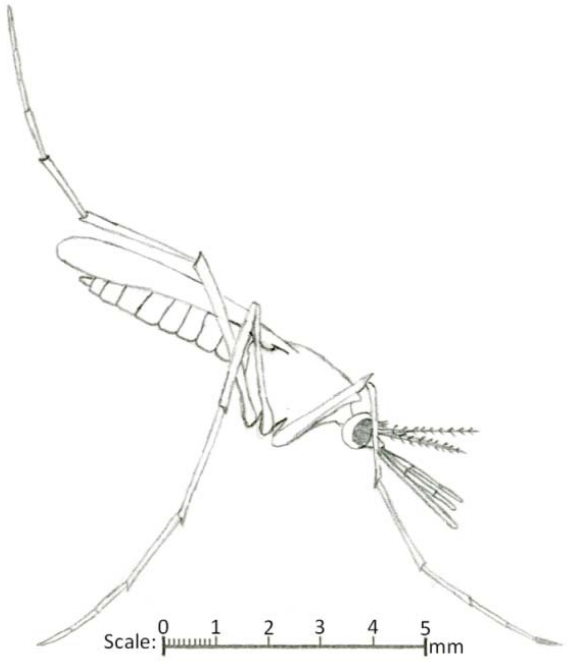


Figure 1.2 The general form of *Anopheles gambiae.* By author from observations. Anophelines show distinctive long palps and a resting position with the body at an acute angle to the surface they are on.

1.1.2 Malaria

Mosquitoes are effective vectors of many human and veterinary diseases and parasites. The endophagy (preference towards indoor feeding) observed in *An. gambiae* is the reason that the insects are of medical veterinary importance, as

this is the mechanism through which they vector the malaria. *Anopheles gambiae* vectors the malaria parasite, a eukaryotic protist in the genus *Plasmodium*. There are over 200 species of *Plasmodium*, with at least 10 that can infect humans. Zoonotic *Plasmodium* species infect a variety of birds, reptiles and mammals: for example, in Southeast Asia, *Plasmodium knowlesi* infects humans as well as the macaques which are its natural reservoir (Lee *et al*., 2011).

Laveran discovered that *Plasmodium* is the infective agent of malaria after examination of blood from an infected patient (Cox, 2010), and since then the five most common *Plasmodium* species responsible for human malaria have been identified. These are: *Plasmodium falciparum*, considered the most deadly to humans (Sarkar *et al.*, 2010) and responsible for approximately 70% of all human cases (Nadjm & Behrens, 2012), *P. vivax* (about 20% of human cases), *P. malariae, P. ovale* (Mueller *et al.*, 2007) and *P. knowlesi* (Collins & Barnwell, 2009), the last of which has recently been identified through molecular identification techniques as potentially being responsible for many cases ascribed to *P. falciparum* through traditional medical diagnostic techniques (Sarkar *et al*., 2010; Oddoux *et al*., 2011, Collins, 2012).

Plasmodium life cycle

The life cycle of *Plasmodium* spp. is complicated, requiring both the mosquito vector and a vertebrate host (i.e. a human) for completion (Figure 1.3). The disease is a consequence of *Plasmodium* reproduction in the human host and symptoms include chills, sweating, a high fever and fatigue with vomiting and

joint pains. In severe and cerebral malaria cases caused by *P. falciparum*, death may occur within as little as 24h after infection.

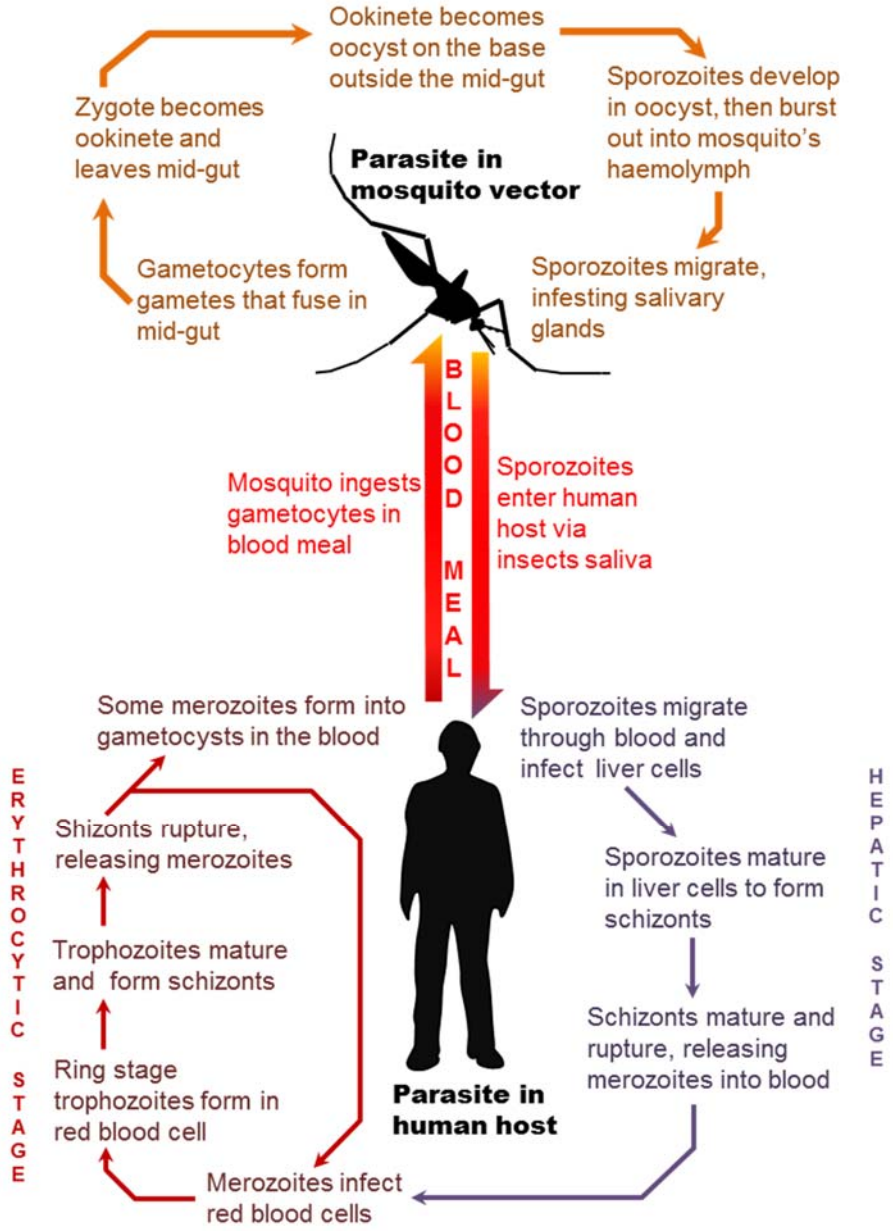


Figure 1.3 The life cycle of the malaria parasite in the mosquito vector and the human host. Drawn by author, based on Cox (1993) & Warhurst (2008).

A female *An. gambiae* requires a blood meal from a vertebrate host, (usually a human host in the case of *An. gambiae*) in order to reproduce. When a female

feeds on the blood of a host that has been infected with malaria, she ingests the *Plasmodium* gametocytes, which are immature haploid cells. Gametocytes mature and fuse in the gut of the mosquito, forming a gamete that soon becomes an ookinete – a motile form that exits the gut through its wall and attaches underneath the base of the mid-gut.

The ookinete becomes an oocyst, from which a large number of sporozoites (the transmitive form) arise, which migrate to the salivary glands. The mosquito salivates while she feeds, and in this way the sporozoites enter the human host.

In the human the sporozoites first move to the liver. These invade liver cells and form a shizont, an asexual reproductive form, from which many daughter cells mature (the hepatic stage). The daughter merozoites emerge *en-mass* and invade red blood cells (erythrocytes), feeding on haemoglobin and serum – the erythrocyte stage (Warhurst, 2008). After 48h incubation more merozoites emerge, causing the characteristic fever every 2-4 days in malaria patients (varying according to the species of *Plasmodium*). Some cells release new gametocytes instead of merozoites, which any *An. gambiae* who feeds on the infected person will ingest, beginning the cycle anew (Cox, 1993; Cowman *et al*., 2012).

1.2 *Anopheles gambiae*

1.2.1 The Anopheles gambiae species complex

Anopheles gambiae was believed, until the 1960's, to be a single species. Suspicions of a species complex arose from the observation that in some areas

what appeared to be *An. gambiae* mosquitoes were not transmitting malaria. Crossing and backcrossing individuals from these populations with those from an area where they did transmit malaria produced sterile males in the F1 generation (Davidson, 1964), showing that the 'species' was in fact a complex of several morphologically indistinguishable, closely related but distinct species, each with a different geographic range, behaviour and vector potential (White, 1974).

Two members of the complex breed in coastal saltwater; *Anopheles merus* Dönitz, which is found on the East African coast and *Anopheles melas* Theobold, which is found on the West African coast. A third species, *Anopheles bwambae* White, breeds only in geothermal springs in Eastern Uganda. These three species are considered mainly zoophilic (animal feeding) (Hunt *et al*., 1998), but both *An. merus* (Cuamba & Mendis, 2009) and *An. melas* (Moreno *et al*., 2004) are also human malaria vectors in some coastal areas, but are not a widespread problem.

A fourth member of the complex, *Anopheles quadriannulatus* Theobold, found in Ethiopia and southern Africa, was considered to be a single species due to similarities in host-feeding behaviour and the non-vector status of the two populations, but is now known to be two species; *An quadriannulatus*, which is found in southern Africa and *Anopheles amharicus* Hunt, Wilkerson & Coetzee, found in Ethiopia. (Coetzee *et al*., 2013).

Three members of the complex, *Anopheles arabiensis* Patton, *Anopheles coluzzi* Coetzee & Wilkerson and *Anopheles gambiae sensu stricto* Giles, have a much larger range than their sister species, largely overlapping and spreading

from the sub-Sahara to Namibia, Mozambique and Madagascar. Along with *Anopheles funestus* Giles, which shares their range, these mosquitoes are the three dominant malaria vectors in Africa (Sinka *et al.,* 2011, Coetzee *et al*., 2013).

Anopheles arabiensis tends to dominate in more arid areas at the northern, north-eastern and south-western extremities of their co-dominant range, but is mostly absent in the area surrounding the Congo basin where *An*. *gambiae* and *An. funestus* dominate (Mahande *et al.,* 2007; Sinka *et al.,* 2011). *Anopheles coluzzi* is the most recently identified of these (Coetzee *et al*., 2013), with an overlap in range west of Central East Africa. Figure 1.4 shows the ranges of *An. arabiensis, An. coluzzi* and *An*. *gambiae s.s* (hereafter*, An. gambiae*).

Anophles arabiensis and *An. gambiae* are responsible for the majority of malaria transmission in Sub-Saharan Africa. They are highly anthropophilic and exhibit high levels of endophily (a preference to rest indoors), and endophagy, respectively, especially in the case of *An. gambiae s.s*. (Kirby *et al*., 2008).

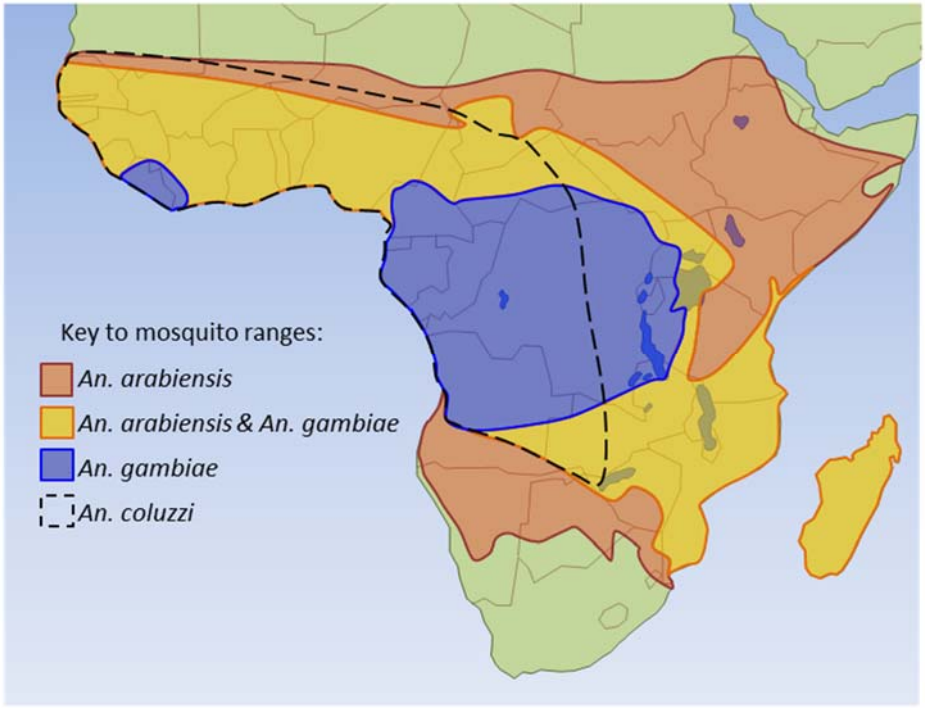


Figure 1.4 The ranges of *Anopheles arabiensis, Anopheles coluzzi* and *An. gambiae* in Africa. Based on data from Sinka *et al.* (2011) and Coetzee *et al*. (2013).

Anopheles gambiae subforms

Until 2013 *Anopheles gambiae s.s.* was believed to exhibit two molecular forms, termed M and S and based on molecular genetic studies. Taxonomy was further complicated by the existence of several "chromosomal forms" based on cytogenetics (the appearance, form and structure of chromosomes; Coetzee *et al.,* 2013).

The differences between M and S form were believed to be not just genetic (Della Torre *et al.,* 2001), but also behavioural (Pennetier *et al.,* 2010; Gimonneau *et al.,* 2012), with one major behavioural difference being oviposition site selection. S-form larvae thrived in temporary pools devoid of predation and M form larvae preferentially oviposited in permanent bodies where predators may exist (Gimonneau *et al.,* 2010). M form was observed in

a small northern belt of semi-desert where S form is absent, suggesting greater drought tolerance (Lehmann & Diabate, 2008). These differences led to a lack of gene flow between the two forms and it was argued that they may be considered as being totally distinct from one another (de Queiroz, 2007). Coetzee *et al.* (2013) demonstrated that this was the case, the S form remaining *Anopheles gambiae s.s.* Giles and the M form becoming *Anopheles coluzzii* Coetzee & Wilkerson. A summary of the taxonomy of the *An. gambiae* species complex is shown in Table 1.2.

Table 1.1 Proposed taxonomies of the *Anopheles gambiae* complex. A summary of the various proposed taxonomies since 1967 through to 2013.

PRE-2013 TAXONOMIES			NEW TAXONOMY
Davidson *et al.* (1967)	**Mattingly (1977) & White (1985)**	**Subforms**	**Coetzee *et al.* (2013)**
Species A	*Anopheles gambiae s.s.* Giles	S form	*Anopheles gambiae s.s.* Giles
		M form[1]	*Anopheles coluzzii* Coetzee & Wilkerson
Species B	*Anopheles arabiensis* Patton		*Anopheles arabiensis* Patton
Species C	*Anopheles quadriannulatus* Theobold	Southern African populations	*Anopheles quadriannulatus* Theobold
		Ethiopian populations[2]	*Anopheles amharicus* Hunt, Wilkerson & Coetzee
Species D	*Anopheles bwambae* White		*Anopheles bwambae* White
East African saltwater breeder	*Anopheles merus* Dönitz		*Anopheles merus* Dönitz
West African saltwater breeder	*Anopheles melas* Theobold		*Anopheles melas* Theobold

[1]Lehman & Diabate (2008), [2]Hunt *et al.* (1998)

1.2.2 Mosquito life cycle

The life cycle of *Anopheles gambiae*, as with all mosquitoes, consists of four major stages: egg, larvae, pupae and imago - the emerged adult (Figure 1.5). *Anopheles gambiae* is a vector of malaria because females must take a blood meal to produce eggs as part of this cycle. Only the female will take a blood meal and only as an adult. The juvenile stages of the mosquito are all aquatic.

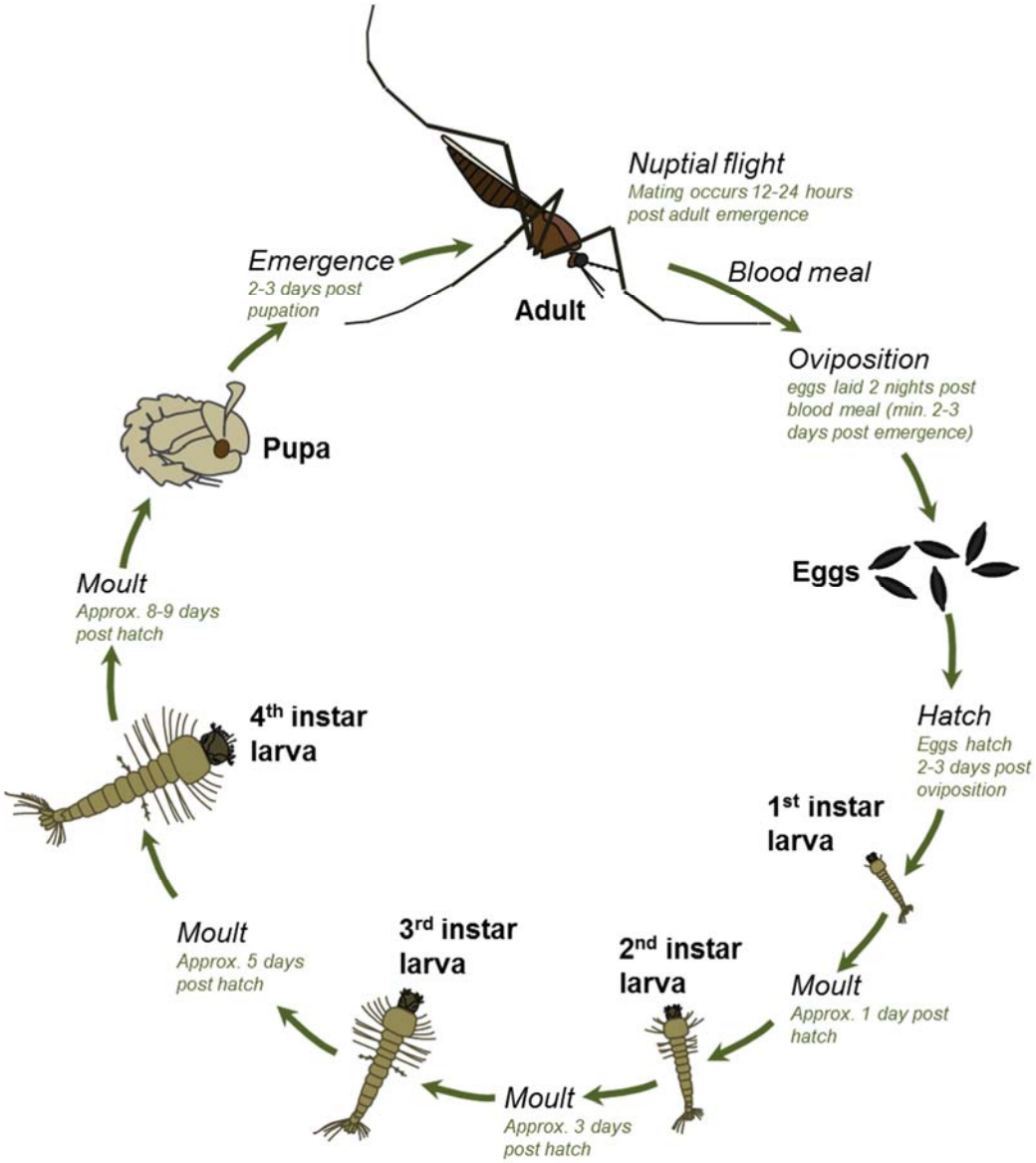


Figure 1.5 The life cycle of *Anopheles gambiae.* Redrawn by author and annotated after Snow (1990).

Reproduction

The reproduction of most medically important mosquito genera has been studied, such as *Culex*, *Aedes*, as well as *Anopheles*, and is similar in all of these genera. However, there are significant specific differences between species, which is why it is important to investigate the oviposition behaviour of individual species, even within the *An. gambiae* species complex. The gonotrophic cycle of *An. gambiae* involves mating, taking a blood meal, egg maturation and oviposition and it may be repeated several times over the course of the mosquito's life. Earlier batches of eggs may need more than a single blood feed per cycle, the female entering a "pre-gravid state" before a second blood feed allows oogenesis to be completed (Gillies, 1955). This takes about 48h under optimal conditions.

Mating occurs shortly after emergence in a "nuptial flight", during which females enter a swarm of males and copulate. The females are believed to be able to use wing tone of the swarming males in determining a male's suitability (Pennetier *et al*., 2010). After copulation sperm is retained by the female and stored in the spermatozoa. The eggs are fertilized during oviposition as they are passed out through the oviduct (Lehane, 1991).

A blood meal is required for egg development, and in order to do so a suitable host is required. Olfaction plays a major role in host seeking (Takken, 1991); the olfactory receptor neurones of *Culex quinquefasciatus* Say, *An. gambiae* and *Aedes aegypti* (L) having been shown to be highly sensitive to volatile chemicals associated with their prey of humans and birds (Davis, 1984; Cork & Park, 1996; Syed & Leal, 2009).

Olfaction may also allow the mosquito to determine the suitability of a host. Studies of *Ae. aegypti* indicate a possible 'masking effect', where a human is less attractive as a potential host, not because they lack certain attractive volatile emanations, but rather the presence of compounds that act to make a potential host 'unattractive' (Mukabana *et al*., 2007).

Behavioural, genetic and electrophysiological studies have shown a range of potential host odours that may attract *An. gambiae* to humans. **Attractiveness**, is the term used throughout this thesis to refer to any sensory cues that **attracts** (draws an organism towards the source of the stimulus, be it, for example, a volatile chemical or the visual appearance of a blood-host animal). Accordingly the attractiveness of infected hosts may be altered by malaria infection, although there is debate as to whether or not malaria reduces (Mukabana *et al*., 2007) or increases (Lacroix *et al*., 2005) attractiveness of the humans to the mosquitoes. Use of repellents, such as DEET or lemongrass may also have an effect as *An. darlingi* has been shown to land less on those wearing repellent than those not in paired tests (Maia & Moore, 2011). Mixtures of compounds, derived from the volatile profiles of human hosts that are relatively less attractive (to host seeking mosquitoes), have been shown to be more effective than DEET at repelling host seeking *An. gambiae* at certain concentrations (Logan *et al*., 2010). Several novel plant based repellents have been examined by Deletre *et al.* (2013), in order to combat behavioural and physiological resistance to current pyrethroid based insecticides.

Blood feeding

Males and females will take sugar from feeders in laboratory cultures, and feed on sugar, particularly fructose, under natural conditions from plant nectar (Gouagna *et al*., 2010). Adult female mosquitoes live about four weeks, whereas male *An. gambiae* live half as long as females, presumably because a sugar water diet is not as nutritious as blood (Gary & Foster, 2004).

Only females take a blood meal and the blood-feeding process begins once a female has landed on a suitable host; she inserts her entire stylet bundle into the host to begin feeding. The stylet bundle is surrounded by the labium, terminating at its tip with the labellum. The pointed feeding tube, the labrum, is the largest stylet and forms a trough up which blood is drawn. To either side of the labrum are the mandibles and beneath these are the serrated maxillae. Under these is a single flattened stylet, the hypopharynx, which is used to deliver saliva to the wound, helping prevent haemostasis and wound closure (Figure 1.6).

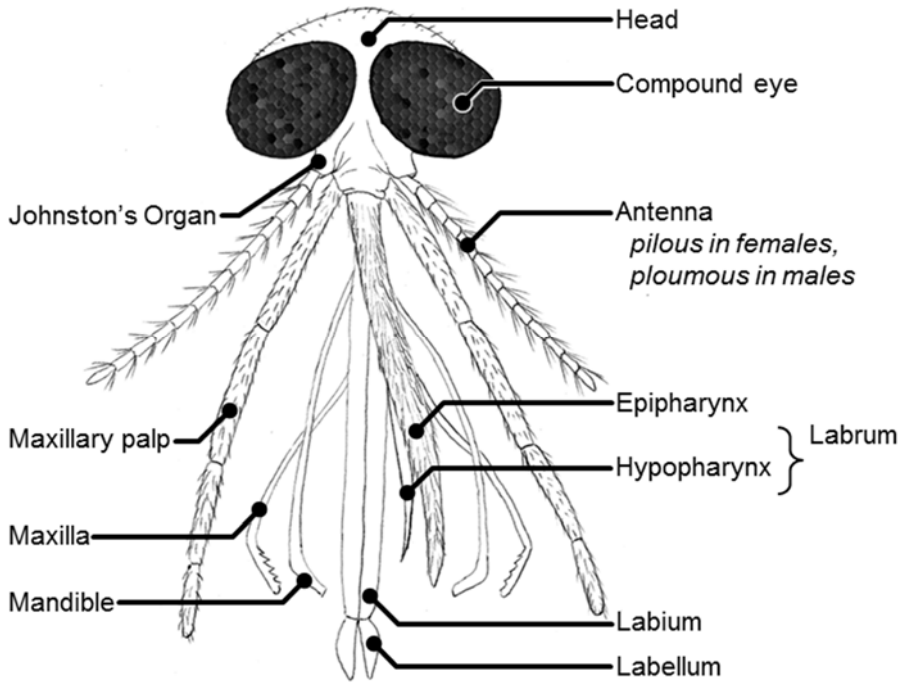


Figure 1.6 The head of a female *Anopheles gambiae.* Shown are the mouth parts and the stylet bundle separated from the labium. The epipharynx forms the ventral surface of the proboscis. Note the long maxillary palps, typical of the genus *Anopheles*, which are involved in olfaction as well as the antennae (by author, based on observations and McGavin (2001)).

Feeding begins with the labia spreading and the maxillae cutting into the host's skin, akin to a saw (Snow, 1990). The labium folds back and the mouthparts are inserted into the host. This is not done by thrusting, but rather by the insect lowering itself closer to the host with its legs. Once the stylets are inserted the insect will feed for several minutes before reversing the insertion procedure and departing.

The ingested blood swells the mosquito's abdomen and the bolus (the ingested material mixed with saliva) is surrounded by a peritrophic membrane, compressing it and releasing digestive enzymes. The compressing of the blood into a bolus necessitates the exit of any ingested *Plasmodium* from the mid-gut.

Many pre-gravid females that take a second meal are nulliparous; as they have never laid eggs (Charlwood *et al*., 2003; Foster & Walker, 2009), and in some cases more than one feed may be required prior to first oviposition (Chadee & Beier, 1997; Clements, 1999, Chadee, 2012). Once blood has been taken, alongside digestion, the ovaries develop and swell, causing the abdomen to enlarge further as the eggs mature.

Development of eggs, larvae and adults

Once eggs are matured the mosquito engages in pre-oviposition behaviour, locating a suitable oviposition site, an activity that may be a form of or related to the foraging behaviour used to find a host (Clements, 1999). Mosquitoes oviposit in a wide variety of aquatic environments and different species have different ecological requirements, displaying differing oviposition behaviour.

Culex spp. will oviposit onto organically rich water, which may contain fermenting grass infusions or other rotting materials (Millar *et al*., 1992), sometimes utilising storm drains or even latrine pits and septic tanks (McCall, 2002). In contrast *An. gambiae*, typically oviposit onto fresh ground pools containing 'clean' water, i.e. with little organic content (McCrae, 1984) or with low levels of accumulated organic waste (Gimning *et al.,* 2001; Minakawa *et al.,* 1999; 2005) .

Eggs are elongated with two buoyant floats on either side, and float on the water surface. Once the eggs are laid, they hatch around 48h later. The larvae develop quickly, moving through four instars in around a week. The larvae filter feed from the water surface on algae and other microorganisms that exist in the surface film.

The pupae are also aquatic. The head and thorax fuse into a cephalothorax with the abdomen curled underneath. The abdomen muscles remain functional and when disturbed the pupae can move quickly by rapidly flexing the abdomen. Air encapsulated in the cephalothorax causes the pupae to float. Breathing trumpets extend to the water surface and their shape can aid in species identification. Emergence is usually nocturnal, taking place between 24 and 72 h after pupation.

Sexual maturation in males takes a minimum of 24 h after which males and virgin females join mating swarms at species-specific sites. Sexual dimorphism occurs in adult anophelines; females are larger and have pilose antennal hairs contrasting with the plumous antennae of the male.

1.3 Mosquito oviposition

1.3.1 Egg maturation and oviposition

Mosquitoes exhibit typical r-selected reproductive strategies, with high fecundity, low parental investment, and grow quickly to maturity with a short generation time (Pianka, 1970). As with most arthropod disease vectors, mosquitoes do not care for their offspring other than perhaps in the selection of oviposition sites (McCall & Cameron, 1995). Females are able to oviposit between 50 and 500 eggs per bout of oviposition and can be expected to survive for about four oviposition cycles (Clements, 1992). This is long enough to potentially become infected with malaria parasites, for those parasites to complete the 'extrinsic' cycle (i.e. outside the human body) of development, and transmit malaria parasites and other pathogens to subsequent hosts.

Oocytes (the immature egg) are produced in the ovarioles (Snow, 1990). Oviposition follows the maturation of the oocyte in the ovarian follicles, which are partially developed prior to the blood meal, before 'resting' prior to their final development after feeding (Christophers, 1911; WHO, 1975). Following maturation the oocyte passes through the oviduct into the vagina, at which point the sperm (stored in the spermatheca since matting) enters the oocyte. Oviposition then occurs when the oocyte is then passed through the gonotreme (located at the terminal abdominal segments), leaving the insect. The oocyte completes meiosis, becoming an egg proper, and fusion of the two pronuclei occurs after oviposition (Clements, 1992; Foster & Walker, 2009).

Anopheles eggs are laid individually by females either directly onto the water surface or the mud that makes up the margin around a water body (Huang *et al*., 2007). The extent to which oviposition occurs in flight or while the female is landed is not known. In the case of *Anopheles melas* the egg drops from the abdomen onto the substratum and after a short period (1-2s) the next egg appears at the gonotrome. *Culex* eggs are laid in rafts, usually onto the water surface. Some species of *Mansonia* attach the raft to aquatic plants. The female constructs the raft by manoeuvring the eggs with her ovipositor as she lays them, with the eggs coat naturally adhering to one another (Clements, 1992). *Aedes* and *Toxorhynchites* lay individual eggs, often into tree holes or moist surfaces (Clements, 1999).

During the photophase, i.e. the light part of the day-night cycle, *An. gambiae* are inactive (Jones & Gubbins, 1979), with their activity peaking at dusk, with a minor peak towards dawn. Oviposition tends to occur at a regular time, peaking during the early part of the scotophase, i.e. the dark phase (Fritz *et al*., 2008).

1.3.2 Cues for oviposition

There are many cues that regulate oviposition in mosquitoes. Internal factors include the circadian rhythm, which determines when in the 24h day a mosquito will oviposit (Sumba *et al.,* 2004a; Fritz *et al.,* 2008), hormones, which regulate the development of eggs and other process (Clements, 1992), and the taking of a blood meal, which can alter gene expression (Rinker *et al.,* 2013). The physiological state of the mosquito is, therefore, important in determining if and when oviposition occurs, but where it occurs is more dependent on external factors. Because mosquitoes do not exhibit brood care, the choice of the oviposition site can have a significant effect on the survival of their offspring (McCall & Pile, 1995).

Oviposition semiochemicals

The oviposition of many mosquitoes appears to be mediated, at least in part, by semiochemicals. **Semiochemical** (a word derived from the Greek for 'signal') is a broad term relating to any chemical (or mixtures of chemicals) which mediate interactions between organisms. Semiochemicals are largely divided into two groups; **pheromones**, which are intraspecific, and **allelochemicals**, which are interspecific and therefore affect species other than their originator.

Pheromones are typically classified by the interaction they mediate, such as alarm pheromones, trail markers or sex pheromones, the last of which is commonly used in monitoring populations of crop-pest Lepidoptera (Dent, 1993). Allelochemicals may be categorised by which organism benefits from its

detection: the emitter (**allomones**), the receiver (**kairomones**) or both organisms (**synomones**) (Brown *et al.,* 1970; Whittaker & Feeny, 1971).

Semiochemicals are extremely wide ranging in their effect on insects. Some may simply be **attractants**, i.e. substances that induces an organism to move towards the source of the chemical, or **repellents**, i.e. substances that induce orientated movements away from the source, or they may act in more subtle ways, such as **arrestants**, which cause the organism to remain in close proximity to the source of the odour, even though the chemical might not have an attractant effect (Kennedy, 1978). Kairomones are semiochemicals emitted by an organism that are affected by an organism of another species, where the receiver gains an advantage by detecting it, e.g. volatile compounds emanating from humans that influence the behaviour of a blood feeding insect, or compounds attractive to ovipositing mosquitoes that emanate from organisms (e.g. such as bacteria) are also kairomones.

Responses to semiochemicals may have a variety of effects on the organisms that detect them. Semiochemicals may stimulate '**taxis**' (i.e., movement, plural 'taxes'), a term describing directional movement in response to an external **stimulus**. Arrestants stimulate positive taxes, i.e. movement towards the stimulus, and repellents cause an organism to retreat from the source.

Responses to semiochemicals do not always involve taxis, as in the case of **deterrents**, which are distinct from repellents, in that they inhibit rather than stimulate behaviour. In the case of oviposition a compound could be an **oviposition stimulant**, if it triggers egg laying without playing any role in bringing a gravid female to a particular place (Clements, 1999).

Responses to a stimulus may also be undirected (kinesis). **Orthokinesis** is an alteration of speed or activation due to the stimulus intensity. **Klinokinesis** is a change in the frequency or rate of turning proportional to the intensity.

It is not known which of these effects are responsible for the oviposition mediation reported in *An. gambiae* (e.g. Sumba *et al.,* 2004; Lindh *et al.,* 2008a), however, as these studies report an increase in oviposition preference towards one target over a less attractive one, it is likely the effect is positive, either stimulating oviposition or acting to attract or arrest the mosquitoes. It is also not clear which compounds are involved and whether it is one or a number of compounds acting **synergistically**, where an effect is seen in the presence of two or more compounds that is not seen or is not as strong in the presence of the individual constituent parts.

Examples of known semiochemicals affecting gravid mosquitoes

Skatole (3-methyl-indole), a breakdown product of rotting material, is attractive to gravid *Culex* spp. females (Blackwell *et al*., 1993). *Aedes triseriatus*, which typically oviposits in tree holes, is attracted to *p*-cresol (4- methylphenol), a breakdown product of lignin from rotting wood (Bentley *et al*., 1979), as are gravid *Cx*. *quinquefasciatus*. These chemicals may signal the presence of water containing plenty of nutrients.

Experienced *Cx. quinquefasciatus* that have previously oviposited into water containing skatole tend to lay successive egg batches in water containing skatole, suggesting prior experience may play a role in site selection (McCall & Eaton, 2001). *Culex* eggs produce droplets which release an aggregation pheromone (Osgood, 1971; Bruno & Laurence, 1979; Laurence & Pickett, 1982)

that promotes further oviposition around existing egg rafts (Laurence & Pickett, 1982; 1985). This compound has been identified (Laurence *et al.,* 1985), synthesised (Dawson *et al*., 1990; Couladouros & Mihou, 1999; Gallos *et al.*, 2000, Sabitha *et al.*, 2006) and used to attract gravid *Cx. quinquefasciatus* (Otieno *et al*., 1988; Dawson *et al.*, 1989). Methods using a plant based oil may show promise, producing a cheap product with activity comparable to the pure synthetic (Olagbemiro *et al*., 1999).

Some effects are enhanced in the presence of additional compounds (Mordue *et al*., 1992), particularly skatole and grass infusions (Mboera *et al.,* 2000; Barbosa *et al*., 2010b, Irish *et al*., 2012), which may be combined with insecticides in traps.

The aggregation pheromone seems to have negative effects on females' orthokinesis but no effect on klinokinesis (Pile *et al*., 1991; 1993; McCall, 2002). It is postulated that the presence of eggs may, therefore, be used as an indicator that a site has previously attracted gravid females and that the eggs are still present, perhaps indicating a suitable site for oviposition by others (Pickett & Woodcock, 1996), but it will also attract competing gravid females. The presence of the pheromone does not indicate survivorship levels of larvae that have hatched.

Artificial lures based on rotting hay infusion to attract gravid *Cx. quinquefasciatus* (Leal *et al*., 2008) have proven to be effective (Hazard *et al*., 1967; Burket-Cadena & Mullen, 2007).

Otieno *et al.* (1988) used effervescent tablets to deliver a combination of oviposition pheromone and an insecticide (specifically an insect growth

regulator – see Chapter 1.4.2) to lure in gravid *Cx*. *quinquefasciatus* and prevent the development of their offspring. Unfortunately, *An. gambiae* has no oviposition pheromone, meaning it cannot be lured in the same way.

***Anopheles gambiae* oviposition cues**

In contrast to the aforementioned culicine mosquito species, the factors affecting the suitability of potential oviposition sites for *An. gambiae* are still not fully understood, and opportunities for exploiting *An.* gambiae oviposition has received far less attention than for the other mosquito groups (Logan *et al.,* 2013). The typical 'clean' oviposition sites *An. gambiae* is known to prefer, i.e. containing little to no growing vegetation and little organic matter (Gimning *et al.,* 2001; Minakawa *et al.,* 1999; 2005), make it difficult to identify compounds, that attract gravid females other than the presence of water, which itself is attractive (Kennedy, 1942; Okal *et al*., 2013). Some evidence suggests that the presence of *An. gambiae* larvae is repellent (McCrae, 1984), however more recent studies suggest *An. gambiae* prefer to oviposit in water containing conspecific larvae (Ogbunugafor and Sumba, 2008). There is no evidence of an aggregation pheromone, and conspecific eggs have no apparent attraction (Sumba *et al*., 2008).

Gravid females are generally found to oviposit preferentially into substrates that contained either mud or water from natural oviposition sites compared to sterilised water (Sumba *et al*., 2004a), suggesting the bacteria contained within a water source may have an indirect effect on *An. gambiae* oviposition. Oviposition responses in the species have also been seen towards the volatiles that are given off from larval pools, perhaps of bacterial origin (Rejmankova *et*

al., 2005). The exact role of bacteria in mediating oviposition site selection is somewhat unclear however, as Huang *et al.* (2006) showed bacteria to have a repellent oviposition effect. Conversely Lindh *et al.* (2008a) showed *An. gambiae* to oviposit preferentially into water containing certain bacteria over uncontaminated water. These bacteria were isolated from mud taken from natural oviposition sites and also the mid-gut of adult mosquitoes.

In laboratory conditions *An. gambiae* oviposition has been shown to be able to transfer gut bacteria to oviposition sites, which may then be taken up by larvae (Lindh *et al*., 2008b). It is currently unknown if this occurs in nature. However it suggests that the presence of *An. gambiae* eggs in a water body alters the site and potentially its volatile profile.

In addition to volatile chemicals, mosquitoes utilise their other senses in oviposition: despite their nocturnal nature, vision appears to play an important role in the oviposition of mosquitoes. Adult mosquitoes, unlike many Diptera, have apposition eyes which gather approximately three magnitudes more light than a comparable Dipteran neural superposition eye, but at the cost of greatly reduced resolution. Adaptations within the eye allow nocturnal mosquitoes to gather an increased level of light (Land, 1997). The majority of ommatidia in the compound eyes of *An. gambiae* females also 'look forward and down', having the greatest sensitivity in the anterio-ventral part of the eye (Land *et al*., 1999). This suggests that the ability to observe the ground at a high level of resolution is more important to the mosquito, compared to other insects which predate or mate on the wing and have enhanced anterio-dorsal areas of high resolution in their eyes (Land *et al*., 1997).

Interestingly, chemical cues may alter the attractiveness of a visual target as well (Snow, 1976), suggesting the mosquito does not rely on a single stimuli when host or oviposition site seeking.

The r-selected strategy, the close anthropophilic habits of *An. gambiae* and the lack of sufficiently protective housing in many malaria blighted areas are all factors that combine to make this mosquito species an incredibly effective and efficient disease vector (White, 1974; Curtis, 1996). Its success can be clearly seen in the malaria burden of sub-Saharan Africa.

1.4 Control of mosquitoes

Control of *An. gambiae* in Africa has, for the last half a century, focused on preventing the adult female from finding a host and taking a blood meal. Current control methods employed are the use of physical barriers such as bed nets - especially long-lasting insecticide treated bed nets (LLIN or with older, less persistent insecticides, insecticide treated nets: ITN) and physically applying insecticide to houses (indoor residue spraying or IRS).

Bed-nets have been highly effective. In 2008 31% of African households owned at least one LLIN, with 21% of children in endemic regions using one regularly (WHO, 2009). Organisations such as Roll Back Malaria partnerships and WHO aim to increase these to 80% by 2015 (RBM, 2005), and report that an estimated 1.1 million child deaths were prevented in sub-Saharan Africa in the first decade of the 21st century due to the rapid scale-up of malaria interventions and increase of resources to combat malaria (RBM, 2011).

1.4.1 Current insecticides

Pyrethroids are the only class of insecticide permitted for use on LLINs and it is strongly feared that resistance to these chemicals by mosquitoes will lead to the LLIN becoming less effective. A physical barrier between host and insect will still provide some protection, but damage and holes develop within a couple of years use, and increased infant mortality occurs if the insecticide is not present or effective (Lengeler, 2004).

Prior to pyrethroids, dichlorodiphenyltrichloroethane (DDT) was commonly used for IRS (but not for LLINs) as an effective control measure for infectious disease vectors as well as all manner of agricultural pests. DDT is an organo-chlorine based insecticide that was used widely in interventions and general insect control since the middle of the 20th century. Unfortunately DDT is a wide spectrum insecticide with a high environmental impact and has been banned in many countries for purposes other than disease vector control. Today it is usually only permitted for use in vector control as the insecticide of last resort in countries where malaria is endemic (Van den Berg, 2009).

DDT is still available for vector control because of the rise in insecticide resistance to modern pesticides. Insecticide resistance occurs naturally through the inadvertent selection pressure towards resistant insects. Through various mechanisms an insect may evolve a physiological or behavioural change that protects it from the pesticide. It survives the pesticide treatment and will pass on these successful traits to its offspring, resulting in a population that is immune to a normal dose of a particular pesticide. Due to the high reproduction rates of mosquitoes, insecticides can quickly become less able to control insect populations than before resistance arises.

Pyrethroid resistance is now acutely observed in sub-Saharan Africa (Ranson *et al*., 2011), and resistance to DDT is also observed (Maxman, 2012). In 1996 South African malaria control programmes switched from DDT to deltamethrin (a pyrethroid). Within four years pyrethroid resistance resulted in a quadrupling of malaria incidence and authorities reverted to using DDT for indoor spraying (Hargreaves *et al*., 2000).

IRS is effective due to the endophilia of species such as *An. gambiae* and *An. arabiensis*. These species often feed indoors and may rest in secluded parts of buildings, such as eaves, when inactive during the day. IRS therefore aims to reduce female density by reducing their life span.

1.4.2 Larval control

In the mid to late 1940s an effective eradication programme took place in Egypt. The methods used were primarily to larvicide the breeding sites of *An. gambiae* using Paris green (copper (II) acetoarsenate), a highly toxic compound previously used as an insecticide and rodenticide. Paris green powder floats and is ingested by the larvae as they feed.

Oil was also used, which acts to smother the water and prevents the larvae from breathing. Additionally, it was found that adult control, using pyrethrum (a natural insecticide manufactured from chrysanthemum flowers) and DDT to control insects inside buildings and to prevent their movement on vehicles would be required.

The campaign was successful and eradication was achieved by the end of 1945 (Shousha, 1948). Prevention has since stopped the insect returning to Egypt in great numbers. Today the use of chemicals such as Paris green, which have a

high toxicity and are unspecific, is not permitted despite their very effective nature and low cost of application (Coosemans & Carnevale, 1995). Pyrethrum has long been noted as having insecticidal properties and is the basis of modern synthetic pyrethroids, and while resistance to pyrethroids has developed (Chapter 1.4.1); none is seen to pyrethrum itself (Chandre *et al*., 1999; Hemmingway & Ranson, 2000; Kristan *et al*., 2003).

In the 1930s and 1940s larviciding was effectively used to eradicate malaria vectors in the USA, Egypt and Brazil (Killeen *et al*., 2002), but it did not play a large role in any other eradication programme and was largely forgotten as a technique until the closing years of the 20th Century.

In today's urban environments, where there is a high human density and limited sites for mosquitoes to breed in, the identification and removal of potential breeding sites can be accomplished, often simply, such as by covering water containers. For *An. gambiae*, which predominantly breed in rural areas, this does not seem to apply.

Biological larval control agents also include the use of the microbial treatment *Bacillus thuringiensis israelentis*, commonly known as Bti, (Das & Amalraj, 1997) and even larvivorous fish (Fletcher *et al*., 1992; Tusting *et al*., 2008; Walshe *et al.*, 2013). These measures can be effective and have the advantages of specificity and low risk to humans and other non-target organisms (NTOs) but are often more difficult to apply and have limits as to where they can be used.

Synthetic treatments, such as larvicidal pyrethroid treatments may be easier to use, but lack the specificity of Bti or juvenile growth hormone regulators (IGRs).

Rather more importantly, the control of larvae, in particular using microbial control measures, has been shown to reduce the burden of malaria due to reducing adult vector populations (Fillinger & Lindsay, 2006).

Limitations of larviciding

One important factor is public perception of vector control. Spraying insecticides looks impressive to a bystander. The adult mosquito is also the most commonly encountered form of this vector – few people think of larvae when a mosquito is mentioned – and so a treatment that appears to kill the adult is very easily accepted by those observing.

Larvicidal treatments using oils are seen to be messy and damaging to the water, while treatments that have toxicity associated with them frighten people. This suggests that novel chemicals and especially biological agents are more acceptable, such as is the case with larvivorous fish (Fletcher *et al*., 1992). A larvicide that is simple to use and does not produce any mess or toxic side effects is most acceptable and can be applied by members of the community or volunteers when required after little training.

Larviciding can be effective, and have been shown to reduce malaria in areas where the habitats are manmade and discrete, such as drains (Fillinger *et al.*, 2004), water containers (Samnotra & Kumar, 1980) or on a large scale, such as rice paddies (Yapabandara & Curtis, 2004). However interventions using larvicide are extremely labour intensive (and therefore expensive), requiring frequent reapplication, surveillance and the evaluation of success to determine when to stop (Tusting *et al*., 2013).

The right larvicide must be used, as a treatment that takes several days, or acts on a specific developmental stage, such as an IGR, will not be effective if applied at too late a stage. It is also impractical to apply a treatment to every potential breeding site as there would simply be far too many. A treatment must be simple, cheap, and acceptable to those that have to live with the consequences and target more permanent breeding sites. But to be effective it is essential that the breeding sites of *An. gambiae* can first be effectively identified. Where human activity has altered the environment this can sometimes be achieved, but often this is not possible. The environment can also prevent effective larviciding, such as where mosquitoes breed in rivers, which are too large to effectively treat (Bogh *et al*., 2003; Majambere *et al.*, 2008).

1.4.3 Alternative control methods

In some areas a reduction has been seen in the efficacy of bed nets treated with pyrethroids due to this resistance (N'Guessan *et al*., 2007). Therefore, although it is important to restrict the use of chemicals that may cause harm to non-target organisms (NTOs), it is also important to limit the use of effective chemicals on target organisms that may develop resistance. It is largely for this reason that nearly 4000 tonnes of DDT were used globally in 2007 (van den Berg, 2009) – so we could reserve pyrethroids for IRS and LLINs.

Creating barriers

The use of traditional and locally available fumigant repellents, such as wood smoke, has been suggested as an inexpensive and potentially effective additional control method (Moore *et al*., 2007). However, only certain plants when burnt act as repellents: Palsson and Jaenson (1999) and Seyoum *et al*.

(2002) report smoke from a number of plants which do act to reduce *An. gambiae* biting and landings. Snow *et al*. (1998) indicate the use of smoke, while effective as a repellent, may not necessarily result in a reduction in malaria. It is not clear if smoke from domestic fuel has a repellent effect (Biran *et al.,* 2007).

Smoke residues and soot do not directly affect the effectiveness of insecticides on LLINs (Kayedi *et al.,* 2007), but may cause users to perceive them as dirty and wash them more, and so (in older net designs) potentially reducing the effective life of the insecticide (Miller *et al.,* 1999). As long as the net remains intact it will still offer some protection as a physical barrier to biting.

Physical barriers such as screens are effective to, but like LLINs, rely on user compliance to be useful, which in 2003 was surprisingly low (Alaii *et al.,* 2003). Since then WHO has recommend LLINs are distributed and used by all people in malarious areas (WHO, 2007) and a rapid increase in LLIN uptake in the poorest nations in Africa has been seen (Flaxman *et al.,* 2010). Compliance is not total, but most are aware of the benefits of LLIN use, as shown by a higher rate of compliance during the high malaria risk rainy seasons (Atieli *et al.,* 2011). Those with more than one net tend to use nets more, especially in the case of pregnant women (Sangaré *et al*., 2012).

Prophylaxis

Chemical prophylaxis of malaria has been suggested as a tool which, in addition to insecticidal control, in eradicating malaria from an endemic area (Roux *et al*., 1983). Anti-malarial drugs may be useful in both preventing and controlling infections, but are limited in their usefulness as it is difficult to sustain treatments

over prolonged periods and can affect or even facilitate drug resistance. Chemoprophylaxis for children can be cheap, as little as $3 per child, but administering the drugs with high coverage can stretch medical resources beyond their abilities (Goodman *et al*., 1999). It is therefore most effective (both in treatment and cost) when used to treat children and pregnant women (Greenwood, 2009). Mass chemoprophylaxis has a role in controlling malaria but cannot eliminate it on its own.

Environmental control

Larval source management (LSM) is the targeted management of mosquito breeding sites, and aims to reduce the transmission of diseases by reducing the number of larvae and pupae. One of the oldest known examples of LSM is the early Romans, who drained wetlands and swamps to prevent malaria, although they thought the fumes were the cause of the disease (Russell, 1955). In the southern US states, swamps were drained well into the 20th Century to prevent arthropod-borne diseases (Adler & Willis, 2003), contributing to the growth of population and life expectancy in these areas (Population Resource Centre, 2003).

The effectiveness of draining wetlands shows that effective control of oviposition disrupts the mosquito life cycle, effectively controlling diseases such as malaria. However, while such projects are expensive and damaging to the environment affecting many more species than the target vector, the benefits in some situations make LSM an effective vector control strategy (Majambere *et al.,* 2008; Fillinger & Lindsay, 2011).

With the effective use of LLINs and IRS having a successful impact on malaria in Africa, LSM is now seeing a resurgence and is encouraged by WHO as an important part of IVM (WHO, 2010; Smith *et al.,* 2013). Malaria 'hotspots' may benefit from LSM, and it may be effective for controlling other vector-borne diseases. LSM may also help manage growing insecticide resistance (WHO, 2012), especially important as resistance has now been observed in all four classes of insecticide recommended by WHO for IRS.

However, what is most abundantly clear is that there is not one single answer to reducing malaria. Direct vector control, parasite control, environmental management and other useful tools are all part of the solution and it is likely that the eradication efforts across Africa and other malaria ridden areas will be successful if they employ large, concerted and integrated control programmes.

Targeting of oviposition sites

The question of oviposition site selection may be an important factor in the future of malaria control. We currently do not know where gravid *An. gambiae* will oviposit and are therefore unable to exploit natural oviposition sites selectively as part of control measures.

In the case of *Cx. quinquefasciatus,* it has been shown that trays treated with an attractant semiochemical (in this case the egg released aggregation pheromone) divert females from similar unbaited trays (Otieno *et al*., 1988). In cages given the same two choices, *Cx. quinquefasciatus* will visit both bowls, but those that visited both tended to oviposit in the treatment bowl (Pile *et al*., 1991; 1993). This olfaction mediated behaviour has also been shown to be an

effective way to deliver insecticidal treatments using insect growth regulators (Otieno *et al*., 1988, Dawson *et al.,* 1989).

The example of *Cx. quinquefasciatus* may not by completely applicable to *An. gambiae*, given no similar pheromone has been demonstrated. Insecticidal treatments are useful, but do not trap or kill the adults, and therefore they may very well oviposit again elsewhere, out of the range of the attractant. CDC larval and 'box' traps have been shown to be useful for monitoring *Culex* (Irish *et al*., 2013), and floating sticky traps and electric nets (Harris *et al*., 2011; Dugassa *et al.,* 2012) have been shown to be effective gravid traps for *An. gambiae* (Dugassa *et al.,* 2013), but are reliant on visual cues and the attractiveness of a water source. The addition of an effective attractant for gravid females of *An. gambiae* would improve these traps considerably and add the capability to monitor as well as trap and kill adults and larvae. Such a trap would likely be an important tool for use in malaria control.

Building on the results of current research projects of Lindh and Fillinger in Kenya, which aim to understand oviposition site selection in *An. gambiae* malaria vector species, specifically the S molecular and chromosomal forms from Kenya (J Lindh and U. Fillinger, personal communications 2010-2014) as a starting point, the experiments described in this thesis examine the oviposition of this insect. Having used mosquitoes from the same mosquito colony as Lindh and Fillinger it is hoped that this body of work will complement their findings and expand our understanding of *An. gambiae* oviposition breeding site selection.

1.5 Aims and objectives

1.5.1 Overview

The burden of malaria in sub-Saharan Africa (as of 2013) is striking (WHO, 2009; 2013a), but great efforts are being made to eradicate the disease. However, despite an estimated reduction in malaria of 49% in Africa between 2000 and 2012, the incidence of resistance to control measures by both parasite and vector remains a large concern (WHO, 2013a).

The vectorial capacity of *An. gambiae* and other mosquitoes is due, in a large part, to their rapid and high levels of reproduction. It appears that a female *An. gambiae* will not oviposit into every available water source, or even into every suitable one, but identifying which sites a mosquito will deposit her eggs into has proven difficult as habitats where larvae are found seem to have few distinguishing features that mark them out easily from un-colonised sites (Majambere *et al*., 2008).

Our lack of knowledge hampers the identification of these sites, which in turn prevents the use of larval sites as a means of targeted *An. gambiae* control. Currently identifying oviposition sites is retrospective and involves a great deal of time, effort and cost (Dongus *et al*., 2007). A preferable scenario would be identifying, and targeting with control measures, a site which is likely to become an oviposition site (Gu *et al*., 2007).

It is, as yet, unknown if sites are actually selected for (due to positive attributes) or against (rejection due to repellent or unattractive factors). If the female mosquito does select sites, what are the cues? Are they visual, chemical or a

combination? Are the factors detectable from some distance or do they require the insect to make contact with the water's surface?

It is also not clear why such site selection would be advantageous. It may be that female mosquitoes lay their eggs more-or-less randomly, and the offspring only thrive in particular water bodies. Vegetation or the presence of bacteria or nutrients may play a role (Bentley & Day, 1989). The egg and the early larval instars are vulnerable life stages for mosquitoes, and their survival into adults can depend on the selection of a suitable location to deposit them. Indeed sites that contain only early larval stages may differ to those where older larvae or pupae are found (Fillinger *et al*., 2009).

As shown previously, there are currently unknown factors that determine mosquito oviposition site selection which have made this a subject one of interest to medical-entomological research for the potential role it may play in the control of *An. gambiae*. A wide and integrated approach was required before the successful monitoring and control of tsetse flies (Diptera: Glossinidae) could be achieved (Torr, 1994), but in a largely piecemeal fashion and focusing on host related olfaction (Gibson & Torr, 1999). As with tsetse, mosquito behaviour study has focused on host-finding, with little thought of the behaviour of the mosquito other than how it finds a meal. It is logical, given that this is the transmission route of malaria that research has focused on this, but by the use of multi-disciplinary methods, this project therefore aims to explore the oviposition behaviour of the mosquito, with the hope that quantification of behavioural responses to oviposition site cues will allow the mosquito to be controlled before it can become an irritation as a biting nuisance, let alone a disease vector.

By understanding what drives the female to lay her eggs where she does it might be possible to modify our surroundings or treat oviposition sites selectively (therefore reducing the cost of treatments) to prevent vector competent mosquitoes from interacting with humans. By means of controlling the vector, the disease can be prevented, improving the lives of millions of people.

1.5.2 Project aims

The overall aim of this project is to increase our understanding of *An. gambiae* oviposition, in order to better understand how the behaviour of the mosquito can be exploited to reduce mosquito populations.

The original specific aim of this project was to identify volatile cues of bacterial origin which could be used to attract gravid female *An. gambiae*, for use either in sampling or lure-and-kill traps. The bacteria from which the volatiles were obtained were previously reported to be oviposition attractants to this species by Lindh *et al*. (2008a).

The initial objectives of this PhD study were to repeat the studies as closely as possible of Lindh *et al*. (2008a) that demonstrated a range of bacteria species to be attractive to gravid *An. gambiae* with the aim of gaining experience in bio-assay methods used to test the effect of volatile semiochemicals on mosquito behaviour. Laboratory colonies of the seven bacteria identified in Lindh *et al*. (2008a) to be most attractive to gravid females of *An. gambiae* were obtained from J. Lindh.

Having repeated Lindh's study, and presumably confirming her results on the relative attractiveness of each bacteria species, the original plan for the first phase of the project was to run three research components largely in parallel to

identify which volatiles are most commonly produced by the most attractive bacteria and which of those volatiles are most attractive. The three research components were; a) produce a chemical profile of the volatiles in the headspace of each of the seven bacteria species by GC/MS, b) test the sensitivity of mosquito antennae to candidate attractant volatile chemicals using electro-antennography and c) determine the attraction of gravid mosquitoes to water containing live bacteria of each species to determine which bacteria produce the most attractive volatiles using a choice-test cage bio-assay. Finally, specific candidate volatiles were to be bio-assayed if they appeared to be likely attractants based on the EAG results and if they were also found in the volatile profiles of the most attractive bacteria. The intention was that this would lead to the identification of oviposition attractants that would be worth testing in semi-field/field conditions in Kenya, where natural populations of *An. gambiae* occur.

These components of the project were undertaken in collaboration with the OviART (Oviposition Attractants Residual Larvicides and Traps) Project, led by Prof. Steve Lindsay (LSHTM) and Dr Ulrike Fillinger of ICIPE (International Centre of Insect Physiology and Ecology), Kenya, with advice from Prof. S. Torr of NRI. OviART aimed to develop more efficient and cost-effective control and monitoring methods for *An. gambiae* by exploiting the mosquito's oviposition behaviour, focusing on the use of oviposition semiochemicals affecting the oviposition site choice of gravid females of *An. gambiae.*

This PhD project aimed to assist OviART by confirming and following-up the outcome of the Lindh *et al*. (2008a) behavioural assays, and to contribute electrophysiological studies of the responsiveness of gravid females of *An.*

gambiae to potential oviposition semiochemicals, adding value to OviART, which did not have the capacity to undertake electrophysiological studies.

However, this approach failed to deliver useful results, as the chemical profiles and cage bioassay results for the seven bacteria did not match the results reported in Lindh *et al*. (2008a). This prompted the OviART team to repeat their studies, but were also unable to replicate the original Lindh findings, for reasons nobody has been able to satisfactorily ascertain.

This prompted the author to widen the nature of the investigation of *An. gambiae* oviposition and question whether or not traditional cage bioassays are actually a suitable tool for investigating the responsiveness of gravid mosquitoes to candidate oviposition semiochemicals. Cage bioassays, as used in studies such as Lindh *et al*. (2008a), potentially involve a number of factors that can vary wildly between laboratories, and may or may not reflect conditions found in nature. The conditions of air movement, for example, affect the distribution of volatiles and therefore the strategies mosquitoes employ to locate their sources. Air flow will be affected by the level of enclosure of a cage. These, and other, variable factors may lead to the high levels of variability in the results obtained by different researchers.

It was, therefore, reasoned that a lack of understanding existed regarding how females locate oviposition sites coupled with the possibility that cage assays do not deliver oviposition stimuli as in natural environments, leading to results that do not reflect what would happen under natural conditions.

The availability of a large wind tunnel at NRI with video apparatus designed to track mosquitoes in flight arose coincidentally at this time and this provided an

opportunity to record the actual behaviour of *An. gambiae* during oviposition in great detail, thereby providing quantitative data about mosquito flight in the vicinity of oviposition sites rather than data inferring oviposition behaviour in retrospect, such as through egg counts.

The project aims were, therefore, reassessed, and it was decided that in order to best contribute original research on mosquito behaviour, the overall project aim should be to characterise their flight behaviour under semi-natural conditions, and in greater detail than previously published. It was decided that an investigation of the flight behaviour of gravid females presented with a choice of oviposition sites would be best conducted, in the first instance, with a test chemical known to have a consistent effect on oviposition behaviour, albeit a repellent effect in this case. The compound chosen, 4-methylphenol, has been shown to be readily detected by female *An. gambiae* (Blackwell *et al*., 1993; Blackwell & Johnson, 2000; Costantini *et al*., 2001), and to play a role in the oviposition of other mosquito species (Bentley *et al*., 1979; Linley, 1989; Kweka *et al*., 2001).

The innovative approach to observing mosquitoes has produced the first detailed characterisation of the oviposition behaviour of gravid *An. gambiae*. Furthermore, the observations have shed light on specific aspects of how *An. gambiae* discriminates between oviposition sites, either by contacting the water and detecting the compound through touch, or due to the presence of the volatile in the air.

Had a biologically active attractant(s) been identified, the study would have also compared the responses of the mosquitoes to attractants and repellents, but

unfortunately, as neither this project nor OviART found an attractant prior to the end of this PhD research project.

1.5.3 Objectives

Initial objectives

In order to address the initial project aims of identifying volatile cues for *An. gambiae* oviposition, leading to development of an artificial lure, the following objectives were identified:

1. Examine the volatile profiles of seven bacteria solutions previously identified in Lindh *et al.* (2008a) to be oviposition attractants, in order to:
    a. Identify the compounds released;
    b. Compare the volatile profiles and identify trends suggesting likely oviposition attractants;
    c. Identify compounds for further examination as oviposition attractants.
2. Identify electrophysiologically active compounds found in the bacteria volatile emissions using gas-chromatography linked electroantennography:
    a. By examining the electrophysiological responses of females of *An. gambiae* to volatiles identified in Objective 1;
    b. Electrophysiological responses of females of *An. gambiae* to potential volatile oviposition attractants were measured. The compounds tested were derived from published research and suggested by OviART colleagues.
3. Identify behaviourally active compounds with cage bioassays to:

a. Determine if the observed response reported by Lindh *et al.* (2008a) can be replicated, in order to confirm which bacterial solution/s are most attractive to gravid *An. gambiae;*

b. Characterise the oviposition response of gravid females of *An. gambiae* to volatile compounds identified in Objective 1 (of bacterial origin) and Objective 2 (electrophysiologically activity)

4. From objectives 1-3, determine the most likely oviposition attractants for further investigation in semi field trials.

Extended objectives

In order to examine and obtain more detailed information about the oviposition of *An. gambiae* the following objectives were identified:

5. Examine the details of oviposition in *An. gambiae* through direct observations of their flight behaviour in a flight arena to:

   a. Characterise the flight behaviour of ovipositing *An. gambiae* with the aid of low light video recordings in a large flight arena;

   b. Characterise the behavioural differences in responses of gravid females to single (no choice) oviposition sites and two (choice) oviposition sites;

   c. Characterise the effects of potential behaviour-modifying compounds on the oviposition behaviour of gravid female *An. gambiae* s.s.

6. Investigate the effect of presenting an identified repellent (4-methylphenol) in water, or in the air above the water.

7. Determine if the effects of active compounds on oviposition behaviour are due to the volatile compound in the air or dissolved in solution.

2 GENERAL METHODS

2.1 Background to common methods

The study of oviposition behaviour of *Anopheles gambiae* requires a wide range of biological disciplines, encompassing chemical ecology, reproductive biology and ethology. Accordingly, this study has employed analysis of bacterial volatiles, electrophysiology, cage bioassays, larger arena assays and direct observations of *An. gambiae* in flight in under natural low-light conditions using video equipment.

However, none of these could be undertaken without first having a colony of insects and uncontaminated colonies of bacteria. The methodologies used to maintain these colonies throughout this project are described here. This chapter does not address any aims, but serves to outline the range of methodologies common to the following experimental chapters.

2.2 Rearing of mosquitoes

Anopheles gambiae s.s. S-molecular form were reared at the Natural Resources Institute, University of Greenwich, Kent, UK. The colony was established in 2010 with eggs from colonies held at ICIPI (formerly International Centre of Insect Physiology and Ecology), Kenya, which were established from wild individuals in 2009 caught in and near Mbita Point, Kenya. In 2012 the colony was confirmed by Karine Moulin of IRD (Institut de Recherche pour le Dévelopment, Burkina Faso) to be uncontaminated by other strains and purely S-form by polymerase chain reaction (method in Favia *et al*., 2001).

Mosquitoes were reared as per Hawkes *et al.* (2012), following consultation with J Lindh (personal communication). Specifically, the mosquitoes were reared in a controlled environment at a constant 26 ± 2°C, 60 ± 10% relative humidity and a 12:12 h light/dark cycle with no dimming between. These environmental conditions are the same as those of Lindh *et al*. (2008a), which in turn are similar to conditions at the ICIPE field station in Mbita Point, Nyanza, Kenya, where ICIPE rears and conducts experiments upon *An. gambiae,* including oviposition assays. ICIPE rears *An. gambiae* at 27±2°C and a relative humidity of 61±7% with a 12:12 h light/dark cycle (Wang *et al.,* 2013).

In order to control for potential microbial pathogens, the water the larvae were reared in and used to prepare sugar-feeders was saline, consisting of deionised water plus 0.9% by weight aquarium salts (Tropic Marin Sea Salts, Dr. Biener GmbH/Tropic Marin, Wartenberg, Germany). Saline, as opposed to pure water, was used because larvae reared in pure deionised water were found to have a high mortality rate, which the addition of aquarium salts was found to prevent (Malaria Research and Reference Reagent Resource Centre, 2014).

Pure water is also rarely encountered in natural environments, especially breeding sites and plant nectar, and can have an adverse effect on cellular osmotic pressure.

Eggs were laid onto wet filter paper and transferred the morning after oviposition to rearing trays containing saline (0.9%) for hatching. First instars were fed baby rice (Organix Organic Wholegrain Baby Rice; Organix Brands Ltd, UK), then ground fish flakes (TetraMin; Tetra GMBH, Germany) as needed.

Pupae were removed as observed and placed into 10 cm dia. dishes of water inside a wire framed cage measuring 30 cm on all sides, covered with medical tube gauze (Tubegauz; Mölnlycka Healthcare, Sweden). The cages were loosely sealed in large, transparent plastic bags to maintain humidity. Adults had access to a 10% sucrose solution wick feeder *ad libitum*. Between 3 and 5 days post eclosion adults were offered a blood feed on a human volunteer's arm approximately 2 h into their dark phase. Feeding was performed by adult volunteers according the guidelines of the University Ethics Committee. Experiments took place two days post blood feeding. Females were selected as close as possible to the beginning of the dark scotophase and were typically 46 h post blood feed. Feeds were planned so that experiments began as close to 48 h post blood feed as was possible.

The decision to use females at approximately 48 h post blood feeding (rather than at 72 h such as in Lindh *et al*. (2008a)) was based on several factors. Firstly, observations through rearing suggest that gravid females of *An. gambiae* will lay eggs from 48- 72 h post-bloodfeeding (WHO, 1975). Although delaying oviposition may ensure larger egg yields, preventing females from laying when they are ready could affect their natural behaviour, and might even stimulate a female that is ready to oviposit at 48 h to resorb her eggs if she could not find a suitable oviposition site for the following 24 h. .It was also felt that this may introduce an additional bias, prompting mosquitoes to oviposit on the sugar feeder or elsewhere in the cage due to a greater biological need to deposit the clutch of eggs.

2.3 Technique for visual selection of gravid females

Experiments required gravid females, which were selected by visual examination of their abdomens The appearance of the abdomen alters significantly from unfed to fed females (Figure 2.1 and Plate 2.1), but can also be used to indicate the state of ovary development, allowing identification of gravid females (WHO, 1975).

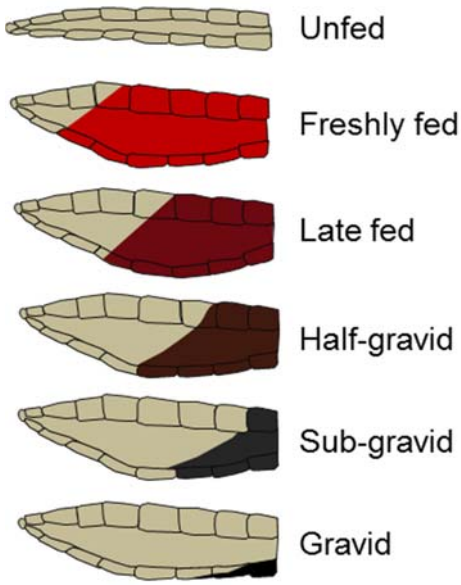


Figure 2.1 Appearance of the abdomen of females at different stages of feeding and ovary development. Redrawn by author from WHO (1975). Progression from freshly fed to gravid is approximately 48 h.

Plate 2.1 Comparison of mated and unmated females at 46h post blood feed. The unmated female (left) resembles an unfed female as her ovaries have not swollen. The mated female (right) shows a swollen abdomen, due to ovary development, and can be seen to be sub-gravid (photographs by author).

The abdomen of females from unfed to gravid are as follows:

'Unfed': females show collapsed abdomens, with the stomach empty and the ovaries taking up no more than one third of the abdomen.

'Freshly fed': the abdomen is greatly swollen and red with blood. Only the final 2-3 abdominal segments are not red, occupied instead by the ovaries.

'Late fed': the meal has darkened and as digestion occurs the stomach shrinks. The ovaries swell to fill the space left, occupying 2½ ventral and 4 dorsal segments.

'Half-gravid': the meal is further shrunken and the ovaries occupy 4-5 ventral and 6 dorsal segments.

'Sub-gravid': the blood meal is greatly reduced and very dark, with the ovaries taking up all but the three dorsal and the first ventral segments.

'Gravid': the blood meal is nearly, if not fully digested. The dark colouration is reduced to a narrow, black line on the still swollen abdomen. The ovaries take up most of the abdomen. The female is now ready to oviposit.

Females selected for experimentation were visually judged to be either gravid, or very late sub-gravid. Unmated females are able to take blood meals. However the ovaries of unmated females do not swell, which means it is possible to clearly distinguish unmated from mated females that have both blood fed at between 44-48 h post blood feeding (the extreme ranges of when females were selected).

2.4 Bacteria storage and culturing

Seven bacteria taxa were maintained, labelled L2, L4, L6, L9, L11, E2.5 and Ps, as per in Lindh *et al*. (2008a), the origin of which are shown in Table 2.1. The bacteria were stored in a glycerol stock solution at -80 °C until required. The bacteria were grown overnight in 50 ml of liquid broth (LB Media, Fisher Scientific, UK) at 37 °C and shaken at 200 rpm. After approximately 12 h the OD_{600} was determined by a visible light spectrometer in order to estimate the bacterial concentration, with a reading of approximately 0.6 indicating the bacteria were in the log growth phase. During log phase cells are most healthy and an adequate OD_{600} level indicates a high level of biomass, and therefore a sufficiently high number of bacteria (Collins *et al.*, 2004).

Table 2.1 Bacteria strains used and their isolation source. Table adapted from Lindh *et al.* (2008a).

Bacteria label	Isolation source	Phylogenic affiliation	Gen bank ascension number
L2	*An. gambiae s.l.* oviposition site (Sumba *et al.*, 2004a)	*Proteus* sp.	EF426446
L4		*Micrococcus* sp.	EF426448
L6		*Bacillus* sp.	EF426450
L9		*Exiguobacterium* sp.	EF426452
L11		*Comamonas* sp.	EF426453

E2.5	*An. gambiae s.l.* midgut (Lindh *et al.*, 2005)	*Vibrio metschnikovii*	AY837747
Ps	*An. gambiae s.l*. midgut (Lindh *et al.*, 2008b)	*Pantoea stewartii*	EF189919

Broth was then centrifuged at 3000 rpm for 6 min and the resulting pellet was suspended in 50 ml of deionised water. The water contained 0.9% b/w NaCl (Fisher Scientific, UK) in order to prevent cell lysis by creating an isotonic solution (i.e. one with equal osmotic pressure inside and outside of the bacteria cells).

The re-suspended solution was then transferred to a 100 ml conical flask sterilised by autoclave and covered with sterile aluminium foil.

2.5 Determination of bacterial concentrations

The levels of bacteria in a sample were recorded in bacterial units/litre, i.e. the number of individual bacterium in a litre of solution, and calculated *post hoc*. Bacterial broth solutions were prepared and grown overnight as per Section 2.4. A 0.1 ml aliquot was taken prior to centrifugation and serial dilutions were made from $1x10^{-5}$ parts per litre to $1x10^{-9}$ parts per litre using 0.9% saline.

The dilutions were each plated onto LB agar three times and incubated for 24 h at 37 °C. The dishes were inspected for growth and the lowest dilution where growth was seen was selected and the number of colonies counted to give the number of colony forming units (i.e. the initial number of bacteria on the plate when inoculated that each went on to form an individual colony). If none were

seen on any dish, the three dishes were re-incubated for a further 24 h and inspected again. If no colonies were seen it was then assumed the plates had not been successfully inoculated. Plates were then disposed of.

The number of colony forming units recorded was then multiplied by the dilution factor to give the number of bacterial units/litre equivalent.

2.6 Statistical analyses

The statistical programme R was used to conduct statistical analyses unless stated. R is a language and integrated suite for statistical computing and graphics which can be supplemented and extended through 'packages' for additional functions. Version used was R 2.15.0 (R Development Core Team, 2012), and packages are indicated where they were used.

Power calculations

Power calculations used the R package 'pwr' (Champley, 2012). This allows prediction of the effect size - the magnitude of the differences between groups - in terms of control standard deviation - which can be detected with the chosen values for significance level, sample size and power. The power of a test is defined as one minus the probability of a type II error (Cohen, 1992; Sullivan, 2012).

2.6.1 Statistical analysis of results

The statistical methodologies used were dependant on the categories or types of data that were being analysed; several methodologies were used, as shown in Table 2.2.

Table 2.2 Statistical tests used in data analysis showing the data distribution, the test used and the null hypothesis (H_0) for each test.

Distribution		Test		H_0
Parametric	T-test	Independent		No difference in means
	F-Test	Regression		No effect of changing X
		ANOVA	2-way ANOVA	Main effects or interactions are not significant
			Tukey	No difference between groups
		ANCOVA		There is no relationship with the x variate
Non-parametric	χ^2 Test	Contingency table		There is no relationship between X and Y. $X^2 = 0$
	Generalised linear models	Analysis of deviance		As for anova and ancova

Parametric tests

Parametric tests are used when the data (strictly, the residuals) - fit a normal probability distribution. A **t-test** is used to test if the means differ between two samples.

Linear Regression is used to determine the strength of the relationship between the dependant and an independent variable, either using a linear regression, (the equation for which is: $Y = a + bX + u$) or multiple regression (i.e. $Y = a_1 + b_1 + a_2 + b_2 + \cdots + a_t + b_t + u$) where Y = the dependant variable, X = the independent variable, a = the intercept, b= the slope and u = the residual.

Linear regression analysis was used to fit the data points for the behaviours analysed in arena experiments (throughout Chapter 6 and 7) (Clegg 1982).

Analysis of variance, or **ANOVA**, allows the statistical significance of main effect factors and their interactions on a dependant variable to be tested using an F test. F is the ratio of the variance due to a factor, or the interaction between two or more factors, divided by the residual variance.

ANOVA is used in Chapter 5.3.1 to test oviposition between targets in cage tests, and in Chapters 6.3.8 (comparison of behaviour towards a single or two oviposition dishes) and 7.3.2 (behaviour towards dishes with repellent-containing sachets adjacent).

Tukey's multiple comparison test compares all possible pairs of means for levels of factors in an analysis of variance. Significance levels are corrected to allow for multiple tests on the same data.

Tukey's test was used to determine the effects of oviposition towards the volatiles emitted by bacteria in solutions (Chapter 5.3.1), while the numbers of eggs laid were analysed by ANOVA. Tukey's test was also used to compare the means in the two dish oviposition experiments described in Chapter 7.

Analysis of co-variance, **ANCOVA**, allows models which combine categorical factors with an independent variable to fit several different regressions to the independent variable. F tests on the resulting ANOVA table distinguish parallel line models from those with different slopes for the different categories (Zuur *et al.,* 2009).

ANCOVA is used to determine the level of difference in behaviours between scenarios in Chapters 6.2.11 and 6.3.8.

Non-parametric tests

Non-parametric tests are not based on probability distributions. A **Chi-squared**, or χ^2 is used to test the relationship between two variables by assessing the goodness of fit between the observed values and the expected values (i.e. is the difference significant or due to sampling variation).

A Chi-squared test analyses categorical data, which is fitted into a contingency table. Contingency tables are a grid of at least 2x2 into which the data is sorted according to the categories to be analysed. Statistically significance results from an interaction between the two factors indexing the rows and columns of the contingency table.

In this study Chi-squared tests were used to in the determination of behaviour differences between each behaviour set in Chapters 5, 6 and 7.

Generalised linear models

Generalised linear models (GLM) allow analysis of data sets with response variables that have other than normal distributions, typically count data, or proportion responding. (Crawley, 2012). The model is equivalent to an ANOVA or ANCOVA, and significance testing is by an analysis of deviance.

The GLM consists of three elements: 1. A probability distribution, 2. A linear predictor, i.e. a linear function of regressors, and 3. A link function, which transforms the expectation response variable to the linear predictor.

The linear predictor is used to incorporate the independent variables into the model. The link function provides the relationship between the linear predictor and the mean of the distribution function. The error distribution determines the link function, which are shown in Table 2.3.

Table 2.2 Error distributions and links for GLMs

Error distribution	Test	Link	Example
Binomial	χ^2 Test	logit	Proportion of insects responding to a stimulus
Poisson	χ^2 Test	log	Count of occurrences in fixed amount of time/space
Quasi-binomial	F-Test	logit	As for Poisson, allowing for over-dispersion.

GLMs are fitted to data by the method of maximum likelihood, rather than least squares (as for ANOVA). This is an iterative process.

GLMs with binomial errors were used in the determination of the effect of dose on behaviours in Chapters 6.2.10 and 6.3.8.

Determination of optimum number of mosquitoes for experiments used a GLM with poison error for relationship of eggs and female visits (Chapter 6.3.5).

A GLM with quasi binomial errors was used to determine the effect of 4-methylphenol on gravid female mosquitoes in 2 dish experiments in Chapter 5.3.2, 6.2.11 and 7.2.3.

3 ANALYSIS OF THE VOLATILES PRODUCED BY BACTERIA INVOLVED IN MOSQUITO OVIPOSITION SITE ATTRACTION

This chapter addresses Objective 1, as described in Chapter 1.5.3. The volatiles produced by seven strains of bacteria were reported to attract gravid *An. gambiae* mosquitoes to oviposit on water containing the bacteria (Lindh *et al., 2*008a). This chapter describes the analysis of the headspace volatiles of the bacteria in solutions in order to identify potential oviposition semiochemicals.

3.1 Background

Anopheles gambiae is a dipteran insect of the Nematocera suborder. It is an obligate haemophage, requiring a blood meal, specifically from humans, in order for eggs to develop (Gillies, 1955; Takken, 1991; Clements, 1999). Gravid females oviposit into a water body, which is the habitat of all the immature life stages of a mosquito (4 larval instars and pupa) larval habitat. These aquatic breeding sites are most often described as small bodies of warm, sunlit and clean (low levels of organic matter) water (Gillies & De Meillon, 1968; Clements, 1999).

The mechanism of oviposition site selection in mosquitoes generally has not been fully described, and *An. gambiae* breeding sites vary to such an extent that they defy simple characterisation (Fillinger *et al*., 2009), making it even more difficult to identify the environmental cues used by gravid females to locate them.

It has been shown that *An. gambiae* oviposits into a wide variety of water bodies, including small temporary puddles, relatively permanent rain-water butts, irrigated rice fields, lakesides and, in certain areas even urban drainage ditches (Majambere *et al*., 2008), with the exception of very heavily polluted, malodourous water (Awola *et al*., 2007).

Oviposition cues and attractants

Larval presence is used as an indicator of oviposition, and as per Darwinian fitness, it can be argued that gravid females will seek the sites most likely to promote good larval development. As larvae are found in such a wide variety of water bodies it is thought that the steps leading to oviposition are mediated by a number of environmental cues (Muirhead-Thompson, 1945).

Water has been shown to be an attractant to ovipositing mosquitoes (Kennedy, 1942; Bentley & Day, 1989); gravid females are more attracted to areas of increased humidity than their unfed counterparts (Okal *et al*., 2013), suggesting humidity could be a pre-oviposition attractant that females use to locate potential oviposition sites. Water vapour is most likely a long range cue (Kennedy, 1942; Bernáth *et al*., 2012).

Long range cues attract females to the general area of potential oviposition sites. Short range cues are probably more complex volatile chemical compounds (Bentley & Day, 1989) with semiochemicals (substances that convey a message from one organism to another) attracting or repelling a gravid mosquito to or away from, respectively, water bodies over short distances (Rejmankova *et al*., 2005). *Anopheles gambiae*, particularly S form, oviposition sites are thought to be mainly temporary (Fillinger *et al*., 2004), in the sense that

they are formed by rainwater collecting in depressions, and dry out before they are colonised with many organisms that could be potential predators or parasites (Gimonneau *et al.*, 2010), which would also have major implications for the range of semiochemical cues that would emanate from such water bodies.

Bacteria as a source of oviposition attractants

Aquatic organisms that produce semiochemicals are thought to provide a 'signal' (see Chapter 1.3.2, Cues for oviposition) indicating to ovipositing mosquitoes that the water is suitable for rearing the immature stages of the life cycle. These organisms are likely to be of microbial origin since microbes colonise and reproduce more quickly than higher organisms in temporary pools (Bentley & Day, 1989). This theory is supported by the results of Sumba *et al*. (2004a) who found that sterilised water from *An. gambiae* oviposition sites attracted lower levels of oviposition than untreated water from these sites. Water from natural breeding sites was found to contain a number of bacteria species, some of which were later discovered to elicit a positive oviposition response in *An. gambiae* (Lindh *et al*., 2008a). Additionally, water with bacteria derived from the mid-guts of mosquitoes was found to attract gravid female *An. gambiae* (Lindh *et al*., 2008b).

Bacteria in larval sites can be ingested during larval development (Merrit *et al.,* 1992), although it has been shown that adult *An. gambiae* can also ingest bacteria that will colonise their gut (Lindh *et al*., 2006). Bacteria ingested as larvae may persist in the gut through pupation and remain in the adult gut (Lindh *et al*., 2005; Briones *et al*., 2008), although the exact mechanism of how this

happens is not fully understood. Evidence from other arthropod orders suggests that holometabolous metamorphosis does not remove everything from larval guts, as shown by the moth *Utetheisa ornatrix* which sequesters toxins from its larval diet and retains them as adults (del Campo *et al*., 2005).

During metamorphosis the alimentary canal of culicids is restructured and the midgut is totally replaced (Clements, 1999; 2012). These tissues are destroyed through targeted apoptosis of cells, and new adult tissues develop in situ, with regenerative cells forming the adult gut (Richins, 1945; Lockshin & Williams, 1965). Not all tissues are destroyed as the musculature around the gut remains through to the adult stage (O'Brien, 1966a; 1966b). The waste formed during pupation in the gut is discharged as meconium upon adult emergence. The process of metamorphosis and emergence is thought by many to sterilize the mosquito gut and alimentary canal of emerging adults which is thus free of organisms, or contains very few (Moll *et al*., 2001; Clements, 2012).

The results of Lindh *et al*. (2005), however, showed that a few bacteria (16 species in 15 genera) persist in the gut through metamorphosis, and Briones *et al.* (2008) suggested that certain bacteria are more likely to survive metamorphosis than others. Clements (2012) concluded that Lindh *et al*. (2005) and Straif *et al.* (1998) showed that the acquisition of midgut bacteria is not extensive until a blood-meal is taken and is largely random with regards to which bacterial species are taken up, although *Anopheles stephensi* Liston have been shown to transmit gut bacteria directly to their offspring (Favia *et al*., 2007).

Thus, despite the massive changes to the mosquito gut that occur, certain bacteria may persist through metamorphosis (although most will not and thus

reinfection must occur in adults directly) (Lindh *et al*., 2006; Riehle *et al*., 2007). This suggests the bacteria in a breeding site may play roles other than or in addition to any effect they may have on oviposition site selection.

These bacteria may also play roles in the larval development of certain *Aedes* and *Anopheles* species; Rozeboom (1935) and Wooton *et al.* (1997) have shown that the use of antibiotics and totally sterile media inhibit larval development in species from both genera.

The great variety of bacteria present in oviposition sites suggests that they may play a number of roles. Some may be present in the water naturally, some may be introduced by ovipositing females and some may even be transmitted from the adult to the larva. The *An. gambiae* oviposition site may well be a complex environment, with many bacteria species, of which only some, if any, play a role in oviposition.

Oviposition mediating bacteria

The attractive bacteria from Sumba *et al.* (2004a) and Lindh *et al*. (2008a; 2008b) were identified using molecular diagnostics as belonging to a wide variety of genera, but all were shown to cause greater levels of oviposition by gravid females of *An. gambiae* in two-way choice tests (Lindh *et al*., 2008a), where a cage of gravid mosquitoes were offered two dishes to oviposit into, one containing treated water (i.e. containing one of the bacteria) and the other untreated water (the control).

Six bacteria, from a total of 18, tested in Lindh *et al*. (2008a), as well as one bacteria from a previous unpublished study (bacteria Ps) appeared to be

oviposition attractants. Five of these were identified from oviposition sites and two were found to originate in mosquito mid-guts. These are summarised in Table 3.1.

Table 3.1 Bacteria found to elicit positive oviposition responses. As reported in Lindh *et al*. (2008a)

Bacteria	Identity	Origin
L2	*Proteus* sp.	*An. gambiae* oviposition sites (field)
L4	*Micrococcus* sp.	
L6	*Bacillus* sp.	
L9	*Exiguobacterium* sp.	
L11	*Comamonas* sp.	
E2.5	*Vibrio metschnikovii*	*An. gambiae* mid-gut (field)
Ps	*Pantoea stewartii*	*Aedes aegypti* mid-gut (lab)

In order to identify candidate oviposition semiochemicals, Lindh *et al*. (2008a) examined the volatile profiles of 17 bacteria, including six of the bacteria (bacteria Ps was not analysed) found to mediate an oviposition response. The headspaces of aqueous suspensions of these bacteria were sampled using solid-phase micro-extraction (SPME) and analysed by gas chromatography linked mass spectroscopy (GC-MS). This identified 47 known and 3 additional unknown compounds.

Sampling and analytical techniques

SPME has been shown to be an effective technique to collect volatiles from plants (Robacker & Bartelt, 1997) and bacteria (Scholler *et al*., 1997; Elgaali *et al*., 2002) that are attractive to insects for analysis. The technique uses a polymer coated fibre to collect volatiles for analysis. The volatiles interact with the matrix of the fibre, which acts to hold the volatiles. This fibre is retracted into a protective needle using a syringe-like apparatus, which protects the fibre

and prevent further volatiles from being collected, allowing a carefully timed collection, which is important as the volatile profiles of can change over time. SPME is an equilibration sampling technique - it captures a 'snapshot' of the sample at the point in time at which sampling is made. Collected volatiles are thermally desorbed from the fibre using the heated injector of a chromatograph or similar analytical device. Typically, a gas-chromatograph (GC) is used.

Natural semiochemical odours are often composed of a blend of chemicals, of which only one or a few elicit an electrophysiological or behavioural response. Because it is impossible to tell which chemicals in a blend are eliciting the response in an EAG as described above, a more sophisticated method was developed to make it possible to determine which compound is associated with an EAG response, EAG linked to gas chromatography (Moorhouse *et al*., 1969; Cork *et al*., 1990).

A gas chromatograph, or GC, is an analytical tool that separates out the individual constituents of a blend to allow their identification and to estimate their relative abundances and even absolute amounts. Chromatography is the process of separating the constituents of a mixture by portioning them between a mobile and a stationary phase. In a GC the chemical mixture is the mobile phase and is injected into a moving carrier stream of inert gas.

In the injector the mixture is rapidly volatilised by heat and forced by the pressure of the carrier gas through a long (typically between 10 and 100 m), narrow (under 1mm internal diameter) wax-lined polymer tube; the "column", which acts as the stationary phase. The sample interacts with the lining of the column, essentially sticking to it. Different types of substances interact more or

less strongly with the column (depending on a number of factors including the rate of adsorption on the column, the length or size of the molecule, its polarity).

The column is located in the oven of the GC, which heats the column according to a pre-set temperature programme. As the column heats up the constituents of the injected mixture dissociate from the column, eluting into the carrier stream. The rate at which a sample travels through the column is directly proportional to the column temperature, so a sample will move more quickly through a hotter column but will interact less with the stationary phase, resulting in lower separation and reduced resolution. The temperature programme is set to compromise between resolution and time taken for a sample to be fully analysed.

The compounds are detected as they leave the column, typically by using a flame ionisation detector (FID), which operates by detecting ions from combusting material in a hydrogen flame. The FID produces a current proportional to the relative concentration of the material being combusted in real time, thereby indicating the quantity of the compound eluting off of the column at a known time.

Each compound travels through the column at a different rate and as individual components of a mixture are separated each component exits the column at a different time, known as the ‘retention time’. Under the same conditions (i.e. the same column, carrier gas flow rate and temperature programmes) the retention time will remain constant and unique to the compound; therefore, once a compound’s retention time has been assessed, the compound can be identified by consulting a table of known standards. A qualitative identification can also

be made if the order in which a series of compounds will elute is known. Mass spectroscopy (MS) returns spectra of the masses of the atoms or subunits of the molecule being examined. It does so by bombarding the molecule with electrons, which cause the molecule to ionize and fragment. The resultant ions are then separated by their mass to charge ratio (m/z), typically by measuring their deflection after passing through a magnetic field. The m/z is then compared to other known spectra, often using large databases, such as the information published by the National Institute of Standards and Technology.

The 47 identified compounds found by Lindh *et al*. (2008a) included a number of alcohols, carboxylic acids, esters, pyrazines and sulphides – a wide range of potential attractant semiochemicals that may be responsible for the positive oviposition response and which would therefore be *An. gambiae* oviposition attractants.

However, a principal component analysis of these results was not able to suggest any single volatile (or group of volatiles) that might be responsible for the observed oviposition responses. There were also volatiles that could not be identified from bacteria L2 and E2.5. Lindh *et al*. (2008a) were able to conclude only that 13 putative oviposition chemicals had been identified and suggested that 3-methyl-1-butanol (identified in 3 out of the 6 bacteria) might be a candidate oviposition semiochemical.

These results were disappointing since bacterial volatiles appeared to be what attract gravid females to oviposition dishes, but no volatile or mix of volatiles was associated with all of the attractive bacteria. The source of the attraction remained undetermined. It was decided, therefore, that in addition to

investigating the oviposition of *An. gambiae* it would be valuable to repeat the volatile analysis of the seven bacteria with equipment in the NRI laboratories to double-check the conclusions of Lindh *et al*. (2008a). The findings of this would also serve as a guide for future studies into the oviposition response of *An. gambiae* as presented in this thesis.

3.2 Materials and methodologies

Seven bacteria samples were provided for this study by J. Lindh, as shown in Table 3.1. These were labelled L2 (*Proteus* sp.); L4 (*Micrococcus* sp.); L6 (*Bacillus* sp.); L9 (*Exiguobacterium* sp.); E2.5 (*Vibrio metschnikovii*) and Ps (*Pantoea stewartii*).

The bacteria were taken from long-term storage samples and solutions were prepared as described in Chapter 2.3. The bacteria were grown in liquid LB broth overnight at 37°C in a shaker-incubator, then centrifuged to remove the bacteria from the broth. The pellet was then suspended in 0.9% b/w saline (NaCl). A sterilised metal loop was used to plate out onto LB-media agar to determine bacterial concentration (as per Chapter 2.3). The resulting solutions each contained a single species of bacteria suspended in 50ml of saline in a sterile conical flask. Each flask was covered with sterile aluminium foil (Plate 3.1).

Plate 3.1 SPME extraction of volatiles from bacterial solution headspace. An SPME fibre is inserted into a flask covered in foil containing the solution to be examined.

The headspace of each flask was sampled using SPME to collect the volatiles emanated from the bacteria in solution. A PDMS/DVB SPME fibre (Supelco, UK) was inserted into the headspace through the aluminium foil and was held by a clamp and stand for the duration of the extraction Samples were taken in the laboratory at normal room temperature of 26 °C.

Sampling times of 15, 30 and 60 minutes were employed in order to determine the optimal length of extraction required. As the fibre was exposed to the headspace of a L2 bacteria solution a countdown timer was started, set to the desired time. At the end of the sampling period the SPME fibre was retracted immediately and injected into the chromatograph as soon as it became available (usually within five-ten minutes).

In order to investigate any changes in volatile profiles of solutions over time, 15 minute samples were taken from a L2 bacteria solution at three intervals after it was prepared: immediately after sample preparation (0 hours), plus 6 and 24

hours after preparation. Solutions were kept at normal room temperature (26°C) between sampling periods.

After this was completed, samples of each species of bacteria were taken individually and analysed by GC-MS. These samples were extracted immediately after preparation and for 15 minutes.

GC-MS analysis was performed using a CP-3800 GC and Saturn 2200 MS (Varian, now Agilent, UK). Non-polar analysis used a Varian VF-5ms (internal diam. 0.25mm, film thickness 0.25μm, length 30m) capillary column (Agilent, UK) and polar analyses used a DB-Wax (internal diam. 0.32mm, film thickness 0.25μm, length 30m) capillary column (Supelco, UK). Helium carrier gas (flow rate 1ml/min) was used. Samples were desorbed from the SPME in the injector at 220°C, operating in splitless mode, with split opening 1 minute after desorption. The heating profile for both polar and non-polar was the same: hold at 40°C for 2 minutes after sample desorption, then increase 6°C per minute to 250°C. Hold at 250° for 5 minutes. The oven then cooled for the next sample. Total run time per sample was 42 minutes.

The MS was operated in electron impact mode, scanning from 30 – 400 m/z.

The results were analysed and the collected volatiles were identified using the National Institute of Standards and Technology (NIST) Atomic Spectra Database version 4.0.

3.3 Results

3.3.1 Volatile Analysis

GC-MS analysis of the volatiles collected from the headspaces of the seven bacteria species detected seven chemicals that were not detected in collections of a clean bottle containing only fresh growth media in deionised water.

The most consistent results were found for the following bacteria:

- L2 – both studies (Lindh *et al*., 2008a/this study) identified high levels of indole and small amounts of 2-n-undecanone and 2-n-tridecanone
- L4 – both studies found high levels of dimethyl disulphide and dimethyl trisulphide.
- L6 – both studies found dimethyl disulphide
- L9 – both studies found medium- high levels of 3-methyl-1-butanol
- L9 and L11 – both studies found low to high levels of dimethyl disulphide and dimethyl trisulphide

Several compounds were found to be present in three or more bacteria samples (bold indicates these chemicals were found in both studies in these bacteria):

- Aliphatic alcohols
    - 3-methyl-1-butanol (l2, L4, **L9**, L11 & Ps)
    - 1-pentanol (L2, L9 & Ps)
- Nitrogen compounds
    - Indole (E2.5, **L2**, L11 & Ps)
- Sulphides
    - Dimethyl disulphide (E2.5, L2, L6, **L4**, **L9** & **L11**)

- Dimethyl trisulphide (**L4**, **L6**, **L9** & **L11**)

In total 8 compounds were identified from the headspaces of the bacteria, 11 matches between the two studies found.

The concentrations of bacteria produced by the study presented here were calculated as per Chapter 2.5, and found to be within the ranges of concentrations used in Lindh *et al*. (2008a) (Table 3.2). The results of the volatile analysis are shown in Table 3.3.

Table 3.2 Concentrations of bacteria solutions sampled

Bacteria	Calculated concentration of sampled solution (bacterial units/litre)	Concentration range of Lindh *et al*. (2008a) (bacterial units/litre)
L2	$7.6x10^7$	$6.9x10^6$ to $3.2x10^8$
L4	$8.2x10^7$	$7.7x10^6$ to $1.8x10^7$
L6	$6.9x10^7$	$6.5x10^7$ to $1.0x10^8$
L9	$5.5x10^7$	$5.2x10^7$ to $5.3x10^7$
L11	$7.6x10^8$	$4.2x10^7$ to $8.1x10^7$
E2.5	$2.4x10^8$	$2.0x10^8$ to $4.0x10^8$
Ps	$8.6x10^7$	n/a (not tested)

Table 3.3 Comparison of volatiles detected from bacteria suspended in saline. Volatiles detected in this study labelled '**JB**' and in Lindh *et al*. (2008a) labelled '**JL2008a**' - part 1 of 2.

Compounds	Retention time (m)			Bacteria																No. of bacteria with chemical
	JL 2008a	JB		E2.5		L2		L4		L6		L9		L11		Ps				
		Polar	Non-polar	JL2008a	JB	JL2008a	JB	JL2008a	JB	JL2008a	JB	JL2008a	JB	JL2008a	JB	JL2008a	JB			
Aliphatic alcohols																				
3-methyl-1-butanol	*3.7*		4.04			T		++				+++	**		*		***			*5*
1-butanol			6.34								*									*1*
1-pentanol		3.32	7.64				****						***				***			*3*
1-nonanol	*15.5*			+++																*1*
1-decanol	*19*			+																*1*
2-methyl-3-decanol	*21.1*			++																*1*
Aromatic alcohols																				
Phenylmethanol	10.8													+						*1*
2-phenylethanol	*13.2*					+								++						*2*
Aldehydes																				
Benzylbutanol	*17*					T														*1*
Carboxylic acids																				
2-methylbutanoic acid	*6.5*											+								*1*
3-methylbutanoic acid	*6*		2.97									+								*1*
Ethers																				
1-methoxy-3-methylbutane	*3.1*					+														*1*
2-ketones																				
6-methyl-2-heptanone	*8.7*									T										*1*
2-nonanone	*13*									++										*1*
2-n-undecanone	*18.8*		15.8			T	*													*1*
2-n-dodecanone	*21.5*					T														*1*
2-n-tridecanone	*24.1*		19.8			+	*													*1*
2-pentadecanone	*26.8*					+														*1*

Table 3.3 continued – part 2 of 2.

Compounds	Retention times (m)			Bacteria														No. of bacteria with chemical
		JB		E2.5		L2		L4		L6		L9		L11		Ps		
	JL 2008a	Polar	Non-polar	JL2008a	JB	JL2008a	JB	JL2008a	JB	JL2008a	JB	JL2008a	JB	JL2008a	JB	JL2008a	JB	
5-decanone	15.4							++						+				2
Pyrazines																		
Diisopropylpyrazine	14.9									+								1
2,5-diisopropylpyrazine	15.7									+++								1
Isopropyl-secbutylpyrazine	18.1									++								1
Isopropyl-isobutylpyrazine	18.3									+								1
Nitrogen compounds																		
2-methyl-1-propaneamine	2.4					T												1
Indole	18	16.35	29.99		****	+++	***								***		**	4
Sulphides																		
Dimethyl disulphide	3.7	3.47	4.54		***		***	++	****		*****	+++	***	+++	*****			6
Dimethyl trisulphide	8.8		11.37					++	****	+	***	+++	*	+++	****			4
Methyl (methylthio)methyl disulphide	13.5													++				1
Number of chemicals identified/bacteria (both studies)				5		12		4		9		6		8		3		
Number of chemicals/bacteria that are the same in both JL & JB				0		3		2		1		3		2		-		

Retention times are shown for L2008a (Supelco SPB-1, non-polar column) and for JB on both polar (Agilent DB-Wax) and non-polar (Varian VF-5ms) columns where compounds were detected. In bacteria columns, the level of each volatile detected is shown. For JL2008a: T <1%, + 1-5%, ++ 5-25% & +++ >25% of total area in GC (Lindh *et al*., 2008a). In JB stars indicate the approximate relative levels of each volatile detected, with 1 star the lowest and 5 star the greatest abundance. Identification of volatiles in JB was by mass spectral data from NIST.

3.3.2 Sampling duration and timing volatile extractions

Six volatiles were detected by GC-MS from solutions of bacteria L2 at all extraction durations. The relative abundance of dimethyl-disulphide, 1-pentanol, 2-n-undecanone and 2-n-tridecanone decreased as the duration of the extraction was increased (Table 3.4).

Table 3.2 Comparison of L2 volatiles from 15, 30 and 60 minute extractions. Relative abundance is the total area in GC readout for each compound (excluding non-bacterial compounds) and shown as follows: T <5%, * 5-10%, ** 11-20%, *** 21-40%, **** 40-65%, ***** 66% and over.

Compound	Retention Time (non-polar)	Relative abundance detected at:		
		15 minute extraction	30 minute extraction	60 minute extraction
3-methyl-1-butanol	4.04	***	****	****
Dimethyl disulphide	4.54	**	**	*
1-pentanol	7.64	**	*	T
2-n-undecanone	15.81	**	*	*
2-n-tridecanone	19.78	*	*	T
Indole	29.99	*	**	***

However the relative abundance of 3-methyl-1-butanol and indole were seen to increase as the duration of the sample period increased, with 3-methyl-1-butanol showing the greatest abundance of any compound at 30 and 60 minutes. Indole was seen at low abundance at 15 minutes, but increased steadily over time (Figure 3.1), suggesting that either the compound had a slow release rate from the bacterial solution (the total amount in solution was the same but volatilised slowly) or that more was produced over time by the bacteria.

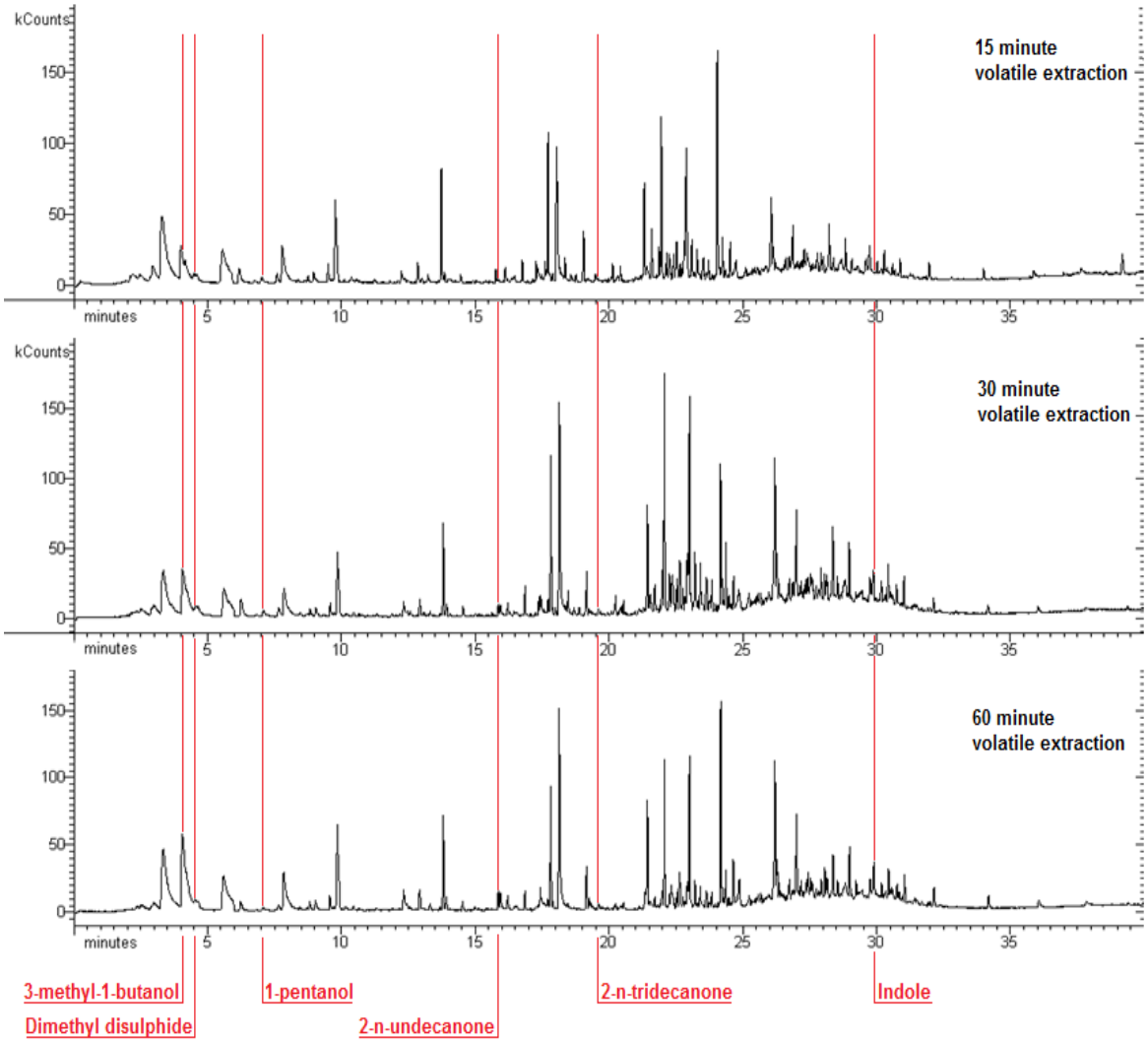


Figure 3.1 GC traces from L2 headspace extractions of 15, 30 and 60 minutes. Y-axis shows GC signal intensity, x-axis is time. Red annotations indicate the retention times of the volatiles identified (shown at bottom of figure) using non-polar VF5 column.

A large number of peaks were observed in the bacteria headspace samples (Figure 3.1) which did not correspond to the volatiles shown in Table 3.4. These peaks were also present in headspace analysis of empty flasks and of flasks containing broth. Many of these compounds were revealed to be silanes, most likely originating from the detergent used to wash glassware (despite thorough rinsing prior to autoclaving). Compounds not found in empty flasks but which

were present in flasks containing broth were also found in the bacterial headspace samples, suggesting that the centrifugation had not been completely effective in removing the broth from the bacterial samples. These compounds were excluded from the final analysis, as their presence in the blanks suggested them to be of non-bacterial origin (or at least present in the absence of bacteria).

Fifteen minute extractions from solutions of L2 also detected the same six volatiles at 0 h, 6 h and 24 h after they were prepared. The relative abundance of 3-methyl-1-butanol was seen to increase with duration after the solution was prepared, and was seen to have doubled in relative abundance by 24 h (Table 3.5).

Table 3.3 Relative abundances of L2 volatiles detected from 0, 6 and 24 hour old solutions. Relative abundance is the total area in GC readout for each compound (excluding non-bacterial compounds) and shown as follows: T <5%, * 5-10%, ** 11-20%, *** 21-40%, **** 40-65%, ***** 66% and over.

Compound	Retention Time (non-polar)	Relative abundance detected at:		
		0h	6h	24h
3-methyl-1-butanol	4.04	***	****	*****
Dimethyl disulphide	4.54	**	*	T
1-pentanol	7.64	**	*	T
2-n-undecanone	15.81	**	*	*
2-n-tridecanone	19.78	*	T	T
Indole	29.99	*	*	*

The relative abundance of all other volatiles was seen to decrease over time (dimethyl-disulphide, 1-pentanol, 2-n-undecanone, 2-n-tridecanone) or remain constant (indole).

Non-bacterial volatiles were most prevalent in real terms in the fresh solution. Those associated with the growth media decreased over time, with their detected levels reducing considerably after 6 hours and falling to low levels by 24 hours (Figure 3.2). Silicates tended to be present at similar levels at all recorded time points.

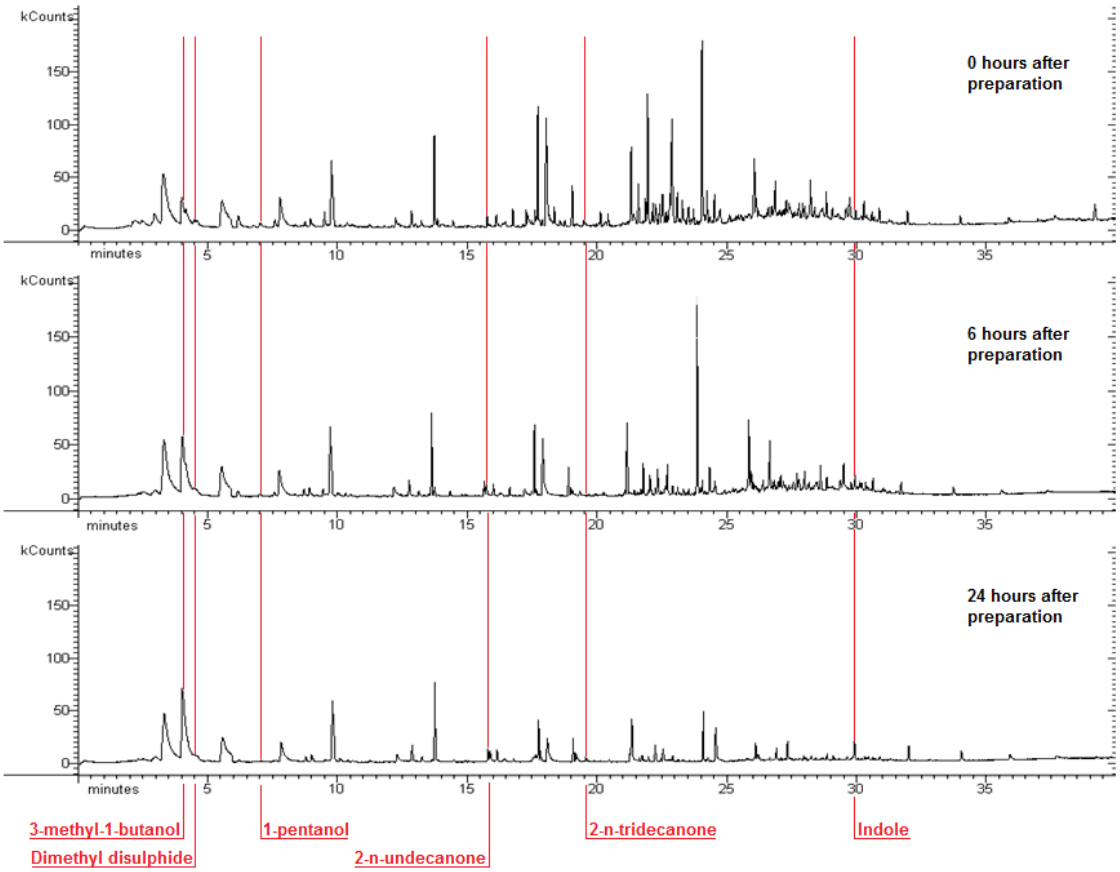


Figure 3.2 GC traces of L2 volatiles collected from extractions of 0, 6 and 24 hour old solutions. Y-axis shows GC signal intensity, x-axis is time. Red annotations indicate the retention times of the volatiles identified (shown at bottom of figure) on non-polar VF5 column. These traces follow the same individual preparation over time.

3.4 Discussion

3.4.1 Detection of volatiles from bacteria headspace

Headspace analysis of the seven bacteria identified volatile chemicals from all seven bacterial solutions. These chemicals were detectable in solutions 24h old at similar or higher levels than fresh solutions, suggesting these chemicals will continue to be detectable for some time after the solution is prepared. It is not clear if the increase seen in some chemicals is due to a slow rate of release from the solution, giving a gradual build-up of the chemical over time, or if the metabolic processes of the bacteria continued after their suspension in saline.

Although the same bacteria were used in Lindh *et al*. (2008a) and the experiment presented here, the two sets of volatile profiles were inconsistent.

For five bacteria solutions, however, the following volatile chemicals were detected in the same bacteria by both studies:

- **L2:** 3-methyl-1-butanol, 2-n-undecanone, 2-n-tridecanone, indole
- **L4:** Dimethyl disulphide, dimethyl trisulphide
- **L6:** Dimethyl trisulphide
- **L9:** 3-methyl-1-butanol, dimethyl disulphide, dimethyl trisulphide
- **L11:** Dimethyl disulphide, dimethyl trisulphide

However, Lindh *et al.* (2008a) reported a number of volatiles from six of the seven experimental bacterial solutions that were not found in the study presented here:

- **E2.5:** 1-nonanol, 1-decanol, 2-methyl-3-decanol
- **L2:** 2-phenylethanol, benzylbutanol, 1-methoxy-3-methylbutane, 2-n-dodecanone, 2-pentadecanone, 2-methyl-1-propaneamine
- **L4:** 3-methyl-1-butanol, 2-methyl-5-nonanone, 5-decanone
- **L6:** Diisopropylpyrazine, 2,5-diisopropylpyrazine, isopropyl-secbutylpyrazine, isopropyl-isobutylpyrazine, 6-methyl-2-heptanone, 2-nonanone
- **L9**: 2-methylbutanoic acid,3-methylbutanoic acid
- **L11:** Phenylmethanol, 2-phenylethanol

Conversely, the following volatiles were not detected in Lindh *et al*. (2008a), but were found in this study:

- **E2.5:** Indole, dimethyl disulphide
- **L2:** 1-pentanol, dimethyl disulphide
- **L6:** 1-butanol, dimethyl disulphide
- **L9:** 1-pentanol
- **L11:** 3-methyl-1-butanol, indole

The analysis of bacteria E2.5 in this study displayed no parity with Lindh *et al*. (2008a), i.e. the two studies identified only unique volatiles which were not detected in the other study.

The detection of dimethyl-disulphide and dimethyl-trisulphide does not seem to differ between the two studies, with both studies identifying these chemicals in the bacteria L4, L9 and L11 and in L6 for dimethyl-disulphide only. Bacteria L2 and L9 were also seen to produce 3-methyl-1-butanol in both studies, but it was detected from L4 and L11 solutions in only one study each.

Direct comparison (see Table 3.3) shows no clear pattern linking which volatiles are detected in one or both studies from any given bacteria, nor is there any pattern in either study that links the seven bacteria by their volatile profiles, other than sulphur bearing compounds. It is therefore difficult to conclude from either analysis which chemicals might be oviposition attractants, either in their own right or as part of a mixture – if indeed any are.

The reason for the observed lack of parity between studies is not clear. The bacteria used in both studies were the same, having been prepared by J. Lindh and identified in Lindh *et al*. (2008a) using molecular techniques. The bacteria provided for this study were sent directly by J. Lindh. Upon arrival to NRI the bacteria were cultured on agar and visually identified and examined using Gram stain to confirm their identity, which matched the information provided by J. Lindh.

The protocol for culturing the bacteria matched that of Lindh *et al*. (2008a) and was devised in consultation with J Lindh (personal communication with J Lindh). The bacteria were stored according to the same procedures, at -80°C in glycerol stock. The same growth media was used in both studies, although it is possible that individual differences between batches may have altered the metabolism of the bacteria, resulting in different chemicals being detected later.

The analysis was also similar; using a GC equipped with a non-polar VF5 column (Lindh *et al.* (2008a) used a non-polar SPB1 column). Care was taken to ensure the water quality and the glassware were the same or equivalent. The SPME fibre used was the same, too (PDMS/DVB), with at least one analysis of

all bacteria lasting 24 hours, the maximum duration of extraction in Lindh *et al*. (2008a).

It is therefore concluded that the bacteria used in these two studies had different volatile profiles from one another, despite efforts to ensure that the present analysis duplicated that of Lindh *et al*. (2008a).

It is possible that the bacteria sent from Sweden to the UK were in fact not the correct bacteria – they may have been subject to mislabelling or the wrong stock cultures were used to prepare the samples. As the identity of the bacteria was not confirmed genetically it is not possible to discount this theory.

Another possibility is that the correct bacteria were sent but they became contaminated at some point prior to headspace analysis and further experiments. Thus, the two studies would have been investigating different bacteria. However, each bacterial solution was plated out onto agar and grown to confirm the bacteria in solution was the correct species. It is also possible that the bacteria may have not been contaminated, but somehow altered in transit or whilst in storage in Sweden. While unlikely, this cannot be fully discounted, again as genetic analysis was not performed.

Since the bacteria were sent by J Lindh directly, with no interference between her preparation from confirmed stocks, and receipt of the un-opened phials at NRI, it was concluded in spite of no genetic analysis being performed that the bacteria were the same as were tested by Lindh.

Finally, while great efforts were taken to maintain the procedure from Lindh *et al*. (2008a), inevitably there may be slight differences between the two studies.

One such factor is that the two laboratories may have been at different ambient temperatures, affecting the volatility of the chemicals – it has been seen that some volatiles are released from the solution in greater amounts as time increases. Note though that this might affect relative or absolute amounts, but not necessarily the qualitative differences reported, such as presence or absence of a given compound. Thus, the bacteria may have indeed produced the same volatiles, but they were not released from the solutions at the same rate, or even at all. It is also possible that factors such as differences between the water used, the presence of growth media or even the detergents used to clean the glassware altered the release rate of the volatiles. Both the cleaning agents and some residual growth media were detected in the GC analysis.

It is not possible to establish precisely if the bacteria were the correct bacteria, although the lack of parity between the volatile analyses of the two studies does make this a possibility. However, there is no evidence that the bacteria were not the same, and therefore it must be assumed that they were. Thus, it must be concluded that the oviposition attraction reported in Lindh *et al*. (2008a) was not seen to be replicated in this study (see Chapter 5).

3.4.2 Effective sampling times of bacterial headspace

The results suggest that the level of detection of compounds within the volatile profile of the bacterial solutions reduced slowly over time, except in the cases of 3-methyl-1-butanol and indole; the level of detection of both increased with sample duration. Extractions made immediately after the solution was prepared and at 6 h and 24 h after the solutions were made also identified the same volatile compounds. The level of 3-methyl-1-butanol detected was seen to

increase as the solutions age increased, but there was no detectable increase in the level of indole detected.

Both 3-methyl-1-butanol and indole are water soluble at room temperature, and 1 mg/ml solutions of each smell strongly. As the duration of the volatile extraction increases, the level of these highly volatile compounds builds up in the headspace. Extractions from 1 mg/ml solutions show the SPME fibre is not saturated by either of these compounds at the levels observed from the bacterial headspaces and so the GC-MS analysis is able to detect the higher levels seen from the longer extractions. Indole is, however, sensitive to both light and air, and will break down over time, possibly explaining why this increase is not seen in the samples taken of older solutions. In contrast, 3-methyl-1-butanol is far more stable, and lingers in the headspace of the older solutions in greater quantities.

It is apparent that there is little to be gained from sampling solutions for a longer period than 15 minutes, as all chemicals present are detected by an extraction of this length. Additionally, a very long duration sample is likely to lead to the SPME becoming saturated by 3-methyl-1-butanol, reducing the resolution of the analysis of the sample.

There is also little to be gained from sampling older solutions, other than to compare the change that occurs over time. This change seems to be limited to the increase of 3-methyl-1-butanol and the reduction of most other chemicals, again leading to reduced resolution when analysing the sample. No novel volatiles were present after 6 or 24 hours that were not seen at 0h. Thus,

optimal sampling of the bacterial solutions is to extract the volatiles for 15 minutes using a freshly made solution.

It is also apparent that the bacterial solutions are suitable for behavioural tests in cage assays (as per Chapter 6), as the volatile profile of the solution will contain the same compounds whether fresh at the beginning of the assay, or at 16 hours old, at the end of the assay.

3.4.3 Potential volatile oviposition attractants

Indole was seen in the headspace of E2.5, L2 and L11. All three bacteria are gram-negative Proteobacteria, although that is where the similarity ends. L11 (*Comamonas* sp.) is an aerobe Betaproteobacteria in the order Burkholderiales. E2.5 (*Vibrio metschnikovii*, order Vibrionales) and L2 (*Proteus* sp., order Enterobacteriales) belong to the class Gammaproteobacteria and are both facultative anaerobes – the three are very different organisms with little in common with one another in terms of their genomes, habitats, pathology or the volatiles they emit (Lindh *et al.,* 2005; 2008a; Garrity *et al.,* 2005).

Indole is a metabolic product of tryptophan common in nature. At low doses it gives off a floral odour, but at higher doses it is unpleasant – indole is one of the compounds that give faeces its odour. The antennae of *An. gambiae* are able to detect indole (Meijerink *et al*., 2001), and the chemical has been suggested as a potential oviposition attractant in this species (Blackwell & Johnson, 2000).

Indole is also present in the odour of humans, being a component of sweat (Meijerink *et al.,* 2000). *Anopheles gambiae* is seen to respond to indole prior to blood meals, but not after blood meals, suggesting indole is a host seeking

cue, rather than an oviposition cue (Takken *et al*., 2001; Meijerink *et al*., 2001; Qui *et al.,* 2011).

Dimethyl-disulphide was the most ubiquitous compound detected, found in the headspace of all bacteria except for Ps. Lindh et al. (2008a) also reported its presence in samples of L2, L4, L9 and L11. Sulphurous compounds are strongly suggested to be toxic to *Cx. pipiens* larvae (Kimbaris *et al.,* 2009), and extracts from garlic and horseradish, both of which contain sulphur compounds (Petrovska & Cekovska, 2010; Tomsone *et al.,* 2013) have been shown to be insecticidal to *Ae. albopictus* 4th larval instars (Tedeschi *et al.,* 2011) and *Culex* larvae (Kalu *et al.,* 2010).

Dimethyl-disulphide is an odour commonly associated with plants of the genus *Allium*, and has a distinctly onion or garlic like smell. It has been used as a fumigant in fruit production (such as strawberries), replacing bromomethane and other banned chemicals (DeCal *et al*., 2004). Anecdotal evidence also suggests that in Ethiopia some people believe that eating or carrying garlic reduces mosquito bites (Legesse & Deressa, 2009). However, laboratory tests suggest eating garlic does not repel *Ae. aegypti* seeking a blood-meal host (Rajan *et al*., 2005). Campbell *et al.* (2011) demonstrated repellence in *Ae. aegypti* to extracts of garlic oil, and garlic has been shown to act as a tick repellent (McHugh, 2001; Hanifah *et al.,* 2012). Allicin, an aliphatic unsaturated sulphur compound found in garlic, also has antibacterial properties, even at low concentrations (Petrovska & Cekovska, 2010).

Dimethyl disulphide has also been suggested as a potential oviposition attractant in mosquitoes, although Trexler *et al.* (2003) found no oviposition

preference over control water in *Ae. albopictus* in field and laboratory experiments. As there is no evidence to suggest an oviposition attraction towards sulphides in *An. gambiae*, and the apparent repellent or even toxic effect in a number of mosquito species, it is probably not likely that these chemicals are responsible for the observed oviposition responses seen in Lindh *et al*. (2008a).

Aliphatic alcohols were also found in several bacteria, both here and in Lindh *et al*. (2008a), but there was little parity between the studies as to which aliphatic alcohols were observed. Here only three of the six compounds observed by Lindh; 3-methyl-1-butanol, 1-butanol and 1-pentanol, were found. None were found in the headspace of E2.5, in which Lindh observed the three not detected here (1-nonanol, 1-decanol and 2-methyl-3-decanol). Of these, only 3-methyl-1-butanol, found in 4 bacteria here and three in Lindh *et al*. (2008a), has been suggested in literature as an oviposition attractant to Diptera: *Drosophila melanogaster* (Becher *et al*., 2012) and *An. gambiae* (Lindh *et al*. 2008a; 2008b).

The comparative results of these two studies might suggest that of all the compounds detected only indole and 3-methyl-1-butanol to be the only likely oviposition attractants. This is by no means certain though; as 3-methyl-1-butanol was detected from L9 and indole from L2 in both studies, and previous behavioural studies suggest that indole is not an oviposition cue (although this is not yet proven conclusively). Lindh *et al*. (2008a) detected both chemicals from L2 and this study detected both from only L11. Again, as no clear pattern emerges from the detection of these two volatiles from the tested bacteria, it is not clear if they are actually oviposition attractants or not.

In summary, Objective 1 was accomplished to some extent: the compounds released by the test bacteria were identified and the chemical profiles of the emissions were compared. However, these profiles did not match those of Lind *et al*. (2008a), and thus, while the initial objectives of this part of the study where met, unfortunately based on the comparative results of these two studies alone, it is not possible to determine which, if any, bacterial volatile is a potential oviposition attractant (Objective 4).

4 ELECTROPHYSIOLOGICAL RESPONSES OF *ANOPHELES GAMBIAE* TO PUTATIVE OVIPOSITION SEMIOCHEMICALS

This chapter addresses Objective 2 (as described in Chapter 1.5.3), by modifying and developing the GC-EAG (gas-chromatography linked electroantennography) technique for examining the electrophysiological responses to volatile potential oviposition semiochemicals of gravid females of *Anopheles gambiae*.

4.1 Background

Environmental stimuli play a major role in the suite of behaviours observed when *Anopheles gambiae* females locate a breeding site and lay their eggs. Therefore, it is important to identify the sensory stimuli that play a role in this behaviour. The detection of volatile chemicals is primarily by sensory neurones in the antennae. The electrical signal that results is sent to the insect's brain, which may stimulate a motor response that results in observed changes in behaviour. Therefore the examination of the electrophysiological responses in the antennae of mosquitoes can provide valuable information as to which volatile compounds, such as those emitted by bacteria, excite the nervous system, and how reliably a given compound elicits a significant response. The work presented in this chapter, therefore, aims to identify and investigate some of these "inputs" (oviposition stimuli).

Previous studies have indicated that oviposition behaviour likely involves volatile chemicals (Takken & Knolls, 1999), which may attract (Blackwell & Johnson,

2000), repel or deter (Omolo *et al*., 2004) gravid females. The particular chemicals that have these effects can be species specific (Millar *et al*., 1992), or general (Clements, 1999; Himedan *et al*., 2013).

Lindh *et al*. (2008a) proposed that volatiles emitted by bacteria are a likely source of oviposition attractants for *An. gambiae*, based on bioassays that found whole-odour emissions from specific bacteria collected from natural *An. gambiae* breeding sites are attractive to gravid females (Sumba *et al.,* 2004a). Therefore, a key early step in these investigations was to test which components of the attractive whole-odour emissions from bacteria reported to elicit a positive oviposition response in *An. gambiae* (Lindh *et al*., 2008a) are detected by the NRI strain of *An. gambiae* used (provided from the Mbita colony by U. Fillingher). One way of doing this, and the method employed here, is to measure the response of the mosquito's olfactory sensory system to candidate chemicals using gas chromatography linked electroantennography (Cork *et al*., 1990), a technique that is well-established, has been used in many studies (e.g. Cosse *et al.,* 1995; Qui *et al.,* 2004; Puri *et al.,* 2006), allows for quantification of the stimulus and the related response (Cork *et al.,* 1990) and does not require the additional skill, time and precision of single cell sensillum recordings (SSR).

The electroantennography (EAG) technique used in this thesis captures the sum of electrical potentials created in activated olfactory sensory neurones across the whole antenna (Roelofs, 1984; Byers *et al*. 2013), whereas SSR identifies responses of individual receptors, (Blight *et al*., 1995; Syed & Leal, 2011). EAG is suitable to identify the capacity to detect a particular odour (particularly those producing strong responses by stimulating multiple neurons in the antennae), whereas SSR is more useful as a technique for mapping receptive ranges of

individual olfactory sensory neurones (Hallem *et al*., 2004; Hallem & Carson, 2006).

4.1.1 Insect olfaction

Insects primarily detect volatile chemicals with sensory apparatus located on their antennae. There are a variety of types of sensilla, the sense organs, distributed across the body of the mosquito, four of which are involved in olfaction (Hallem *et al*., 2006). Three types of olfactory sensilla are found on the antenna; single-walled multiporous hairs, double-walled multiporous pegs and the sunken double-wall multiporous pegs. A fourth type, the single-walled multiporous peg sensilla, is found on the maxillary palps alongside the proboscis (Figure 1.6, Chapter 1 shows the location of the antennae and palps). Sunken double-wall multiporous peg sensilla are unique to *Anopheles* (McIver, 1982).

The insect uses these four types of chemoreceptive sensilla to sample volatile chemicals in the air around them. The morphology of a sensillum consists of the outer layer, which is composed of cuticle, the sensory neurone protected within the cuticle, and the auxiliary cells that are positioned alongside the sensory neurone. In olfactory chemosensilla the cuticle takes the form of a porous hair or peg. The cuticle surrounds the dendritic branches of the neurone.

Each chemosensillum is composed of between two and five sensory neurones, which are responsible for detecting stimuli (by the cell dendrites), and

generating and transmitting the nerve impulses via the axon (Figure 4.1).

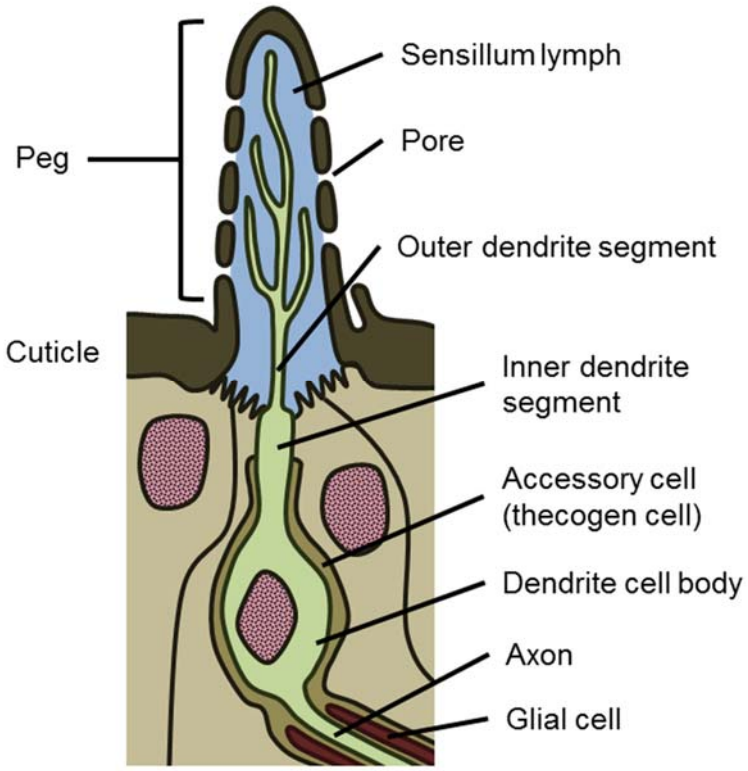


Figure 4.1 Simplified diagram of a single walled multiporous peg sensillum. Drawn by author, after McIver (1972), Clements (1999) and Ache & Young (2005).

The third type of cell is auxiliary or accessory cells, of which there are a variety of types, such as the cogen cells, which sheath the neurone. Glial cells are also present, although they are not considered part of the sensillum. These cells form a neurolamella, or neural sheath, around neurone axons, protecting it and playing a role in nerve signalling (McIver, 1972; Steinbrecht, 1996; Clements, 1999; Qui & van Loon, 2005).

The process of sensory reception involves three basic steps:

’Coupling’: Odour molecules enter the sensillum lymph through pore tubules, where they bind to odour-binding proteins (OBPs). OBPs bind to an odour molecule which increases its solubility, as many odour molecules are hydrophilic. The OBP transports the odour molecule through the lymph, releasing it in close proximity to the odour receptor (OR) located on the neurone dendrite. Excess, stray or used odour molecules are hypothesised to be inactivated by molecular traps or enzymes, but this is not certain (Leal, 2013).

‘Transduction’: ORs consist of two parts, the binding protein and the co-receptor. The odour molecule binds to the OR and activates a chain of proteins in the dendrite wall that alter the flow of ions into and from the neurone creating a receptor potential (Rubenstein *et al*., 1998; Sato *et al*., 2008).

‘Encoding’: The receptor potential is a depolarisation of the cell due to the altered ion balance at the receptor. If this is greater than the required threshold potential, an action potential is triggered, activating the nerve cell, causing a nerve signal to travel to the antennal lobe where it is processed.

The depolarisations across the receptors create a measurable dip in the voltage between the base and the apex of the insect antenna. This difference in voltage represents the sum of all of the depolarisations of all of the activated olfactory neurones across the whole antenna. Odour sensilla contain multiple ORs and are able to detect more than one odour molecule. Different odour molecules and ORs bind with differing degrees of affinity, meaning the receptor potential can vary across a range – some odour molecules require more ORs to activate and produce a greater level of receptor potentials to reach the threshold potential (Hallem *et al*., 2006).

ORs differ greatly between insect orders and between species, but at least one OR is conserved within the order Diptera (the *An. gambiae* AgamGPRor7 is homologous with *Drosophila melanogaster*; Hill *et al*., 2002) as well as having analogues with other orders (Krieger *et al*., 2003). There are approximately 80 *An. gambiae* odour receptors (AgORs) and a similar number of gustatory receptors (Hill *et al*., 2002). Several ORs have no *D*. *melanogaster* orthologues and may be unique to heamatophagous Diptera, or may play a role in aquatic oviposition (Fox *et al*., 2001; Carey *et al*., 2010).

4.1.4 Using GC-EAG to study insect olfaction

Electroantennography

The neural responses in the insect antennae can be detected and recorded using the technique of electroantennography (EAG), which is an analytical procedure that detects the electrical activity in an antenna by recording the voltage difference between the apex and the base of the antenna, and was first devised in the late 1950s by Dietrich Schneider for the detection of moth pheromones (Schneider, 1957). The change in receptor potential across an antenna indicates the activation of nerve cells, and while EAG it is looking for the detection of a volatile compound, these may also be caused by mechanical or temperature changes, which the antennae can also detect.

An EAG response is not necessarily indicative of any behaviour response, however; it simply shows that the sensilla of the insect can detect a particular odour. A volatile compound that elicits a high EAG response may not lead to a behavioural effect, just as a strong behavioural response to a chemical will not necessarily be associated with a high EAG response. Thus, although EAG is a

useful technique for screening potential candidates for behavioural activity, it must be borne in mind that it can appear to exaggerate the apparent significance of some compounds detected and underestimate the significance of others.

When EAGs are performed on an antenna, an 'indifferent' electrode is inserted near the base of the antenna, typically through the eye or the base of the antenna. The recording electrode is attached to the apex of the antenna. Alternatively, both antennae may be used in whole head preparations, or a single antenna can be excised and recorded from on its own. The volatile chemical is passed over the antenna, often by puffing clean air over a filter paper ('filter paper method', see Section 4.4.1, below) impregnated with the chemical onto the antenna and the response recorded. Natural or synthetic compounds may be used as long as they are sufficiently volatile. The electrical activity can then be amplified, displayed and recorded using computer software.

Gas chromatography

As described in Chapter 3.1, gas chromatography (GC) is an analytical technique that separates the constituent compounds of a mixture to allow their identification by means of temperature and the interactions of the compounds with the internal coating of the GC column. The material is vaporised and passed through the hollow column by the pressure of the inert carrier gas, which is ultimately delivered to the flame ionisation detector (FID), allowing determination of the retention time of the compound on the column. This is then compared to known standards to identify the compound.

By splitting the effluent stream after the end of the column, but before the FID, one chemical at a time can be introduced to an insect prepared for EAG at the

same time as it is detected by the FID. A separate inert carrier allows the airstream to be manipulated, for example, by controlling humidity, flow rate, or delivery pattern (e.g., pulsed to allow the chemical to build up while maintaining separation of parts of the mixture).

The combination of gas chromatography and electroantennography, or GC-EAG (Cork *et al*., 1990), allows the direct comparison of a known chemical from a mixture with the electrophysiological response of the insect's antenna as it elutes from the GC (see Figure 4.2).

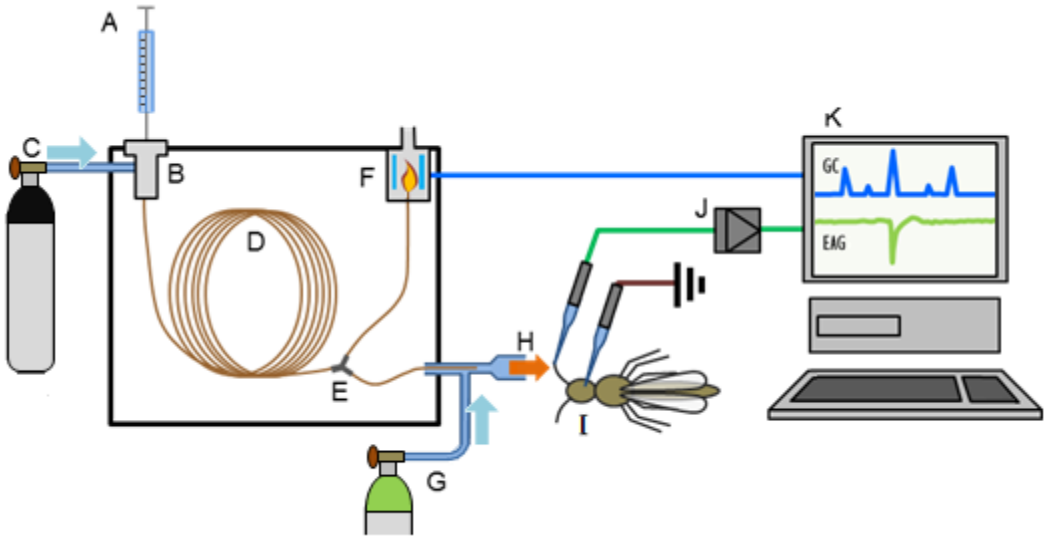


Figure 4.2 Simplified diagram of GC-EAG technique. A sample (A) is injected into the GC and volatilises (B) as it enters the inert stream of carrier gas (C). This mixture enters the capillary column (D), where individual compounds separate, and leave the column by splitting in two (E). Half travels to the FID (F) and half is mixed with a second airstream (G) which passes over (H) the prepared insect (I). The EAG signal is amplified (J) and displayed against the FID readout on the computer (K). After Cork *et al.* (1990).

4.1.3 Rationale for choice of odours tested

It was decided to test the EAG responses of gravid females of *An. gambiae* to 10 chemicals. Six of these compounds were candidates selected from the range of putative oviposition attractants found by Lindh *et al*. (2008a) and four were suggested from other literature.

As described in Chapter 3, Lindh *et al*. (2008a) used dual choice cage bioassays to investigate the attractiveness to gravid females of bacteria isolates collected from natural breeding sites (Sumba *et al.,* 2004a) and from the midguts of field-caught adult *An. gambiae* and a laboratory colony of *Ae aegypti* (Lindh *et al*., 2005; Chapter 3). The logic of this approach was that since these bacteria were isolated from water bodies where immature stages of *An. gambiae* were present (Sumba *et al.,* 2004a), or from the midguts of *An. gambiae* adults reared in the same water, the original gravid *An. gambiae* females that laid eggs in associated breeding sites may have been attracted there by the volatile emissions of bacteria in the water. To test this hypothesis, Lindh *et al*. (2008a) conducted bioassays in the laboratory to test whether gravid *An. gambiae* (from the same mosquito strain as used here) were attracted to the whole-odour volatile emissions of each of these individual bacteria isolates, and found seven of the 19 bacteria isolates were attractive; five isolates from breeding sites and one each from the midgut of *An. gambiae* and *Ae. aegypti (*Chapter 3, Table 3.1).

Lindh *et al*. (2008a) went on to identify 50 volatile compounds that were emitted from the headspace of the most attractive bacteria isolates and, using Principal Component Analysis (PCA), concluded that the following six compounds were the most promising candidate oviposition semiochemicals: 3-methylbutanol (isoamyl alcohol), 3-methylbutanoic acid (isovaleric acid), 2-tridecanone, 2-

phenyl ethanol (PEA), benzyl alcohol and indole. None of these compounds was emitted by all of the bacteria that were attractive to the ovipositing females, however, suggesting there may be more than one compound contributing to the observed attractancy of particular strains of bacteria.

However, Lindh *et al*. (2008a) did not test the EAG or behavioural effects of these six most promising compounds, so it was decided that EAG studies might help to determine which compounds are detected by gravid females, and therefore most likely to be oviposition semiochemicals.

Three of the chemicals identified by Lindh *et al*. (2008a) that scored highly in the PCA have already been shown to be detected by gravid *An. gambiae* by EAG responses (indole, 3-methylbutanol and 3-methylbutanoic acid; Blackwell & Johnson, 2000; Qiu *et al*., 2006), and, therefore, positive EAG responses were expected for these chemicals.

Positive EAG responses to these three chemicals and another of the Lindh *et al*. (2008a) chemicals, benzyl alcohol, have also been found in non-blood-fed (i.e., 'host-seeking') *An. gambiae*; (Cork & Park, 1996; Meijerink *et al*., 2000; Meijerink *et al*., 2001), which suggests these chemicals are detected by *An. gambiae* irrespective of their physiological state (host-seeking or ovipositing).

When testing the effect of environmental stimuli on insect behaviour, it is important to bear in mind the physiological state of the insect (e.g., non-blood-fed or gravid). Generally, while physiological state may not affect the ability of insects to detect chemicals, the behavioural responses of gravid females may differ to those of non-blood-fed females to the same chemicals.

Behavioural responses to mixtures containing one or more of these four chemicals (indole, 3-methylbutanol and 3-methylbutanoic acid and benzyl alcohol) have been reported in non-blood-fed mosquitoes (Meijerink *et al*., 2000; Costantini *et al*., 2001), but no behavioural assays of the response of gravid mosquitoes to these chemicals has been published.

In addition to the six chemicals found by Lindh *et al*. (2008a) in 'attractive' bacteria emissions, the following four chemicals were chosen for the EAG study because they have been found to give positive EAG responses in other studies on blood-fed *An. gambiae*; 4-methylphenol (*p*-cresol) (Blackwell & Johnson, 2000), and on non-blood-fed female *An. gambiae;* 6-methyl-5-heptene-2-one (6-methylhept-5-en-2-one), geranyl acetone (Meijerink *et al*., 2001) and octenol (1-octen-3-ol; Cork & Park, 1996; Qui *et al.,* 2006). Qiu *et al*. (2006) report a positive EAG response to 6-methyl-5-heptene-2-one in both non-blood-fed and gravid females.

4-methylphenol has been tested on gravid female *An. gambiae* and found to be electrophysiologically active at lower doses than in *Cx*. *quinquefasciatus* (Blackwell *et al*., 1993; Costantini *et al*., 2001). 4-methylphenol has also been shown behaviourally to be an oviposition attractant for *Aedes triseriatus* (Bentley *et al*., 1979), *Toxorhynchites brevipalpis*, *Tx*. *splendens* and Tx*. amboinensis* (Linley, 1989) and *An. arabiensis* and *An. gambiae* s.l. (Kweka *et al*., 2011). However, 4-methylphenol is a constituent of cattle urine (Bursell *et al*., 1988) and, therefore, may be an indicator of contaminated water, suggesting its role in *An. gambiae* oviposition (if it has one) may be repellent.

Since it is possible that strains of a species may vary in their neurophysiological and behavioural responses to given chemicals, it was decided that the EAG response of gravid females of the NRI strain of *An. gambiae* s.s. (S-form) from Mbita Point, Kenya to these chemicals should be tested, alongside bioassays to test their behavioural responses to the same chemicals (Chapters 5, 6 and 7).

Therefore, GC-EAG runs using synthetic solutions of the six chemicals identified by Lindh *et al*. (2008a) and the four identified in the literature were carried out to test for biologically active compounds, from which putative semiochemicals, or even oviposition attractants could be identified.

Cork & Park (1996), Blackwell & Johnson (2000) and Meijerink *et al.* (2001) all found that these four chemicals elicit EAG responses, although in different physiological states. These are all components of human sweat and it has been suggested that the volatiles electrophysiologically active in human sweat may also play a role in oviposition site location as well host seeking (Cork & Park, 1996, Meijerink *et al*., 2001), given the limited range of *An. gambiae* ORs. It is, however, possible that any chemical cues involved in oviposition site identification may differ to those used in host finding, and that the distinction prevents gravid females searching for hosts and *vice versa.* If the first three chemicals also detected by gravid females, it would suggest that the corresponding ORs are not down-regulated post blood feed, suggesting a possible post blood feeding role in the mosquito's ecology (Fox *et al*., 2001).

4.2 Methodology

4.2.1 Insect preparation for EAG

Insects were reared as per Chapter 2.2. A gravid female was selected, placed in a glass culture tube and sedated using carbon dioxide gas. The wings of the sedated female were fixed to the abdomen to prevent movement during EAG recordings using a solvent free adhesive (Tipp-Ex Ecolutions correction fluid, Tipp-ex GMBH Germany). The legs were attached to a small piece of filter paper using the correction fluid, presenting the insect in a standing position. The tip of the abdomen and the palps and proboscis were fixed to the paper, and the head was immobilised using the correction fluid (Plate 4.1).

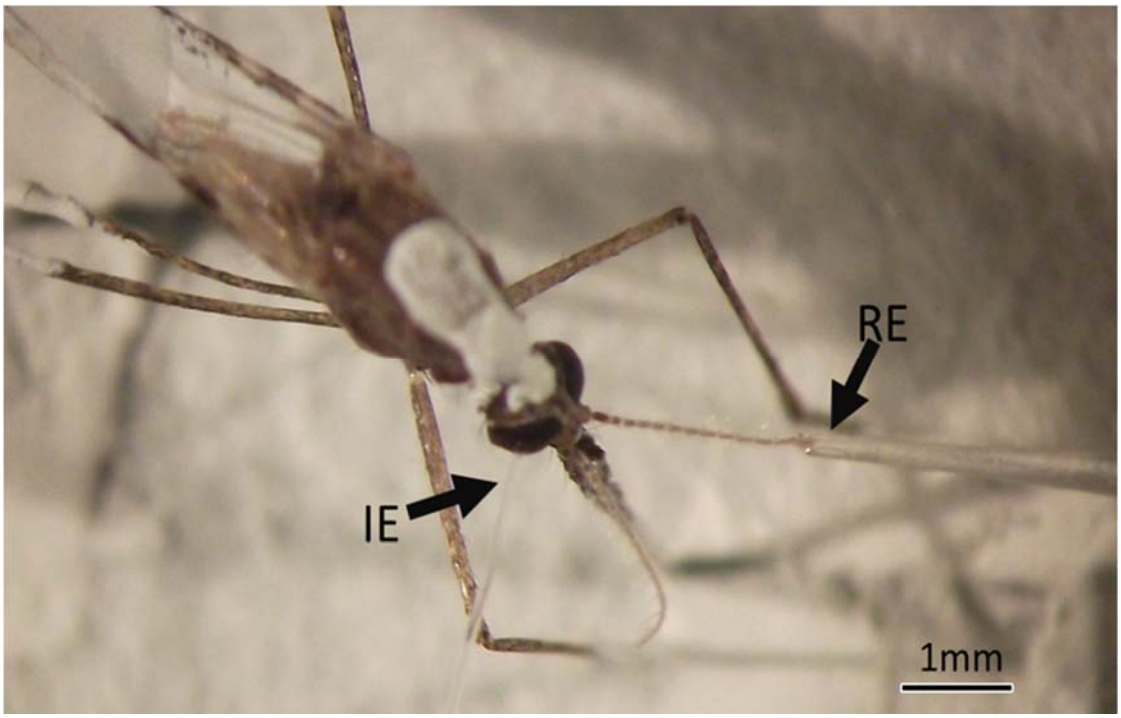


Plate 4.1 A gravid female *Anopheles gambiae* prepared for GC-EAG. The indifferent electrode (IE) enters the eye and the recording electrode (RE) contacts the antenna tip.

Electrodes, formed from borosilicate glass capillary tubes (1.5 mm external dia., 0.86 mm internal dia. with filament; Harvard Apparatus, UK) were heated and pulled to a fine point, and filled with electrolyte. The electrolyte was made by dissolving 1/4 ringer solution tablets (BDH Ltd, UK) in water to give a solution containing NaCl (2.25 g/l), KCl (0.105 g/l), $CaCl_2$ (0.12 g/l), $NaHCO_3$ (0.05 g/l) at pH 7.0. The electrodes were fitted to an EAG recorder unit (Syntech INR-02, Syntech, Netherlands) by positioning them onto silver wire within the recorders electrode holders.

The electrodes were then manipulated so that the recording electrode was positioned over the terminal segment of one antenna. The indifferent electrode was then inserted into the eye (taking care to not tear the surface of the eye) on the opposite side of the insect's head (Plate 4.1). Electrical contact was indicated by a light on EAG recorder.

4.2.2 GC-EAG setup and method

The EAG recorder was connected to an Agilent Technologies 6890N gas chromatograph, fitted with a capillary GC column (30 m x 0.32 mm internal diam.) coated with polar DBWax (0.25 µm film thickness; Supelco, UK) (Plate 4.2).

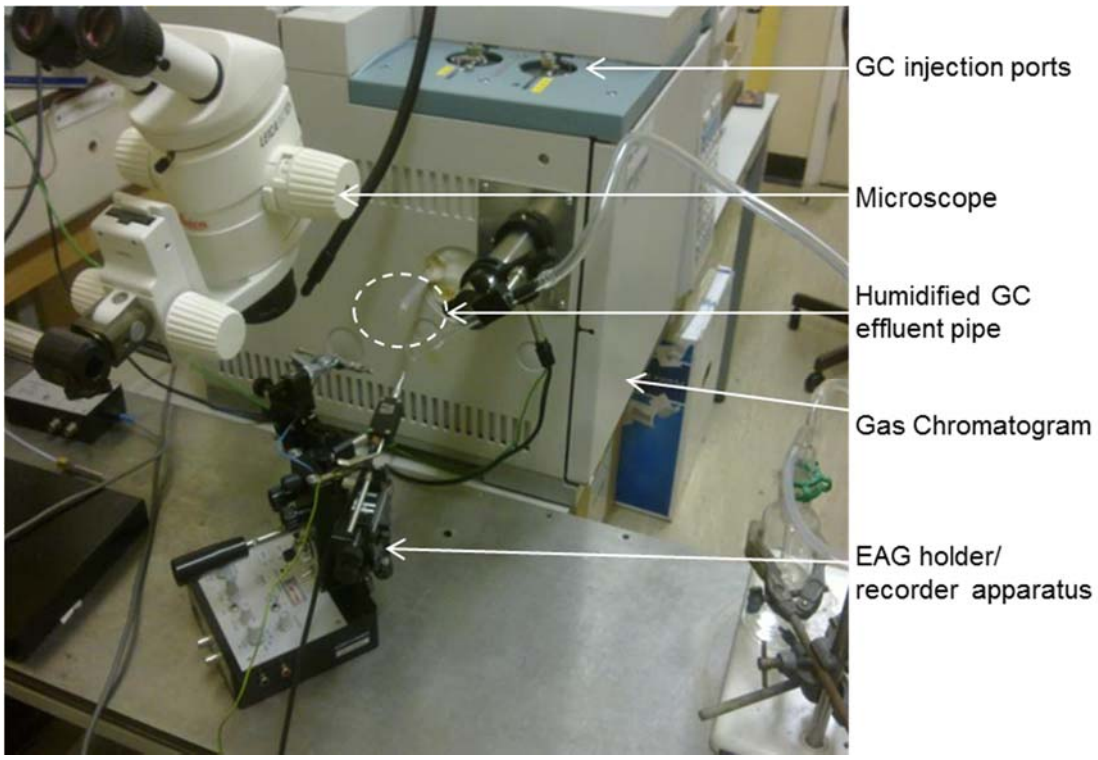


Plate 4.2 The GC-EAG apparatus. The insect is fastened to the EAG holder/recorder and the electrodes fitted under microscope. The insect is then moved into the effluent airstream of the GC for recording volatile responses.

The column effluent stream was split 50:50 between the FID before entering a humidified air stream (300 ml/minute, air) and then delivered over the insect being recorded. The GC was set to the following programme for all runs: from start hold at 50 °C for 2 min, then the temperature was increased 20 °C/min to a holding temperature of 250 °C for 5 min for a total run time 17 min.

The GC was programmed from and controlled by a PC running EZChrom Elite (Version 3.3.1, Build 3.3.1.902, © Agilent 1988-2008). EZ Chrom Elite also recorded and displayed the out-put of the GC and the EAG.

During method development 4-methylphenol was observed to elicit a strong and repeatable EAG response. It was decided, therefore, to include 4-methylphenol

in every run as an internal control to ensure the set-up was working and the mosquito preparation was still responding to volatiles passed over it.

Peaks in the EAG responses were matched to peaks that occurred at the same time in the chemical eluted and the size of the EAG response was recorded. The size of an EAG response was calculated by subtracting the amplitude of the baseline 'noise' from the absolute amplitude of the response, measured in millivolts (Cork & Park, 1996). Means of responses were then calculated and compared as percentages of the mean 4-methylphenol response size. It was decided that any run deemed to be unsuccessful (i.e. having no 4-methylphenol response) would have to be rejected, even if responses to other chemicals were seen.

The sizes of EAG peaks for particular chemicals were then compared across all runs in which a response was seen to that chemical and a mean taken of all positive responses. Means of all runs, including where no response was seen to the chemical, were also calculated, as explained in Results, below.

Each solution was tested multiple times; S1 was tested on 9 runs, S2 on 8 runs, S3 also on 8 runs and S4 on 7 runs.

4.2.3 Chemical solutions tested

Solutions of the chemicals to be tested were prepared prior to experiments. Each solution contained three-four of the chemicals to be tested dissolved in a solvent. The components of the mixture were selected with care to ensure that the retention time of each component chemical in a particular mix would allow a clear separation to be seen when eluting from the GC. Retention times were established prior to EAG tests by injecting 1µl into the GC for each compound.

To prepare the mixtures, a 1mg/ml solution of each chemical to be tested was made initially. These were then diluted in a solvent (hexane) by taking a 50 µl aliquot of each initial solution and diluting it into 5 ml, less the total volume of chemicals added, of pure hexane. Four such solutions were prepared and labelled S1 through S4 (Table 4.1). All solutions contained the internal standard, 4-methylphenol. The combination of the components of the solutions was selected based on their miscibility together in hexane and also the separation of GC retention times, in order to give the insect a recovery period and to allow clear identification of responses to peaks, which may have been hindered by very close retention times (i.e. <5 seconds apart). Preliminary results indicated that 10 seconds was a sufficient recovery period for antennae between stimulations.

The chemicals were sourced from Sigma-Aldrich, UK, and were >98% purity according to the manufacturer. 4-methylphenol is mostly in crystalline solid form at room temperature with a small liquid fraction. The 4-methylphenol used in these experiments was drawn from the liquid fraction and found to contain 98% 4-methylphenol and 2% 3-methylphenol (*m*-cresol).

Table 4.1 The four solutions tested by GC-EAG, including all 10 chemicals tested. Retention times given are for the GC used, taken from preliminary data. Kovats retention indices are calculated from observed retention times compared to those of n-alkanes. 4-methylphenol was used as a positive control and present in all solutions.

Solution	Chemicals	GC retention time (m:s)	Kovats retention index
S1	1-octen-3-ol	6:41	982
	Indole	11:52	1293
	4-methylphenol	10:11	1179
S2	6-methyl-5-hepten-2-one	5:57	914
	geranyl acetone	9:05	1110
	4-methylphenol	*10:11*	*1179*
S3	3-methylbutanol	4:55	846
	3-methylbutanoic acid	8:03	1044
	4-methylphenol	*10:11*	*1179*
S4	2-phenylethanol	8:51	1094
	2-tridecanone	9:11	1115
	benzyl alcohol	9:25	1039
	4-methylphenol	*10:11*	*10.19*

As the GC- EAG technique delivers a known amount of the compound being tested a negative control is not required, as is often employed with studies using the 'puff technique'.

4.3 Results

GC-EAG recordings were successfully made of the EAG responses of gravid *An. gambiae* to the 10 volatile chemicals tested. The level of EAG response varied between no detectable response to response peaks of 1.91 mV. Stable baselines of between 0.1 mV and 0.9 mV were obtained in runs that gave successful EAG responses.

Comparison of the EAG traces with the GC output allowed the determination of relative EAG responses to the candidate chemicals (Figure 4.3), with responses

seen as the chemical eluted or immediately after, suggesting near instant detection.

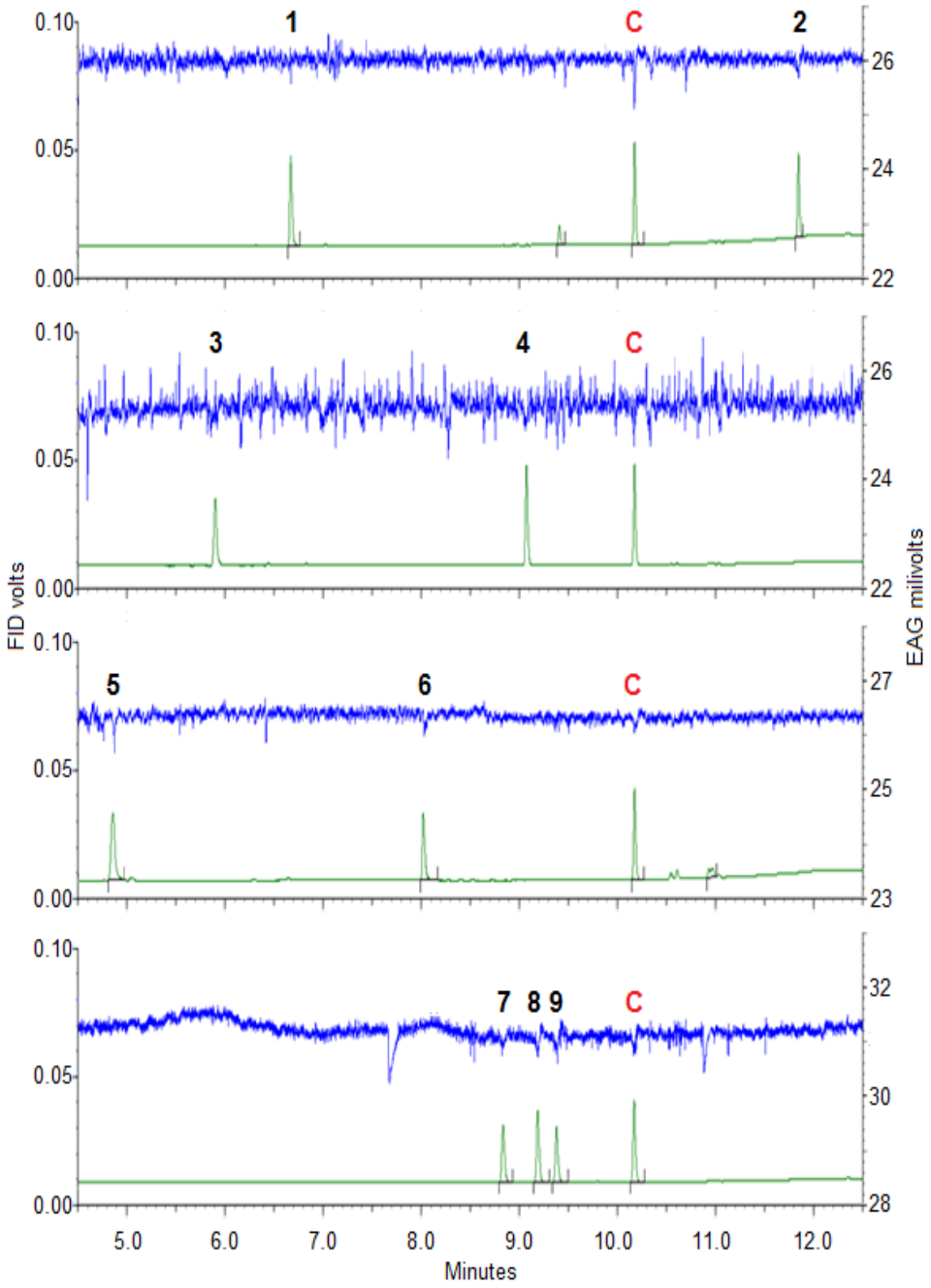


Figure 4.3 GC-Electroantennograms of typical *Anopheles gambiae* responses to the four chemical blends tested. GC traces are shown as green lines, EAG responses are coloured blue. 4-methylphenol is indicated by C; 1-octen-3-ol (1); indole (2); 6-methyl-5-hepten-2-one (3); geranyl acetone (4); 3-methylbutanol (5); 3-methylbutanoic acid (6); 2-phenylethanol (7); 2-tridecanone (8); benzyl alcohol (9).

In all 32 runs responses to 4-methylphenol were recorded, and it elicited the most consistent and in all but two cases, the largest absolute responses. Solutions were each run between 7 and 9 times (see Figure 4.4) The mean response to 4-methylphenol was greater than the mean response of any other chemical tested (Figure 4.4).

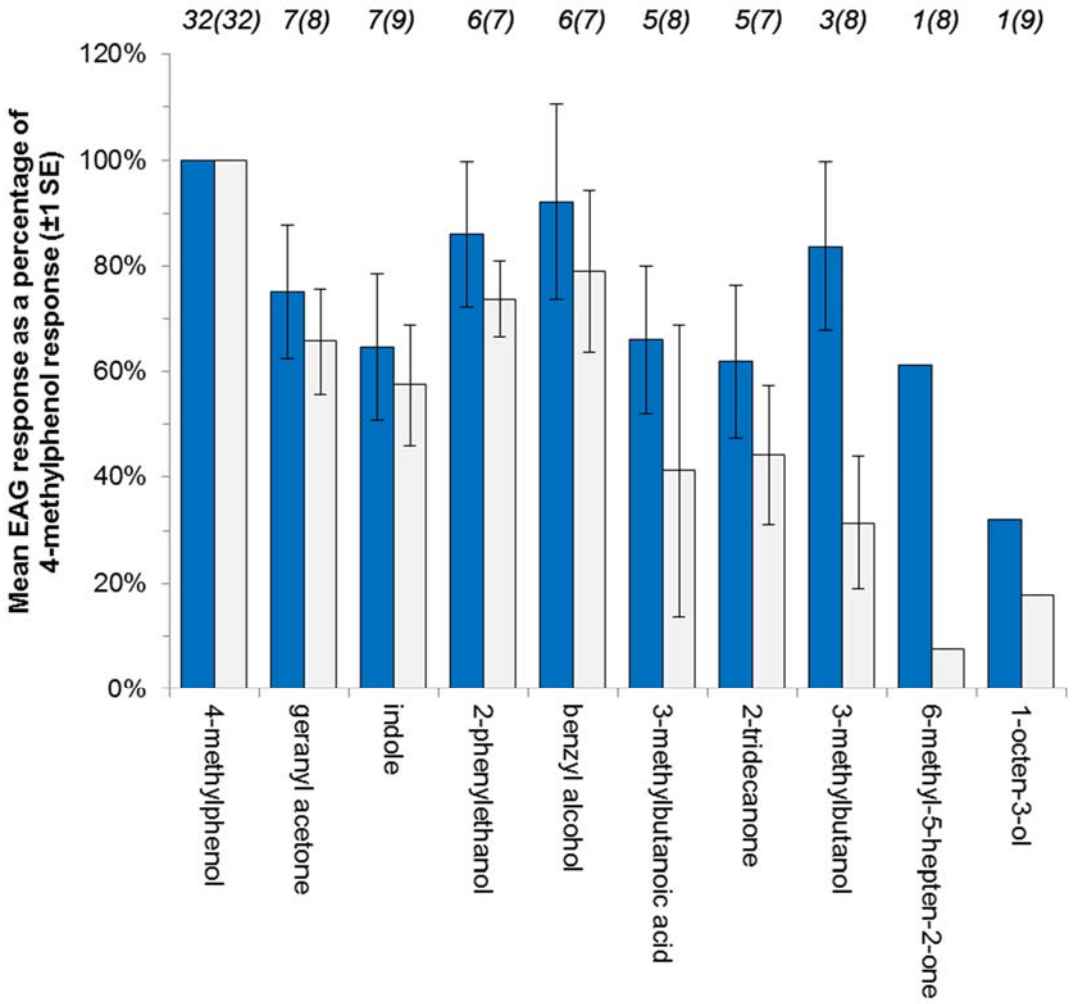


Figure 4.4 Mean EAG responses as a percentage of the mean 4-methylphenol response ±SEM. For each chemical the mean response in relation to 4-methylphenol (i.e. including where no response was seen) is shown in grey. Mean responses, where responses were greater than zero are shown in blue. Indicated above are numbers of runs where a response was seen, with total number of runs for each chemical in parenthesis, to give an indication of consistency of response (e.g. a response was seen to geranyl acetone 7 times; it was tested 8 times in total).

Clear and consistent EAG responses were elicited by geranyl acetone (75±10% (mean ±SE) response strength of 4-methylphenol, in 7 out of 8 runs), indole (65±11% (mean ±SE) 4-methylphenol, 7/9 runs), 2-phenylethanol (86±7% (mean ±SE) 4-methylphenol, 6/7 runs) and benzyl alcohol (92±15% (mean ±SE) 4-methylphenol, 6/7 runs), all of which gave strong responses in at least 75% of successful runs.

3-methylbutanoic acid (66±28% (mean ±SE) 4-methylphenol, 5/8 runs) and 2-tridecanone (62±13% (mean ±SE) 4-methylphenol, 5/7 runs) gave reasonable responses (i.e. 50-74% 4-methylphenol response) in over half of their runs. A high but inconsistent response level was seen to 3-methylbutanol (84±12% (mean ±SE) 4-methylphenol, 3/8 runs).

The lowest and least consistent responses were seen for 6-methyl-5-hepten-2-one (8% 4-methylphenol, 1/8 runs) and 1-octen-3-ol (18% 4-methylphenol, 1/9 runs). In some cases a response may have occurred but was obscured by the baseline, despite it being as low as could be achieved.

A total of 69 insect preparations were tested, many of which did not yield results due to difficulties establishing a clear EAG baseline which lasted for the duration of a run. Ultimately only 17 prepared mosquitoes yielded useful results and produced 32 successful runs (where both a response to the tested compound and the 4-methylphenol control were observed). Mosquitoes still responsive after the first run were tested again using a different solution in an attempt to ensure responses were not affected by individual differences between mosquitoes. Eight mosquitoes gave a single successful run, five gave two runs

and two gave three runs. Two mosquitoes gave four runs and were tested against the full set of solutions.

4.4 Discussion

4.4.1 The use of EAG in investigating olfaction

Measuring EAG responses in *An. gambiae* has been conventionally achieved using a puff of air over a volatile source to deliver the odour to the insect. Typically, this is done by impregnating a filter paper with a known amount of the candidate chemical (Cork & Park, 1996; Meijerink *et al*., 2001). While this permits the detection of EAG responses from a volatile chemical, it is not possible to quantify the delivery of the volatile. That is to say, one does not know with any degree of certainty how much volatile is delivered to the insect, just how much has been put on the paper.

In the puff method (opposed to the GC-EAG technique used in this thesis) the volatility of the chemical, the air temperature, the solvent used and even the thickness and absorbance of the paper can all affect the rate at which the chemical volatilises. It is impossible to know the amount of volatile passing over the insect, so it is not possible using this method to make any reasoned comparisons of sensitivity of the insect to particular odours. All that can be concluded is that the insect is responsive to the volatile, without proper quantification of the response.

The use of solutions of known doses of chemicals in GC-EAG assays eliminates this uncertainty and allows the quantification of the EAG response of the insect

to a volatile, and furthermore allows the discrimination of the constituent parts of a mixture. The previous method would require the analysis and separation of the mixture components prior to examination, thus there are distinct advantages to using GC-EAG over the traditional system.

But despite the advantages of GC-EAG, a number of other factors must be considered when using the technique. A lessening of responses to certain odours may occur over time and through generations as a result of constant laboratory culture. Factors required for survival in a cage will be different to those needed in natural environments and a long established culture may result in receptivity to particular compounds no longer encountered being bred out or suppressed (Lecomte *et al*., 1998).

There is little that can be done to obviate this problem, and it is not addressed in this study. It was assumed that the insects would respond as wild mosquitoes would, and as the mosquito strain used by Lindh *et al*. (2008a; 2008b).

It is also important to bear in mind the assumption that the odour receptors involved in the detection of the volatiles are located mainly on the antenna may be wrong (Moorhouse *et al*., 1969). Since EAGs refer specifically to recordings from the antenna, they do not detect activity in the palps or the tarsi, for example. However, the results of previous studies using EAG have shown that antennae detect all of the chemicals that were chosen to be tested here.

The most crucial false assumption to overcome is that the detection of an electrophysiological response implies a behavioural response. Furthermore, a large electrophysiological response is not necessarily more likely to elicit a

behavioural response than a weak electrophysiological response to a stimulus at the same dose (Marion-Poll & Thiéry, 1996).

These cautions highlight the importance of taking care when analysing data obtained from EAG assays. A small response, especially to a compound for which there are relatively few odour receptors can still play a role in mediating behaviour.

Finally, compounds that might naturally occur at high doses will not necessarily give strong electrophysiological responses because the 'swamping' effect of the compound might result in a reduced sensitivity in order to prevent sensory overload. Previous studies indicated electrophysiological activity would be detected at the concentrations levels used here; reducing the likelihood that swamping would occur (Cork & Park, 1996; Meijerink *et al*., 2001).

4.4.2 The difficulties of using EAG to study mosquitoes

In this study an internal standard was added to the test mixtures. This was possible because the volatiles used in the mixtures were separated sufficiently by GC. Additionally, several chemicals were investigated on a single EAG run, thereby saving considerable time and helping overcome the limitations of the smaller than expected number of mosquitoes that gave stable baselines.

The GC-EAG technique is not as simple as it may sound, and it takes time to develop a standard protocol for each type of insect. The success rate of EAG runs during the early stages of this study were not high, with only 17 out of 69 giving at least a single successful run. Prior to the use of correction fluid, great difficulty was found in preparing a mosquito for mounting in the EAG apparatus. Initially ice was used to sedate the mosquitoes, but this proved somewhat too

effective, killing a large number of the insects. Carbon dioxide gas was found to be more reliable in sedating the insects, which revived within a few minutes after exposure to the gas.

Mounting the insects also proved problematic. Initially, small pins were constructed from copper wire. These were used to mount a sedated insect onto a block of plasticine. With practice this could be done rapidly and with minimum damage to the insect, but properly immobilising the wings was often impossible. Additionally, the heat from the GC equipment often caused the plasticine to soften, resulting in the insect wriggling a leg free. Either of these resulted in the insect moving, causing a great deal of interference to the EAG reception, or even dislodging the electrodes completely.

Attempts were made to mount insects with their legs and wings removed, but unfortunately this resulted in the insect quickly desiccating and expiring after a single run, if not earlier. Attempts using a decapitated head or single antennae met with similar results.

Ultimately the technique described, using correction fluid as an adhesive, was found to be effective at holding the insect upright in an immobilised position. It also meant the insect could be used on a number of EAG runs, not just a single one. As stated in Section 4.3 above, the 32 successful runs were obtained from 17 prepared mosquitoes.

Unfortunately, due to the preparation of the insect, it was not possible to dissect each mosquito to determine if it had mated, as the correction fluid used prevented dissection. Females were given the chance to do so in rearing cages prior to the experiment (see Chapter 2.4). Blood feeding took place 2-3 days

post emergence to give the mosquito sufficient time to mate. Ordinarily it would be possible to tell if a female was mated by dissection. Careful extraction of the spermetheca allows the detection under microscope of sperm and therefore whether or not mating had occurred (WHO, 1974). Fixing the abdomen with a layer of adhesive and the eventual desiccation of the insect meant that this was not possible.

However, there are clear differences in appearance of blood fed but not mated females blood fed mated females, most noticeably the lack of abdominal swelling from the developing ovaries in the former. Since gravid females were chosen for the experiments on the basis of the appearance of their abdomens, it is highly likely that all females tested were gravid. What can be said with some degree of certainty is that all females were blood fed. Females were visually selected on the basis of their abdominal morphology. Selected females had a partially digested bolus in the front part of their abdomen, showing they had blood fed. Those that only sugar fed were observed to have much lighter coloured or even translucent bulges in their abdomens. Any females where the blood fed state was questionable were disregarded.

Females were selected as described in Chapter 2.3, approximately 44-48 h after a blood meal. The females were fully gravid, and therefore ready to oviposit, the conditions under which a female would become responsive to appropriate oviposition site cues.

Gravid, or at least blood fed females were selected for this study, because the bloodmeal can alter the odours the mosquito will respond to (Takken *et al*., 2001). This alteration is genetic in basis, with proteins for host associated odour

receptors down regulated after blood feeding (Fox *et al*., 2001). Olfaction is critical to the behaviour of *An. gambiae* (Takken, 1991), and while the model of olfaction is widely conserved across many phyla (Hildebrand, & Shepherd, 1997), insect olfaction is fundamentally different to other animals and each probably arose uniquely (Wistrand *et al*., 2006).

4.4.3 The responses of AN. GAMBIAE to potential oviposition semiochemicals.

The results of this study show a range of strength in EAG responses, with some volatiles eliciting a relatively strong response consistently across runs (4-methylphenol and benzyl alcohol), and others eliciting a low EAG response, which was not clearly evident in all runs (1-octen-3-ol).

4-methylphenol was the most strongly and consistently detected chemical tested, although other compounds (3-methylbutanoic acid and geranyl acetone) gave at least one response equal to or greater than 4-methylphenol in the same run. Taken alone this may tell us little though individual EAG response strength is not indicative of the strength of any behavioural response. It should be noted that while 4-methylphenol was used as a control to define a 'successful' run, it is also the case that a response to 4-methylphenol was seen in every run where a response to another chemical was also detected. That is to say, no runs were rejected due to a lack of response to 4-methylphenol.

4-methylphenol has been investigated as a bait blend for detecting mosquito vectors of encephalitis (Silver, 2008), an oviposition attractant for *Aedes triseriatus* (Bentley *et al*., 1979), *Toxorhynchites moctezuma* Dyar & Knab, *Toxorhynchites amboinensis* Doleschall (Linley, 1987) and *Culex*

quinquefasciatus (Millar *et al*., 1992). The compound is a component of cattle urine (Bursell *et al.,* 1988), perhaps playing a role in the oviposition of *An. arabiensis* and *An. gambiae* s.l. (Kweka *et al*., 2011), most likely as a repellent, given the high organic content of urine.

The OR AgOR1, which detects 4-methylphenol, responds strongly to the compound, suggesting the compound has strong ecological roles (such as in host finding or oviposition site selection) and has a very narrow tuning curve (Wang *et al*., 2010), suggesting that AgOR1 is used to detect a narrow band of chemicals, again highlighting the importance of 4-methylphenol in *An. gambiae* ecology – evolutionary pressure has driven the expression of a single olfactory receptor to this and other very similar compounds. This suggestion is enhanced by the role 4-methylphenol is thought to play in host selection of other heamatophagous Diptera feed (Hassanali *et al*., 1986, Torr *et al*., 1995), along with 1-octen-3-ol (Cork & Park, 1996).

1-octen-3-ol has been identified as an*. gambiae* host attractant (Takken & Klein, 1989; Takken *et al*., 1997), and has been shown to elicit a strong EAG response in host-seeking females (Cork & Park, 1996). However, unlike 4-methylphenol, here EAGs showed a low responsiveness to 1-octen-3-ol by gravid mosquitoes. This contrast between host seeking and gravid females suggests that 1-octen-3-ol detection appears to be correlated with the physiological state of the mosquito.

1-octen-3-ol strongly binds to AgOR8, which again has a very narrow tuning curve (Wang *et al*., 2010). Again, the narrow band of receptivity shown in AgOR8, suggests this compound has a role an important role in *An. gambiae*

ecology and is not simply an odour detected as one of many in a suite. The ability to detect 1-octen-3-ol is quite specific.

Gene down-regulation of AgOR8 after a blood feed appears to suggest that 1-octen-3-ol is not strongly detected by females at 24 h post-blood feeding (Rinker *et al*., 2013). A similar change in AgOR response is predicted for 6-methyl-5-hepten-2-one (Rinker *et al*., 2013), matching the low EAG responses seen in this study. Rinker *et al.* (2013) also suggests little difference in AgOR response to 4-methylphenol, indole and geranyl acetone, post blood feed, also matching these results.

Indole, a product of bacteria found in the bacteria L2 by Lindh *et al*. (2008a) and in this study, and by this study in E2.5, L11, and PS (Chapter 3), is bound by AgOR2, another narrowly selective OR, and mediated by *An. gambiae* OBP1 (Eliopoulos *et al*., 2010), which also appears to have a role in 3-methylindole detection (Biessmann *et al*., 2010).

Indole has been identified as a potential oviposition cue in *An. gambiae* (Blackwell & Johnson, 2000). It elicited a strong EAG response in *An. gambiae* females here (Figure 4.4), supporting Blackwell & Johnsons (2000) suggestion. Further studies into the role of indole as an oviposition seem to be a most logical course of action for future studies. It is anticipated that behaviour assays would quickly determine the effectiveness of indole as an oviposition attractant. Regrettably, this was not done as part of this thesis following the results of Chapter 5, which led to a change in direction of studies away from oviposition attractants to general oviposition behaviour.

Other tested compounds, such as benzyl alcohol (an attractant in *Culex quinquefasciatus* (Puri *et al*., 2006), were detected by gravid females of *An. gambiae* in this study, suggesting that along with any role in host location, these may also be oviposition semiochemicals. 6-methyl-5-heptan-2-one has been examined as both a host seeking repellent (Logan *et al*., 2010) and also in traps as a component of an artificial human lure (Mboera, L. E. *et al*., 2000). Geranyl acetate has been shown to be repellent to mosquitoes (Omolo *et al*., 2004), in presumably host seeking states.

Due to the weak and inconsistent EAG response seen here it is not likely that compounds that scored lowly in this study, such as 1-octen-3-ol and 6-methyl-5-hepten-2-one, play a role in oviposition site selection. It is more likely that these compounds, detected strongly in pre-bloodfed mosquitoes (Cork & Park, 1996; Meijerink *et al*., 2001) are involved in host selection. That they cease to be attractive post blood-feed, further suggests these compounds are either not found in, or play no role in the location of *An. gambiae* oviposition sites.

The results of this study suggest that several compounds thought to be involved in *An. gambiae* host seeking are also involved in the location of oviposition sites. This raises questions as to how the mosquito discriminates between a potential host and a potential oviposition site. It is, therefore, likely that the mosquito does not rely on olfactory cues alone when in ether a host-seeking or gravid state, instead using the olfactory resources as a complement to other sensory information such as visual cues (see Chapter 6).

The alteration of gene expression and the apparent behavioural changes in *An. gambiae* post blood feeding shows that these two areas of the insect's life are

truly very distinct. While the insect utilises the sensory apparatus in both states, the event of blood feeding marks a huge change in the insect's ability to detect certain compounds, and most likely the changes in responses to these when detected. While the sense organs, such as the antennae and the sensillum do not alter structurally, the genetic transcription of odour reception compounds does, allowing the insect to change its ability to detect relevant compounds while in different states. What might work to trap host-seeking mosquitoes will most likely not attract gravid females - a suggestion borne out in reality by the current lack of an effective *An. gambiae* gravid/oviposition attractant baited trap.

Finally, EAG responses are not indicative of behaviour, but merely show that the insect can detect a given compound. However, while EAG results cannot determine what, if any, behavioural response would be associated with a given weak/strong EAG response, Rinker *et al.* (2013) demonstrated that the change in transcriptome profiles post blood meal can be used to predict potential oviposition cues. Such changes could be reflected in the comparison of EAG responses of insects to chemicals pre and post blood feeding.

EAG is a useful guide, and can be used to screen chemicals by determining the insect's sensitivity to them, but only further investigation using bioassays (such as utilised in Chapters 5, 6 and 7) can determine if the actual behavioural response of an insect to a chemical.

In summary, this chapter has achieved the main objectives: the electrophysiological responses of females of *An. gambiae* to volatiles found in the volatile profiles of the bacteria (see Chapter 3) and a range of other chemicals were identified, revealing that *An. gambiae* females respond most

strongly to 4-methylphenol and benzyl alcohol, with other chemicals eliciting a low EAG response, which was not clearly evident in all runs (1-octen-3-ol. 3-methylbutanoic acid and geranyl acetone) gave at least one response equal to or greater than 4-methylphenol in the same run.

5 TWO-CHOICE CAGE BIOASSAYS OF *ANOPHELES GAMBIAE* OVIPOSITION

This chapter addresses Objective 3 (as described in Chapter 1.5.3) to determine if the observed response reported by Lindh *et al.* (2008a) can be replicated to confirm which bacterial solution is the most attractive to gravid females of *Anopheles gambiae,* and also to characterise the response of gravid females of *Anopheles gambiae* to a) volatiles identified in Chapter 3 to be promising attractive components of bacterial solutions, and to b) 4-methylphenol, the compound that produced the strongest electrophysiological response in Chapter 4, by means of two-choice cage oviposition assays.

The results of Chapters 3, 4 and 5 would then be used to address Objective 4, to determine the most likely oviposition attractants for further investigation.

5.1 Background

Mosquitoes are known to use volatile chemicals as cues in host seeking (e.g. Takken & Knolls, 1999; Zweibel & Takken, 2004) utilising kairomones, and are also believed to use semiochemicals as cues to successfully navigate around their environment to subsist, survive and to reproduce.

Anopheles gambiae males can be said to exhibit only three states during their adult life; resting, swarming (i.e. mating) and feeding (Clements, 1999). Resting is primarily driven by circadian rhythms and vision (Hecht & Hernandez-Corzo, 1963) and swarming is believed to involve auditory stimuli (Pennetier *et al*., 2010). The third, feeding, involves olfaction and it is believed that the males

utilise plant nectar to subsist, using the plants volatiles to orientate themselves to food sources (Gary & Foster, 2004).

Females also use olfaction to identify potential food sources, be that from plants and nectar (Impoinvil *et al*., 2004) which may provide sustenance to females as it does to males, or to locate hosts in order to take a blood meal (Clements, 1992; Cork & Park, 1996; Smallegange & Taken, 2010). *Anopheles gambiae*, for example, has been shown to be attracted to the "cheesy" smell of human feet (De Jong and Knols, 1995), and indeed the odours of certain cheeses (Knols and De Jong, 1996). In both of these cases the odours are secondary volatiles, produced by bacteria present in both environments.

Females have an additional set of behaviours to the three previously mentioned; those associated with the development and laying of eggs. Just as host seeking strategies differ between mosquito species (Clements, 1999), there are also intra-species differences in oviposition cues. Chemical cues from a water body are thought to serve as semiochemicals, indicating the suitability of the body as a habitat for larvae (Bently & Day, 1989; Beehler *et al*, 1993; Allan & Klein, 1995; Dhileepan, 1997).

Oviposition attractants

Mediation of a positive oviposition response in *Culex quinquefasciatus* and *Aedes aegypti* has been observed towards substrates infused with hay; due to secondary volatiles from metabolic processes of the bacteria present in the infusion (Hazard *et al*., 1967). However, *Anopheles albopictus* oviposition does not seem to be affected by infusions that prove extremely attractive to *Cx. quinquefasciatus* and *Culex restuans* (Burket-Cadena & Mullen, 2007). The

secondary volatiles of bacteria seem to be important to mosquitoes at various stages of their lives and have important roles as behavioural cues, but with different volatiles having different effects in different species.

Oviposition attractants may be of bacterial origin (Ikeshoji *et al*., 1975) and the bacterial mediation of oviposition has been demonstrated in a number of mosquito species, including, *Ae*. *aegypti*, (Pavlovich & Rockett, 1988) *Ae*. *albopictus* (Hasselschwert & Rockett 1988), *Culex pipiens* (Rockett, 1987; Poonam *et al*., 2002) and *An. gambiae* (Sumba *et al*., 2004a). These bacteria are most likely to originate in the soil which oviposition sites form in (Sumba *et al*., 2004a) as it was shown that their removal from substrates taken from oviposition sites rendered them less attractive to gravid females, or at least, that is to say, reduced the oviposition towards them (Sumba *et al*., 2004a; Navarro *et al*., 2003).

Anopheles gambiae has traditionally been thought to preferentially oviposit in relatively clean water sources free from organic contaminants, as evidenced by laying eggs preferentially in rainwater (Clements, 1999; Howard, 2006), but in fact almost any water body that is not too polluted may contain the larvae of *An. gambiae* or other anopheline species (Fillinger *et al*., 2004; Majambere *et al*., 2008; Awolola *et al*., 2007). Water vapour itself is a strong attractant to gravid *An. gambiae* (Okal *et al*., 2013), but some of the characteristics of the water body, such as temperature (Munga *et al*., 2005), are not thought to play a strong role in water finding. Again, the micro-biota of the water body appear to have a role as a number of bacteria have been shown in laboratory tests to mediate positive oviposition response in laboratory tests of *An. gambiae* (Lindh *et al*., 2008a).

Potential oviposition attractants of bacterial origin

In 2010 seven bacteria which had previously been found to elicit a positive oviposition response towards them were provided by Dr. J Lindh. This was done in order to confirm their positive oviposition mediation and to enable studies of the volatiles produced by the bacteria. The exact compound/s that acted as oviposition attractants were unknown, but the volatile profiles of the bacteria had been examined in Lindh *et al*. (2008a) and again by the author at NRI using GC-MS (see Chapter 3), a technique used in several previous studies to identify unknown bacterial volatiles.

Results obtained by GC-EAG analysis of potential oviposition attractant volatiles (see Chapter 4) suggested that several of the chemicals, which gave strong EAG responses, may have a biological effect and as such showed potential towards being oviposition mediators.

One of the chemicals tested using GC-EAG was 4-methylphenol, which was found to elicit the most consistent and strong EAG responses in gravid *An. gambiae* females. 4-methylphenol is usually a by-product of rotting organic material, and is also associated with pig odour, human sweat and cattle urine (Bursell *et al*., 1988). 4-methylphenol has also been associated with host-seeking in tsetse flies (such as *Glossina morsitans* Westwood), and is attractive to males, increasing catches in traps when used as an attractant (Vale *et al*., 1988; Torr, 1994; Torr *et al*., 1997; 2008). In mosquitoes including *Culex* spp. and *An. gambiae*, cattle urine, of which 4-methylphenol is a constituent, has been associated with increased levels of oviposition activity during rainy seasons in natural habitats (Kweka *et al*., 2011), but it is not clear if 4-

methylphenol contributes to the effect. The compound was not found in the headspaces of bacteria tested either in this study (Chapter 3) or by Lindh *et al*. (2008a).

The response of gravid mosquitoes to 4-methylphenol is not universal: *Ae. albopictus* is not attracted to baits that contain 4-methylphenol (Trexler *et al*., 2003) and the chemical appears to be a repellent on its own (Wiwatanaratanabutr *et al*., 2010). Repellency to 4-methylphenol is also shown by gravid *An*. *albimanus* (Torres-Estrada *et al*., 2005). Conversely oviposition attraction is seen in *Cx*. *quinquefasciatus* (Millar *et al*., 1992; Geetha *et al*., 2003), *Ae*. *aegypti* (Bentley *et al*., 1979; Allan & Klein, 1995; Baak-Baak *et al*., 2013) and several *Toxorhynchites* species (Linley, 1987; 1989; Collins & Blackwell, 1998). Those species attracted will breed in water bodies that contain decaying matter (i.e. tree holes), and not the 'clean' water *An. gambiae* oviposition is usually associated with, suggesting 4-methylphenol may be an attractant to species whose larval habitats are associated with wood, trees or organic waste, but not *An. gambiae*.

Despite this large volume of literature, it is still uncertain which volatiles might be *An. gambiae* oviposition semiochemicals. The differences in volatile profiles of the bacteria tested in Lindh *et al*. (2008a) and Chapter 3 further compound this. It was decided, therefore, to repeat the cage experiments described in Lindh *et al*. (2008a) in order to confirm the oviposition attraction reported, as well as test the response to 4-methylphenol to see if the strong EAG response was indicative of it being an oviposition semiochemical.

It was hypothesised that in two-way assays *An. gambiae* would be seen to preferentially oviposit into bacterial solutions over control solutions of sterile saline. It was, secondly, hypothesised that some bacterial solutions may be more effective oviposition attractants than others and that a dose response may be found to exist. Thirdly, it was hypothesised that the results would, in addition to Chapter 3 and Chapter 4, suggest which chemicals might be responsible for the oviposition attraction, allowing further investigation of their effects. Lastly, given strong EAG responses in gravid females, it was hypothesised that 4-methylphenol may act as an oviposition semiochemical, albeit one not produced by the bacteria examined in Chapter 3. These hypotheses were tested using a cage assay.

5.2 Materials and methodology

5.2.1 Preparation of mosquitoes and test solutions

Mosquitoes were reared as outlined in Chapter 2.2. Bacterial concentrations were calculated by plating out serial dilutions of each solution and counting the resultant colonies to determine the number of colony forming units (Chapter 2.5). As this method calculates the dose post hoc, concentrations were not known at the time of experimentation. Calculated concentration ranges are shown in Table 5.1.

Table 5.1 The concentration range of bacterial solutions used in cage tests. Showing the ranges of concentrations and sample sizes of the two studies and the predicted effect sizes.

Bacteria	Concentration range (bacteria/ml):		Total sample size:		Predicted power	Predicted effect size, power=0.8
	Lindh *et al*. (2008a)	This study	Lindh *et al*. (2008a)	This study	(Effect size=3.8)	
L2	$6.9x10^6$ to $3.2x10^8$	$1.6x10^7$ to $2.0x10^7$	14	21	1	0.65
L4	$7.7x10^6$ to $1.8x10^7$	$4.3x10^7$ to $7.59x10^7$	6	14	1	0.81
L6	$6.5x10^7$ to $1.0x10^8$	$4.97x10^8$	9	4	0.99	2.12
L9	$5.2x10^7$ to $5.3x10^7$	$4.41x10^7$ to $3.7x10^8$	6	9	1	1.07
L11	$4.2x10^7$ to $8.1x10^7$	$7.48x10^7$	7	3	0.89	3.26
E2.5	$2.0x10^8$ to $4.0x10^8$	$9.15x10^7$	7	3	0.89	3.26
PS	*not tested*	$9.22x10^7$	*not tested*	3		
Average sample size (excluding PS)			**8.2**	**9**		

As for Lindh *et al*. (2008a), the number of cages run on any given night depended on the amount of bacterial suspension and availability of mosquitos. As Table 5.1 shows, average sample sizes were the same for both studies. Lindh found a just significant difference (p=0.046) for the L4 data with N=6 replicates, which enables an estimate of the effective size needed for this system. Using Cohen's method a large ("big") effect size of 3.8 is found (Cohen, 1992). Note that some loss of power is evident for the smaller samples, with

the predicted effect size increasing (i.e. the system will only detect a larger effect as the predicted effect size increases due to a smaller number of replicates).

Solutions of 4-methylphenol were prepared from stocks of 4-methylphenol (98% pure, 2% 3-methylphenol; Sigma-Aldrich, UK) diluted by weight into deionised water containing 0.9% NaCl. Concentrations prepared were 1g/l, 0.1g/l, 0.01g/l, and 0.001g/l.

A solution of deionised water containing 0.9% NaCl (Fisher Scientific, UK) was prepared and used as control and as a zero dose for both the bacteria and 4-methylphenol. NaCl was added to prevent egg lysis and to match the salt content of the water used to make the bacterial solutions.

5.2.2 Cage testing oviposition response

Approximately 1 hour prior to their scotophase 12 gravid female mosquitoes were selected visually from stock cages (Chapter 2.3). The females had been fed two days previously and were selected visually by inspecting their abdomen. Suitably fed females were assumed to have been mated and transferred to an identical fresh cage of the same dimensions (wire fame, 30 cm all sides, covered in stretched tube gauze), inside a transparent plastic bag.

Oviposition targets consisted of tapered plastic dishes measuring 9.5cm dia. and 5cm deep. The dishes were transparent and no visual cues, such as dark backgrounds, were added. Tests were conducted in closed controlled environment rooms (26±2 °C, 60±10% RH in total darkness.

Two plastic dishes were selected and labelled by pen on the underside, to identify the treatment and control dish from one another. As experiments were

performed under conditions of total darkness, it was assumed the labels would not act as visual cues. The treatment dish was filled to approximately 1/3 full with 50 ml of test solution (either a bacteria solution or a 4-methylphenol solution, suspended in 0.9% sterile saline). The control dish contained 50 ml of 0.9% sterile saline.

A corner of the cage base was randomly selected and the control dish was placed there, approximately 1cm away from the cage interior walls. The treatment dish was then placed in the diagonally opposite corner (Figure 5.1). The edges of the dishes were placed approximately 15 cm apart, with the distance between the centres of the dishes approximately 26 cm. These distances were approximate, as precise measurements were not taken in order to minimise the time cages were open to prevent mosquitoes escaping and the cages loosing humidity.

Dishes were inserted five minutes prior to the scotophase commencement. The cages were then sealed by knotting the cage opening and the plastic bag covering was closed using a “bulldog” style metal binding clip (Plate 5.1). Plastic bags for experimental cages were washed in warm water and thoroughly dried between experiments to prevent further contamination from volatile odours.

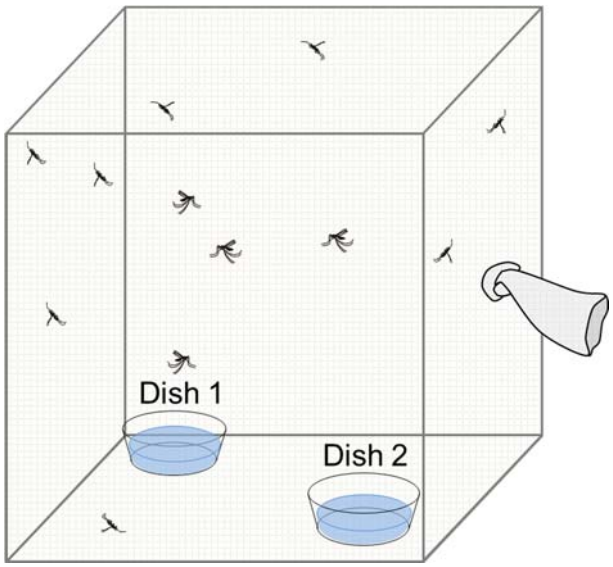


Figure 5.1 The design of cage assay used in experiments. Stretch bandages cover a 30 x 30 x 30 cm (approx. 0.027cm^3 internal volume) cage containing 12 gravid females and two dishes. The treatment dish was placed in a random corner of the cage and the non-treatment dish positioned diagonally opposite.

Plate 5.1 View of a typical experimental cage. The stretch bandage over the fame is tied at the front and the entire cage is kept in a clear plastic bag to maintain internal humidity. A control dish can be seen in the top-left corner of the cage.

The dishes were left in the cage overnight and removed the following morning. Due to the timing of the light cycle this meant the dishes were in the cages for 19 hours. The number of eggs deposited into each dish was then counted and recorded. Cages that yielded no eggs were recorded but not used in the statistical analysis, as it was not clear why no eggs were laid, i.e. was a third choice made (to not oviposit at all) or did no oviposition occur for other unaccounted factors?

The results were examined by analysis of variance (ANOVA) using the statistical programme R (as per Chapter 2.6). Tukey's test was used to determine the effects of oviposition towards the volatiles emitted by bacteria in solutions compared to each other.

5.3 Results

5.3.1 Oviposition towards bacterial targets

Between April-June 2010 and August-September 2011 a total of 78 two-choice cage tests were performed to examine the oviposition preference of gravid females *Anopheles gambiae* towards bacteria-in-water suspensions. From these a total of 21,745 eggs were counted, equating to a mean ±SE) of 23.2±19.36 eggs laid per female. Overall 9,516 eggs, 43.76% of the total, were laid in the treated dish containing bacteria.

Eight tests yielded no eggs in either dish; 2 tests of L2 (bacterial concentrations of $6.3x10^6$/ml and $6.3x10^7$/ml), 4 tests of L4 (1 at dose $4.3x\ 10^7$/ml, 3 at $8.5x10^7$/ml), 1 test of L9 (dose $8.5x10^7$/ml) and 1 control v. control test.

Where eggs where laid, the number of eggs laid into treated dishes was not found to be significantly greater than the control dishes (ANOVA: F=1.69, df=1, 68, p=0.1325). None of the seven bacteria tested were found to be more attractive than the control (Figure 5.2). One bacteria, L4, received significantly less than 50% of eggs laid in treatment dish, with a mean of 30.2% (χ^2=662.46, df=1, p<0.001), suggesting it to be a repellent. L6 showed the lowest range of choices, with a total of 619 eggs laid in the treatment (50.4%) and between 69 eggs (48.5%) and 218 eggs (54.5%) laid in the treatment dish in individual tests.

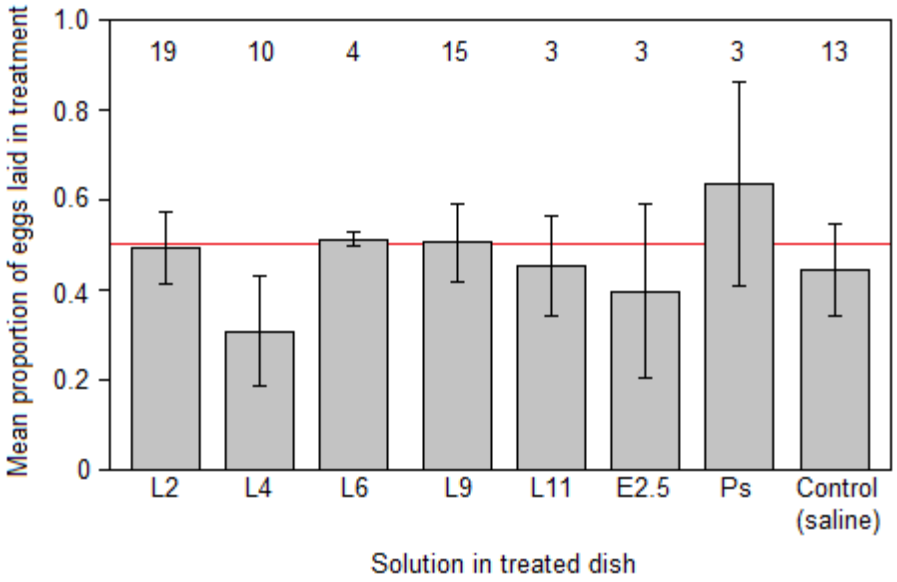
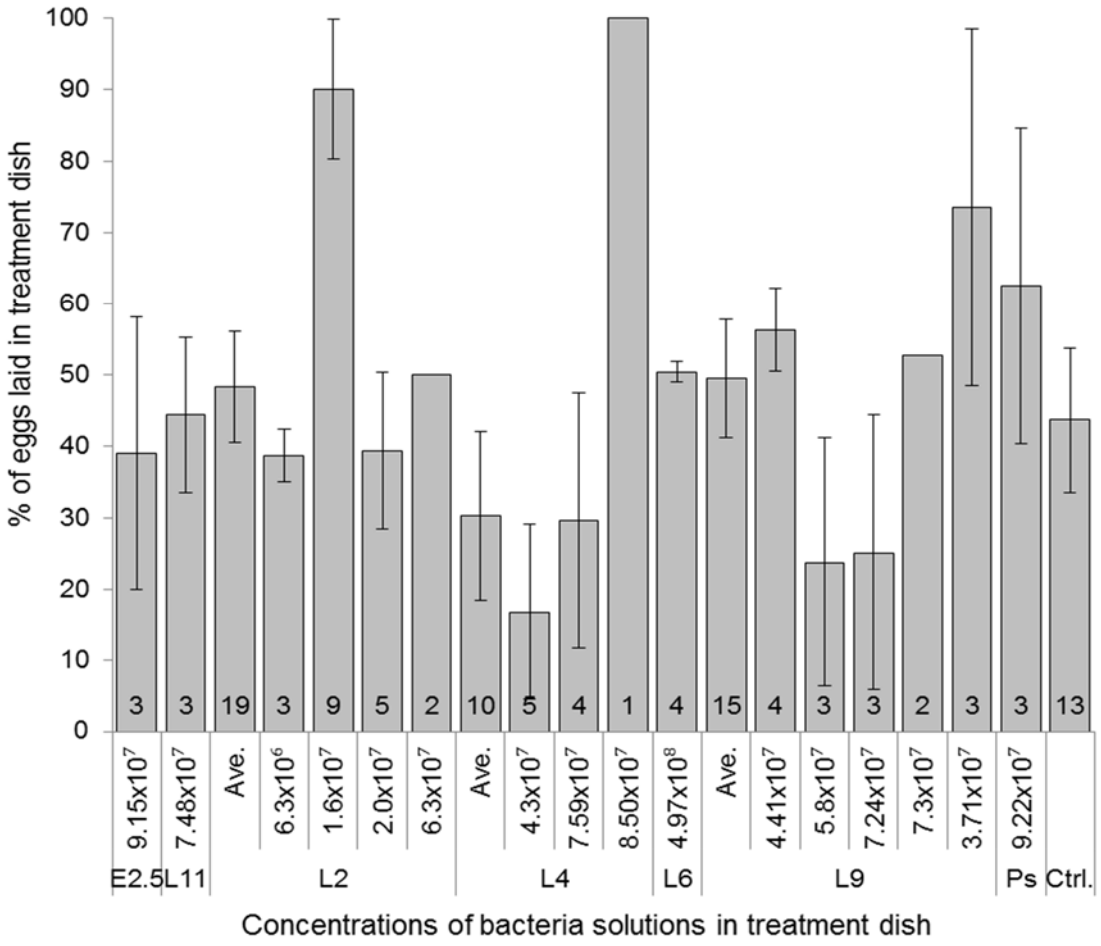


Figure 5.2 Proportion of eggs laid in treatment dish ±SEM. Numbers above bars indicate the number of successful repetitions with that treatment. Red line indicates a proportion of 0.5 (i.e. 50%).

Bacteria L11, E2.5 and Ps were tested only during the second tranche of experiments (August-September 2011) and time constraints limited the number of tests of these bacteria to three each.

Three or more doses of bacteria were tested in the case of bacteria L2, L4 and L9 (Figure 5.3), but no clear response to dose could be found. Bacteria L2

shows a strong peak in oviposition dish selection towards the treatment at a bacterial concentration of 1.6 x 10^7/ml.



<u>Figure 5.3</u> The mean percentages of eggs laid in treatment dishes at all concentrations ±SE (where applicable). Numbers inside columns indicate the n. of replicates at each concentration. Means of all concentrations for a bacteria are shown in column "Ave.". Column "Ctrl." shows saline vs. saline results.

From 9 experiments at this dose a highly significant 90.0±9.46% (mean ±SE) of eggs were laid in the treatment dish (χ^2=30.72, df=8, p<0.005). However, no overall significant positive oviposition was observed effect towards bacteria L2 (ANOVA: F=0.099, df=1,1, p=0.754).

Bacteria L4 elicited a strong oviposition response at the highest dose tested. At a bacterial concentration of $8.5x10^7$/ml 100% of eggs (count = 123 eggs) were laid in the treatment. This, however, contrasts sharply with the three other tests at this dose where no oviposition was recorded. At a concentration of $3.71x10^7$/ml, bacteria L9 was observed to be attractive at near significant levels (χ^2=4.78, df=2, p=0.11 (p=0.05 at 2df = 5.99)).

The results suggest there is no relationship between the bacterial dose and the proportion of eggs laid in the treatment dish (Figure 5.4).

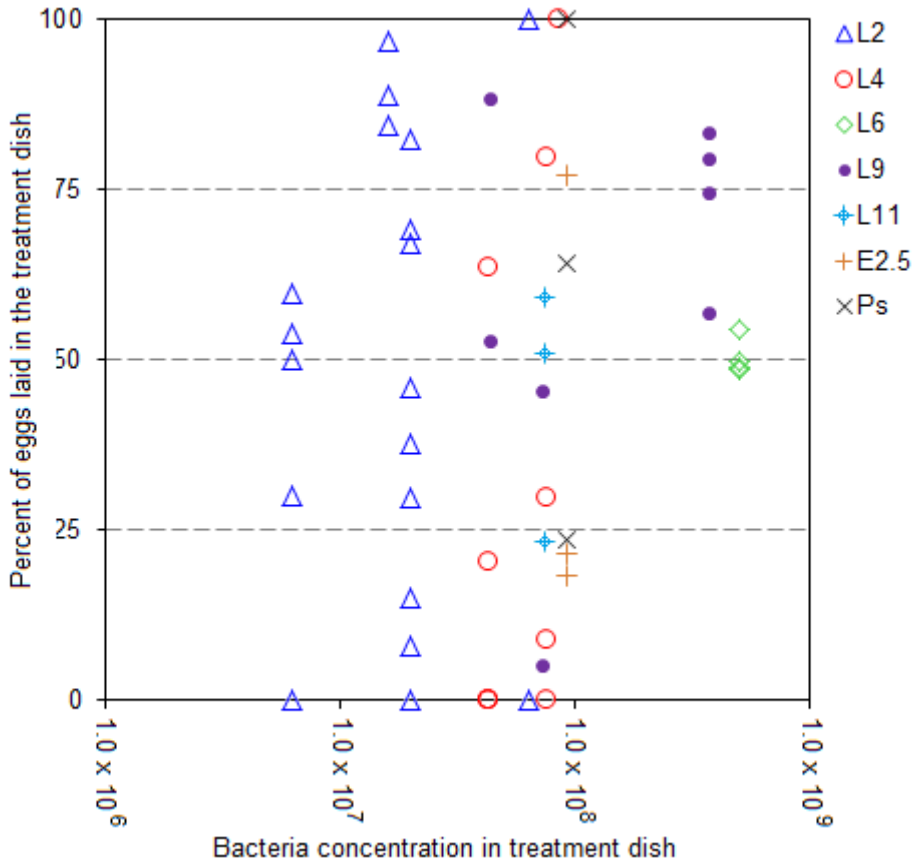


Figure 5.4 The dose of bacteria in all cage tests in relation to oviposition. The results of cage tests are shown against the concentration of the bacteria solution used in the treated dish. Results where no choice was made (i.e. no eggs laid in control or treatment) are omitted.

In most experiments eggs were laid in both dishes, and in these situations the dish with the greatest proportion of eggs was deemed to be most attractive. Total egg numbers in assays varied greatly, with L2, for example, yielding an average sum of 226.82±11.5 eggs (mean ±SE, range 17 min. -740 max. eggs per test) per assay where eggs were laid (Figure 5.5). Significant differences were seen between the numbers of eggs laid in total, i.e. the sum of eggs laid in control and treatment (ANOVA: F=3.2218, df=1,7, p=0.00561). E2.5 and L11 had significantly higher numbers of eggs, whereas Ps had markedly fewer eggs laid. The mean saline result was slightly below 50%, with approximately 45.0%±9.21% (mean ±SE) of eggs in saline vs. saline eggs laid in the nominal treatment dish (ANOVA: F=9.030, df=1,13, p=0.006).

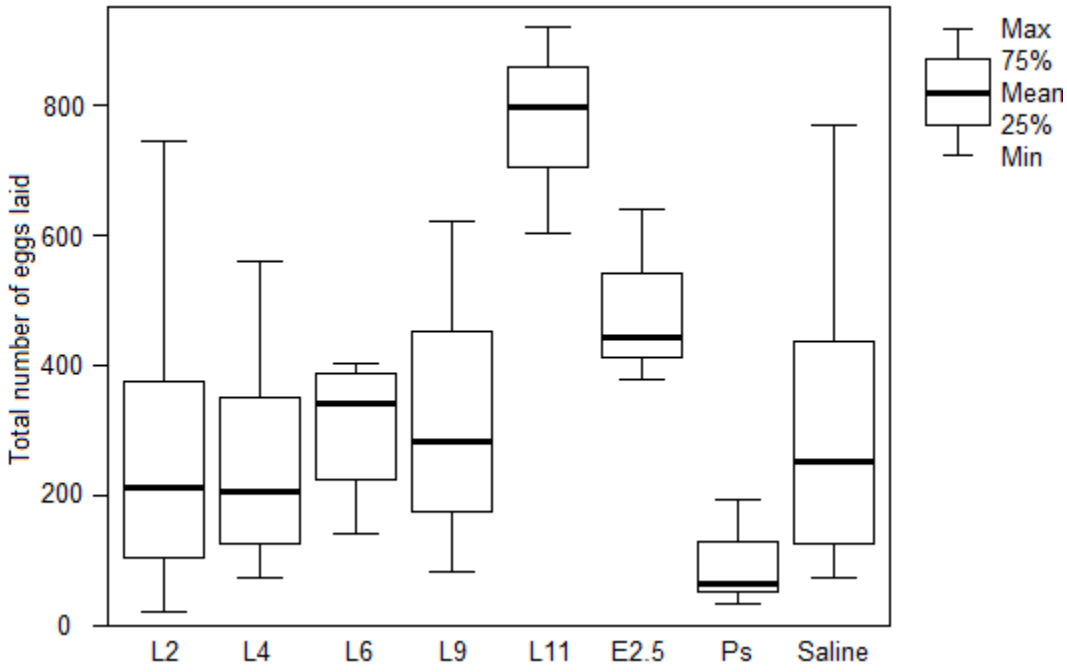


Figure 5.5 Total numbers of eggs laid in assays. Each bar shows the mean, min/max and 25%/75% range for the number of eggs counted (sum of treatment and control) in cage assays.

5.3.2 Oviposition towards 4-methylphenol containing targets

A total of 32 cage tests (6 tests per dose at doses 0, 0.001, 0.01 and 0.1mg/ml, 8 tests at 1mg/ml) were conducted, giving a total yield of 11520 eggs.

Six control vs. control (i.e. 0mg/ml 4-methylphenol in treated dish) tests gave a total of 1,394 eggs (range: 93 min. – 405 max. eggs per test) with a mean 55.2±26.84 % (mean ±SE) in the nominal treatment dish.

At 0.001mg/ml, 4,783 eggs were laid (range: min. 503 – max 1250), of which 43.5%±10.0 (mean ±SE) of eggs were laid in the treatment dish, a significant difference to the control (χ^2=26.3, df=5, p<0.001). Most eggs were laid in cages where this concentration was present.

At 0.01mg/ml the treatment dish received 29.2±12.8% (mean ±SE) of eggs laid (total: 2,166 eggs laid, range: 153 min – 574 max). This proportion fell to 15.8±8.0% (mean ±SE) of eggs laid in the treated dish at 0.1mg/ml (total: 1606 eggs range: 181 min. – 437 max.).

At 1mg/ml a total of 1571 eggs were found to have been laid from a total of 8 tests (range: 66 min. – 312 max.). No eggs were found in the treatment dish in 7 out of 8 tests. In the remaining test 1 egg out of 263 laid (0.38% of eggs in this test) was found in the treated dish, suggesting that at 1mg/ml 4-methylphenol elicits almost total oviposition repellence in gravid *An. gambiae*.

Analysis using a Generalised Linear Model with quasi-binomial errors (compensating for over-distributed residuals) showed the oviposition of females is significantly affected (GLM, 1df, F=44.54, P<0.001) by the dose of 4-

methylphenol in the treated dish. The percentage of eggs laid into the treated dish reduces as the dose increases (Figure 5.6).

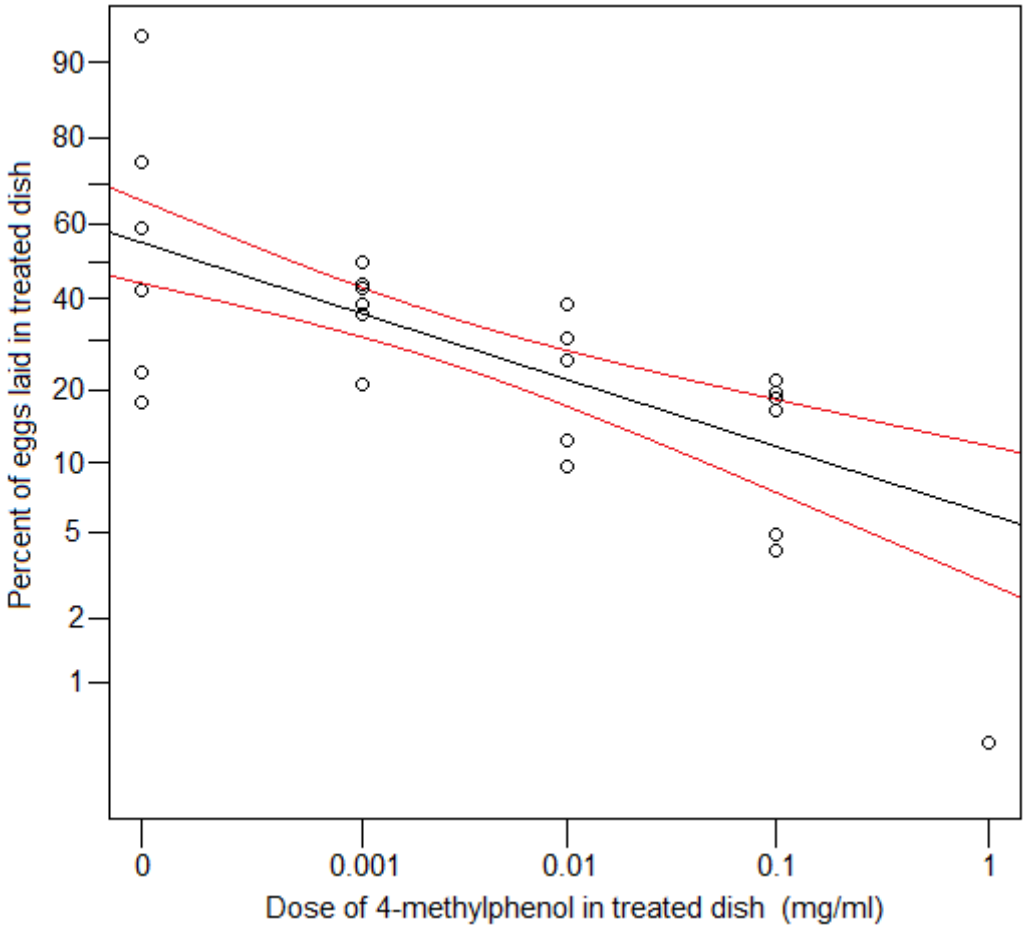


Figure 5.6 Dose response of 4-methylphenol on *Anopheles gambiae* oviposition. Logit regression of the percentage of eggs laid in the treatment dish is plotted against dose. The black line indicates GLM regression (model fit), with 95% confidence intervals (red lines).

5.4 Discussion

5.4.1 Oviposition response to bacterial solutions

A primary objective of this study was to confirm the oviposition response reported in Lindh *et al*. (2008a). This could not be achieved, despite using the same bacteria, sourced from and kindly provided by Dr J. Lindh, the lead author

of that study. Care was taken, after consultation with Lindh to replicate the conditions of the study and it was expected that a similar response would be seen. In this study it was not possible to detect any overall positive oviposition response towards any of the bacteria, despite all of them showing a positive response previously (in Lindh *et al.,* 2008a).

Some attraction was seen to bacteria L2 and L4, but only at specific doses. Only L2 was found to have a dose attractive in more than one cage test, with solutions of 1.6×10^7/ml yielding significantly more than 50% of eggs in the treatment in 9 replicates. At higher concentrations this effect was not seen. In Lindh *et al*. (2008a) L2 showed a positive oviposition response at a range of concentrations between 6.9×10^7 and 3.2×10^8.

The bacteria used in this study were provided by Dr J. Lindh, and as previously stated in Chapter 2.7.2 and Chapter 3.4, were assumed to be the same as were tested by Lindh *et al*. (2008a).

The range of concentrations of the bacterial solutions used in the cage tests was within an order of magnitude in all cases (see Table 5.1, in section 5.2.1) – better accuracy was not possible as a limitation of the experimental design. However, from the results obtained, there seems to be little relationship between dose and the proportion of eggs laid in the treatment dish (Figure 5.4).

Despite the care taken, there were a number of necessary differences in the two methodologies. First, the mosquitoes were from different sources. Lindh *et al*. (2008a) used a colony of G3 *An. gambiae*, which was begun in 1975 from material taken from the Gambia (Malaria Research and Reference Reagent Resource Centre, 2008). In this PhD study *An. gambiae* eggs were taken from

a younger colony (approximately two years old) originating from Lake Victoria, Kenya to begin a daughter colony at NRI. Both colonies were of *An. gambiae* S-form.

The cages in Lindh *et al*. (2008a) were placed into an incubator to maintain a day-night cycle, whereas in this study they were kept in controlled environment rooms in total darkness, and enclosed in transparent plastic bags to keep the appropriate level of humidity within the cages. It is possible that the bags, which were thoroughly cleaned between uses, may have altered the environment sufficiently to explain the results, but it is also likely that the small enclosed still air environment was very similar to that of an incubator.

The preparation of the bacterial solutions involved the centrifugation and pelleting of the bacteria to remove the growth media, LB-broth, before resuspending the bacteria in 0.9% saline. This was done as LB-broth has been reported to elicit a response of rejection by gravid females (Lindh *et al*., 2006). However, as Chapter 3 shows, it was not possible to completely remove the LB-broth prior to re-suspension of the bacteria, and the volatiles associated with the LB-broth were still present in the headspace of the solution. This may have caused some mosquitoes to reject the treatment oviposition target in the cage tests, although this is speculation. The centrifugation and re-suspension may also have damaged the bacteria, altering the volatile profile compared to Lindh *et al*. (2008a), although this methodology was conserved between the previous study and the work of this thesis.

Previous studies have suggested that indole; a chemical seen in the volatile profile of E2.5, L2, L11 and Ps may be responsible for oviposition responses

seen to these bacteria (Lindh *et al.,* 2008a). *Anopheles gambiae* antennae do have receptors for this chemical (Blackwell & Johnson, 2000; Meijerink *et al*., 2000; 2001; Qiu *et al*., 2006). As seen in Chapter 4 indole does elicit an electrophysiological response in *An. gambiae*. Another candidate for an *An. gambiae* oviposition semiochemical is 3-methyl-1-butanol, found in the headspace of bacteria L2, L9, L11 and Ps (Chapter 3). 3-methyl-1-butanol also elicits an electrophysiological in *An. gambiae*, and has been suggested as a likely oviposition attractant for bacteria L2, L4, L9 and Ps (Lindh *et al*., 2008a). However, the results of this study and the disparity between volatile analyses of Lindh *et al*. (2008a) and here (See Chapter 3) mean no conclusions can be drawn as to which, if any volatiles are responsible for a positive oviposition response in *An. gambiae*.

Future studies to test the oviposition responses of gravid females of *An. gambiae* to indole may reveal if indole is indeed an oviposition attractant, however the difficulties experienced in reproducing the oviposition effect reported in Lindh *et al*. (2008a) resulted in a shift in focus away from oviposition attractants, and towards 4-methylphenol, which, despite being repellent, was the only compound tested found to be behaviourally and electrophysiologically active, and hence of greater importance to the aim of the project, which as stated in Chapter 1 is "to increase our understanding of An. gambiae oviposition, in order to better understand how the behaviour of the mosquito can be exploited to reduce mosquito populations". The response to saline vs. saline also varied wildly, with an average of 43.8±34.74% (mean ±SE) of eggs laid into the "treatment" dish that contained only saline. Water is an oviposition attractant (Okal *et al*., 2013), but the results suggest that in the cage experiments

mosquitoes show a high level of variability in their choice of oviposition dish when there is no other stimulus. This underlying high level of oviposition choice variation may have been a contributing factor to the inability of this study to reproduce previously published findings.

It is therefore, difficult to draw any conclusion from the results obtained other than the mosquitoes did not behave in a way which suggests that any of the bacteria tested elicit a positive oviposition response.

5.4.2 Oviposition response to 4-methylphenol

Using cage tests, 4-methylphenol was found to be highly repellent to gravid females, showing a highly significant effect of dose on oviposition, with increasing 4-methylphenol dose reducing the proportion of eggs deposited into the treated dish. The response at 1mg/ml was better than predicted by the fitted GLM, with a single egg laid in the treatment dish compared to 1570 laid in the control, suggesting high 4-methylphenol repellency at this dose.

A high level of variance in the control vs. control tests was observed, with an average of 55.2±26.84% (mean ±SE) eggs laid in the treatment, comparing favourably with the bacterial tests, suggesting that (in this style of assay at least) control vs. control experiments can result in a highly variable choice of oviposition site, with no overall bias to one dish or another.

In Chapter 4 it was demonstrated that 4-methylphenol produces a strong EAG response in gravid *An. gambiae*, showing the capacity to detect this volatile. EAG is a useful tool in that it allows us to understand which compounds are electrophysiologically active and therefore detected by an organism, but not what the response (if any) of the organism will be. Here, by cage assay, a

behavioural response has been identified to this compound, demonstrating the useful application of multiple techniques to study the ethological responses of *An. gambiae*.

Limitations of the cage assay mean that it is not clear if the mosquitoes are repelled from the dishes containing 4-methylphenol, or if they are simply deterred from oviposition into the solutions, and this is further explored in Chapter 6 and 7.

5.5.3 Conclusions

The results obtained in this study have given two contrasting pictures. The first is that no overall oviposition preference to any of the bacteria tested can be seen, in spite of the fact that all were reported to elicit a positive oviposition response in Lindh *et al*. (2008a). It is not clear why the results of these two studies are so different.

Secondly, the cage assays used in this study (and in Lindh *et al*. (2008a)) show an oviposition response to a volatile chemical, in this case repellence from a known chemical, 4-methylphenol, with mosquitoes preferentially ovipositing in sterile saline over the chemical as dose increases.

From the high levels of variation in egg distribution seen in the saline 'control vs. control' experiments, it seems that the oviposition behaviour of *An. gambiae* is not as simple as might be expected, with half the eggs laid in one dish and half in the other. It is not possible to determine why the mosquitoes did not deposit eggs evenly in this situation. It may be that the mosquito lays all her eggs in one place, but there is evidence to suggest that "skip oviposition", where a female distributes her eggs over more than one site, occurs in *An. gambiae*

(Sumba *et al*., 2004a). It is also possible that in the small and totally dark environment of the cage the cues associated with the two water sources become indistinct from one another.

The results of these experiments also do little to shed light on the actual behaviour of the mosquitoes during oviposition. The assay cannot tell if the eggs are laid by one or several females, meaning we are unable to tell how reliable the results are – are they due to an average across the 12 females, or just a selection, or even one? Chapter 6 aims to explore this by directly observing the females as they oviposit.

The assay can also not tell how the eggs were laid, i.e. in flight, or by landed mosquitoes, raising questions as to how the mosquitoes detect repellents or attractants in the water (olfaction or contact) and, if contact is made with the water then perhaps this could be useful in IVM to deliver pesticides to adult mosquitoes. Chapter 6 and 7 investigate these questions further.

The assay cannot tell us if the mosquitoes sample the water in each dish prior to oviposition or compare the two dishes, so it is still unclear exactly how the mosquitoes discriminate between sites and if they can transfer material from one potential oviposition site to another (even if they just sample it rather than ovipositing), opening the potential for baiting one site and using this to disrupt or kill mosquitoes at others. This is also investigated in Chapter 6 and 7.

The lighting of the arena is another concern, as it is not likely that an *An. gambiae* would encounter completely dark environments in nature. They are adapted for low light conditions, such as found outside, at night when the mosquito is active (Brady, 1987). Thus, the mosquitoes will have visual cues

when seeking an oviposition site, not just olfaction or moisture detection. It is believed that mosquitoes do use vision to orientate themselves towards hosts (Kennedy, 1940) and that the visual appearance of an oviposition target can affect its attractiveness to gravid females (Kennedy, 1942). Concerns such as these have led to the use of lit assays in Chapters 6 and 7.

The cage assay appears to be a simple two choice arena, but it clearly is not. It takes no account of which mosquitoes lay eggs, their behaviour (outside of expected norms) or their engagement with the environment other than by inference through the physical evidence of the eggs which have been laid. Some of these problems, such as the question of how many mosquitoes laid, could be dealt with by using fewer, or even a single mosquito (see Okal *et al.* (2015b) for a discussion of improving two-choice assays). However, this may not be practical (as in this study) due to time, space and availability of gravid mosquitoes. The use of video to monitor such behaviours is explored in Chapter 6.

Thus, the cage assay, while useful in the case of 4-methylphenol (a compound that elicits a strong choice in gravid *An. gambiae*), is still a useful tool, assuming all one wishes to know is the oviposition repellence (or the attraction) of a compound. In order to explore the behaviour of the mosquito more deeply, a more complicated assay, such as demonstrated in Chapter 6 and 7 is required. More practically, it may prove that 4-methylphenol might have a role in deterring females from ovipositing in suitable sites that are undesirable from a human perspective – a repellent that does not alter the water of a potential oviposition site might be a useful LSM tool. Chapter 7 investigates this aspect further.

In summary, this chapter has not fully achieved the original Objective 3, of identifying behaviourally active compounds through cage assays. The cage assays could not replicate observed responses reported by Lindh *et al*. (2008a), and showed no bacterial solution to be attractive to gravid *An. gambiae* for oviposition. This outcome lead to none of the compounds of bacterial origins identified previously being tested in cage assays, as there were no clear candidate oviposition bacteria or semiochemical/groups of semiochemicals to test, resulting in Objective 4 remaining unfulfilled. 4-methylphenol, an electrophysiologically active compound, was shown to be repellent to gravid mosquitoes at doses as low as 0.001mg/ml, the effect increasing with dose, with gravid females almost totally repelled at 1mg/ml.

These results led to a major shift in research focus from oviposition attractants, to examining and obtaining more detailed information about the oviposition of *An. gambiae*, using a known electrophysiologically and behaviourally active compound (4-methylphenol), as detailed in the following chapters.

6 OBSERVATIONS OF THE EFFECT OF 4-METHYLPHENOL ON THE OVIPOSITION BEHAVIOUR OF *ANOPHELES GAMBIAE*

The aim of this chapter was to obtain detailed information about the oviposition behaviour of females of *An. gambiae* given a choice of sites with or without the presence of an electrophysiologically active compound, 4-methylphenol, by achieving Objective 5 (as described in Chapter 1.5.3); the characterisation of the flight behaviour of mosquitoes in response to single (no choice) and two (choice) types of oviposition site under semi-natural lighting conditions. This was accomplished by video-filming individual ovipositing females of *An. gambiae* in a large lit flight arena to provide the data required to determine differences in behaviour of gravid females of *An. gambiae* when one or two oviposition sites are presented, and to characterise the response to an 4-methylphenol.

6.1 Background

It has been shown that oviposition site selection by *An. gambiae* is likely to be mediated by specific volatile chemicals (Takken & Knolls, 1999) and several studies have proposed that some of these chemicals may be of bacterial origin (Sumba *et al*., 2004a; Lindh *et al*., 2008a; Rinker *et al*,. 2013). However, this line of investigation is still in an early stage; although water colonised by several species of bacteria appears to be more attractive to ovipositing females, the active bacterial compounds have not been identified, and the details as to how they affect mosquito behaviour are not known.

6.1.1 The use of bioassays to study behaviour

From the results of cage bioassays presented in Chapter 5, it is possible to infer only that bacteria have an effect on oviposition and then only from indirect evidence, such as the presence or absence of eggs in one or other oviposition dish. Alternative assays commonly used to identify behaviourally active compounds, such as olfactometers, give results limited by the experimental design, which allows for only a post hoc determination of the binary response – e.g. mosquitoes move towards a chemical or not towards a chemical. As such, the behaviour of the insect is observed in a highly artificial context, so these experiments offer little by way of revealing the underlying processes by which mosquitoes choose one breeding site over another.

Broadly speaking, the underlying processes of resource finding in insects are based on the environmental cues that are detected by an insect through their senses, and a specific motor output (response) from the central nervous system when it receives a particular stimulus. The array of stimuli and responses changes continuously as the insect moves through the environment.

The overall aim of the PhD research project presented here is to increase our understanding of *An. gambiae* oviposition, in order to better understand how the behaviour of the mosquito can be exploited to reduce mosquito populations.

The most commonly investigated oviposition-mediating chemicals are generally those that have an attractant effect (e.g. Sumba *et al.,* 2004; Lindh *et al.,* 2008a), but it is not clear what effect oviposition mediating compounds actually have. There may be no effect on the insect's taxis and the compound may act as an oviposition stimulant, or the compound may affect the insect's taxis, being an

attractant or arrestant, or conversely a repellent or deterrent (Kennedy, 1978; Clements, 1999).

After reviewing the outcome of the cage assays, it was clear that this assay was not ideal for gaining a full understanding of the role that chemicals play in any of the behaviours described above that bring mosquitoes to a breeding site, although the findings (i.e. the numbers of eggs laid in treatment and control dishes) could be a useful guide as to what might be expected when mosquitoes are given a chance to express the full range of behaviours involved with choice of oviposition sites.

The main problems limiting the usefulness of the cage assays were:

1) The results are based on indirect evidence (number of eggs laid) of a simple attraction to or repulsion from the chemical stimulus offered in each oviposition dish, offering little further insight.

2) The confined space of the cage was also potentially problematic; high doses of the test odour might create steep odour gradients, or the confined small spaces and close proximity of the walls of the cages to the oviposition dishes might lead to saturation of the arena with the test chemical very quickly. The techniques required to map the distribution of test odours in the cages to distinguish between these two extremes were not available.

To partially overcome these limitations, it would be beneficial to expand the dimensions of the experimental arena, allowing greater space for the insect to move through and to allow volatiles and local odour plumes to distribute the stimuli in a more natural analogue to real world equivalents.

3) Finally, it was not possible in the cage assays to observe the mosquitoes during their active scotophase. Since the mosquito is crepuscular/nocturnal, observation of behaviour needs to be done with appropriate lighting within the range of light intensities expected in their natural environment, which is not likely to be completely devoid of light.

Unfortunately, it is not possible to simply watch and record their behaviour directly, because these insects are haematophagous and respond strongly to the stimuli present in human odours. Therefore, observation of behaviour needed to be done remotely. A large wind tunnel constructed at NRI, Chatham, UK by Frances Hawkes, Gabriella Gibson and Ian Dublon was originally designed to film the flight of mosquitoes responding to host odours and visual stimuli (personal correspondence). With modifications to this arena, video recordings of gravid *An. gambiae* in flight and their responses towards oviposition targets were possible.

6.1.2 Oviposition timing and visual cues

Anopheles gambiae has evolved eyes suited to low light conditions. The photoreceptors within the ommatidia are modified in shape to allow them to capture more light from greater angles than diurnal mosquito species, but at the expense of resolution (Land *et al*., 1997). Their ommatidia are arranged so that the largest ones face anterio-ventrally i.e., looking forward and to the sides (Grenier, 1996; Land *et al*., 1999), which has been shown experimentally to aid navigation by following high contrasts patterns on the ground in very low light levels (Gibson, 1995) such as might occur in the environment when *An. gambiae* is ovipositing at night. They have also shown a preference for dark oviposition sites that contrast strongly against brighter backgrounds (such as

black on white) irrespective of colour (Huang *et al*., 2007). Other species of *Anopheles* mosquitoes, such as *Anopheles atroparvus* Van Tiel, have been shown to preferentially oviposit on targets on cage floors that are darker than the surrounding area, such as a black target on a white cage floor (Kennedy, 1942). Caged *An. gambiae* have been shown to oviposit preferentially into muddy water rather than clear water as light levels diminish (McCrae, 1984).

From personal observations, a puddle (about 1 cm deep), with a muddy substrate and surrounded by dry bare soil, when viewed from ~30 cm from the centre and at ~30° from ground level, the water surface is dark and glass like, reflecting the sky and stars (plus the moon if at correct angle). At high angles (i.e. from above) this changes: the substrate beneath is visible, i.e. the viewer sees through the water, rather than a reflection of the dark night sky. Under these conditions, a mosquito flying at a height of ~30 cm and ~30 cm from the puddle would see a dark object against a lighter background. As it flew over the surface of the puddle, the mosquito would then see a change in the relative brightness of the puddle and the area around it, depending on the relative darkness of the reflected sky and the puddle substrate compared to the surrounding area. This change in appearance of the puddle from a reflective surface to a translucent 'window' on the underlying substrate may be a property unique to water within the mosquito's environment, and aid the mosquito in visually identifying a water body to be a potential oviposition site.

6.1.3 Anticipated effects of an oviposition repellent

As 4-methylphenol has been shown in Chapter 5 to act as an oviposition repellent, it was hypothesised that the responses of gravid females would differ in their behaviour to oviposition sites dosed with this chemical compared to untreated sites, with treated sites being rejected over control sites. The following methodologies were devised to test this hypothesis.

It was also hypothesised that the 0.9% saline in the control dish would act as neither a repellent nor an attractant, allowing any discrimination to be made solely on the presence of 4-methylphenol in the control dish at different doses.

The anticipated differences were:

- When no choice is offered it is likely that the mosquitoes will reject the oviposition site, which will be reflected by a reduction in eggs laid, visits to the dish and duration of visit to the dish, compared to the control dish.
- When a choice, in the form of two dishes, are offered - with a clean control and a treatment dish dosed with 4-methylphenol - the mosquitoes will preferentially oviposit into the control dish. It is unclear what their behaviour near the treated dish will be, as it is not clear if the repellency is through gustation or olfaction. If it is the first, it is hypothesised that the number of visits to both dishes will be approximately even, but eggs will be preferentially laid into the control dish, and behaviours such as number and duration of landings, and the number of eggs laid per female will be reduced in respect to the treatment dish.
- If the second scenario, olfaction based repellency, is true, then it is hypothesised that fewer visits to the treatment dish than the control will

be recorded, with more eggs laid in the control but the other behaviours and number of eggs per female unaffected by the choice of dish.

6.2 Materials and Methods

6.2.1 The flight arena

The arena, shown in Plate 6.1, consisted of a large bolted metal frame (Handy Angle, Link 51, UK), to which Perspex® sheets were fitted to four sides, making a box measuring 200 cm deep x 120 cm tall and wide. The 'floor' and side walls of the box were constructed from translucent, frosted Perspex® (Polar white, cast 3 mm sheet, from The Plastic Shop, UK), allowing diffuse illumination. The roof was clear to permit filming from above.

The arena was designed to have a laminar air flow, as required, so the ends were not sealed. The upwind end of this arena consisted of a 50 cm deep x 120 cm tall and wide chamber separated from the main chamber by a fine, white nylon mesh net, affixed using hook and loop style adhesive nylon strips (i.e. Velcro®). This chamber contained a water bath (Grant JB Aqua 12 plus; Fisher Scientific, UK) and a thermostatically controlled heater (Bionaire Model BFH912; Grant, UK) to control humidity and temperature within the arena. A sheet of cardboard measuring 120 cm x 120 cm over the open end of the chamber sealed this end of the arena. As there was no air flow, terms such as down or up-wind were not suitable, and so the chambered end of the arena was thereafter referred to as the 'enclosed' end, and the opposite end was termed the 'open' end.

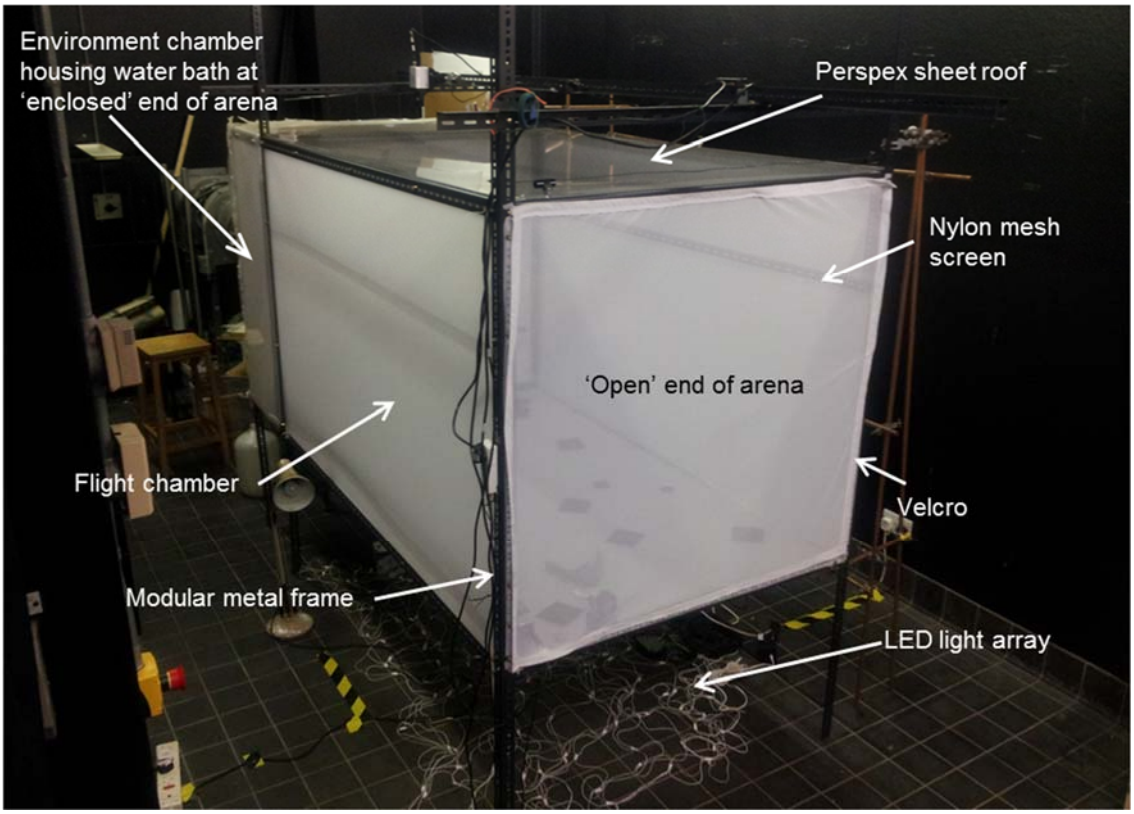


Plate 6.1 The flight arena showing the frame and Perspex construction. The mosquitoes were released into the main chamber of the arena from the 'enclosed' end. The environment section at the 'open' end contained the heater and water bath.

The open end of the arena was sealed by 120 x 120 cm nylon mesh, affixed by Velcro strips, with a sleeve towards the bottom, allowing mosquitoes to be introduced. This was covered by clear plastic sheeting, fixed with adhesive tape to maintain humidity within the arena.

An ethanol spray was used to thoroughly wash the inner surfaces of the arena after each experiment, to prevent volatiles contaminating the plastics. The cardboard was removed and air passed through to prevent accumulation of volatiles from the experiment or cleaning.

The environmental conditions within the arena were maintained by the water bath, which was set to heat the water to 90 °C. The bath was regulated by a timer (Masterplug 24 h mechanical timer, Robert Dyas, UK), which switched it on for 30 min, then off for 30 min, as required. The temperature in the main room was controlled centrally. This system kept the environmental conditions within the arena at 27°C±1°C and relative humidity at 70%±10% over a period of up to 8 h, after which the water in the bath fell to below a safe level and needed to be refilled. These conditions reflect those of Lindh *et al.* (2008a), and at the ICIPE field station in Mbita Point, Nyanza, Kenya (e.g. Wang *et al.,* 2013).

6.2.2 Illumination in the arena

The arena was illuminated from beneath by 3 m x 3 m web of 208 clear LEDs (Kontsmite, Sweden) positioned evenly on the floor of the room 60cm beneath the translucent floor of the arena (Plate 6.2). This was done to simulate the level of light that might be experienced during nocturnal conditions; specifically the light level was designed to be similar to that of moonlight reflected off a soil background about half an hour after dusk, with an approximate ambient light intensity of 0.01 Lux. The environment which a gravid female of *An. gambiae* will oviposit under natural conditions will not be in total darkness, due to illumination from star and moonlight (Brady, 1987).

Plate 6.2 The flight arena as lit for experiments. The light-emitting diode (LED) net on the floor can be seen illuminating the translucent Perspex base of the main flight arena.

Appropriate levels of illumination were determined using a hand-held light meter as described by Young *et al.* (1987), to record the natural light levels during sunset. This light meter measures the level of illumination evenly across at wavelengths from 420-900 nm, and can be used to calculate the total illumination in W/m^2.

This is not ideal, because the sensitivity of mosquito eyes to wavelengths of light varies across the spectrum. However, a flat spectral response photocell is less biased than the more commonly used 'lux meter', which is matched in spectral sensitivity to the human eye and quite different to that of mosquitoes.

A lens (4 cm aperture, 20 cm focal length, Kern Model H16 RX, supplier unknown) was fitted over the detector to approximate the mosquito field of view (of approximately 40° (Land *et al*., 1997)). The reading from the meter was then divided by the solid angle of the lens to give the radiance of the substrate being observed (expressed in watts per meter squared per steradian).

Recordings were also made of the radiance of the sky and several substrates using the photometer in late summer at St Mary Hoo (north Kent, UK). Substrates measured were sand, sand submerged in 1cm of water and short grass.

To permit filming of the mosquitoes, in addition to the dim white light described above, the arena was lit from beneath and from the side with infrared emitting LEDs (Tracksys, UK). These allowed cameras sensitive to infrared (such as used in security applications) to view the mosquitoes as backlit silhouettes against the arena interior, without altering the visual environment of *An. gambiae* which, like *Aedes aegypti* (Snow, 1971; Muir *et al*., 1992), appear to not be sensitive to light at wavelengths in the infrared region of the spectrum (Gibson, 1995).

Seven arrays emitting light at 880 nm, with a beam angle of 40° illuminated the arena floor from beneath. These were positioned to give an even illumination across the floor of the arena. Three further arrays were positioned, facing away from one wall of the arena, illuminating a brushed cotton sheet suspended 30 cm from the lights. The reflected infra-red light provided a diffuse background to one arena wall, permitting a camera to film horizontally against this background lighting.

This arrangement of lights permitted the filming of the arena from above and the side.

6.2.3 First preliminary study to establish experimental protocol

Preliminary work by the author suggested that in the large arena *An. gambiae* oviposits a greater number of eggs in muddy water than clean, but this was inferred through the number of larvae counted later, as it was not possible to determine the number of eggs laid in the muddy water. On the day of the experiment 20 gravid mosquitoes were placed in the arena with two identical clear glass oviposition dishes (15 cm diameter, 1 cm depth) placed 15 cm apart (30 cm distance between centres of the dishes). This is approximately the same distance as between oviposition dishes in cage tests (Chapter 5), but the surface area of liquid in each dish is increased by approximately 250% in this experiment (approximately 70 cm^2 in cage tests vs. 177 cm^2 here).

Both dishes contained 0.9% saline to the brim of the dish, but one dish also had a layer of mud on the bottom (collected from a garden in Chatham, Kent), that had been allowed to settle, so that the water itself was clear. Lighting approximated nocturnal starlight conditions and the dishes were removed after 4 h. This experiment was not repeated.

6.2.4 Second preliminary study to establish experimental protocol

A second preliminary assay was conducted to determine whether the observed preference for muddy water was due to its visual appearance (i.e. the effect of 'dark' water contrasting more against the white floor of the arena than clean water against the white floor) vs. odours associated with organic matter that caused the muddy appearance.

For the first test of this assay, both dishes contained 0.9% saline to the brim of the dish, but had no visual cues distinguishing them from one another or the floor of the arena.

This assay was repeated, but with one dish placed on top of a sheet of black plastic measuring 2.5 mm thick x 5 cm wide x 25 cm long (Instrument Plastics Limited, UK), and the other on the arena floor with no backing. This sheet appears opaque in visible light, but is transparent to infrared light, providing a sharp visual contrast between the black sheet and the white floor visible to the mosquitoes, but also permitting filming under infrared light through the sheet without obscuring the area being filmed.

6.2.5 Third preliminary study to establish experimental protocol

It is known that mosquitoes oviposit during a limited period of the 24 h day. Sumba *et al.,* (2004b) found that *An. gambiae s.s*. colonies exhibit peak oviposition activity 1-2 h after sunset, with a second bout of activity 2 h later. However, it has also been shown that the time mosquitoes oviposit is affected by factors such as the time of day at which blood feeding took place, temperature and the geography of the area (McCrae, 1983).

To determine the optimal oviposition period to observe the strain of *An. gambiae* used for this research project, the behaviour of 20 gravid females was recorded per night, with a different set of females each night for three nights. The gravid females were placed in the arena with a water-filled dish (25 x 15 cm and 1 cm deep), which was replaced each hour for the first 6 hours of the scotophase. The dish was also replaced at 12 and 24 h. The scotophase, 'night' conditions,

were maintained for 12 h, after which the arena was illuminated fully with the external room lights on for the photophase.

6.2.6 Fourth preliminary study to establish experimental protocol

In order to determine the optimal number of mosquitoes for filming, a video camera was placed in the arena (see Section 6.2.7 for details), facing an oviposition dish containing 0.9% saline which was positioned centrally in the arena on a black background.

Video recordings of the dish were made for the first four hours of the scotophase, with five, 10 or 20 mosquitoes released in the arena. After four hours the dishes were removed, the number of eggs laid recorded and the arena was cleaned. The recordings were reviewed and the number of flight tracks (i.e. visits) recorded. No other behaviours were recorded from these observations at this point.

Determination of optimum number of mosquitoes for experiments used a GLM with poisson error for relationship of eggs and female visits (Chapter 6.3.5).

6.2.7 Arrangement of cameras in the arena

To film oviposition behaviour the cameras were placed inside the arena, with a camera filming from directly above and a second filming from the side, aligned so that the optical axis of the camera lens was level with the top of the oviposition water level, so it would be easier to tell how close the mosquitoes came to the water.

Handy Angle, as used to construct the arena frame, was bolted to the interior of the arena, with two parallel beams positioned 90 cm from the arena base along

the length of the arena, to which a cross piece was attached. A third length-wise span was positioned on the opposite side of the arena to the oviposition dish, 15 cm from the arena base. Adjustable camera mounts were secured to these and Samsung SHC-735P analogue, high resolution, wide dynamic range cameras (Samsung, Korea) were attached to this framework (see Plate 6.3).

Samsung SLA-550D lenses were fitted to the cameras (Samsung, Korea) and adjusted so that the whole dish was in focus. Camera 1 (side view) was positioned to film directly along the meniscus of a filled dish, and Camera 2 (top view) was positioned to film directly down onto the dish. When a second dish was added for two-dish recordings, an additional cross bar and camera mount were bolted into place and a third camera (Camera 3) was added next to Camera 2, looking down on the second dish. The cameras were focused and levelled using spirit-levels along three axes and locked into position.

Small clear tape markings (two intersecting strips approximately 0.5 cm x 1.5 cm) were used to align the black target bases on the platforms they sat on, so the cameras did not need to be realigned or refocused for each experiment. Dishes were positioned centrally on the dark backgrounds.

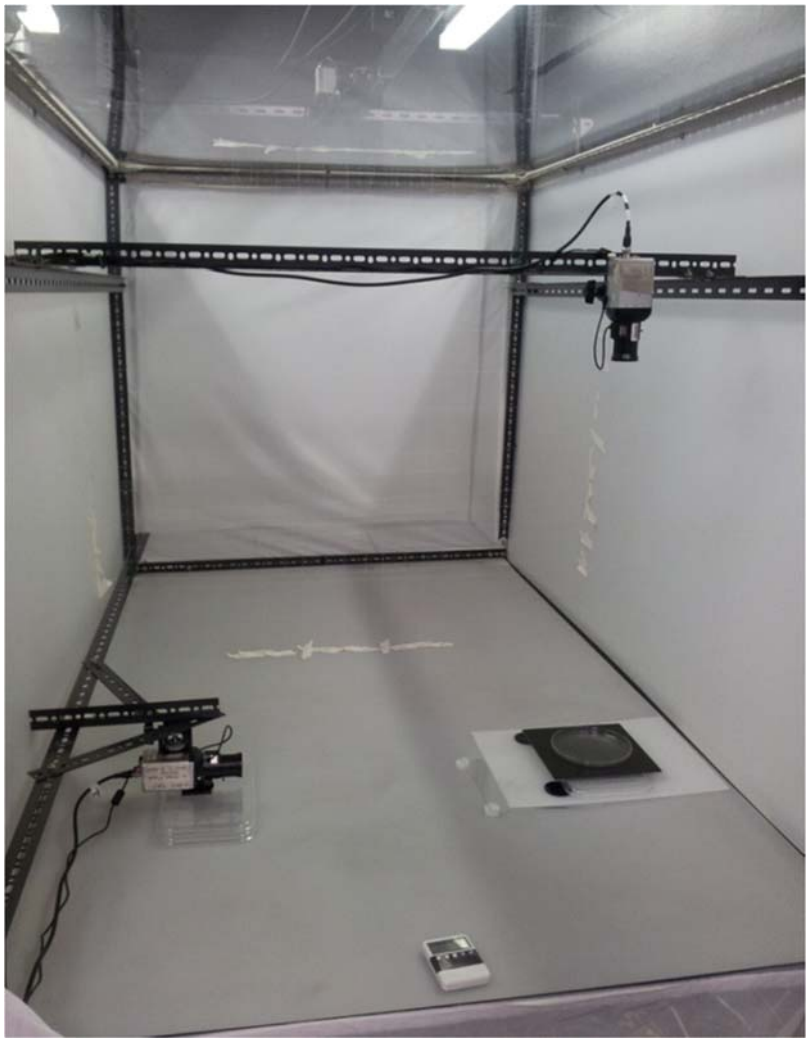


Plate 6.3 The interior arrangement of the arena, looking towards enclosed end. The positioning of the cameras and the one-dish scenario oviposition dish. For two-dish scenario experiments the dishes are positioned in the same place as the single dish, with a second camera looking down from above. Camera views are centred on the dish (or dishes). A humidity meter is also present (seen at the bottom of the plate).

6.2.8 Chemicals tested

As shown in Chapter 5, 4-methylphenol acts as on oviposition repellent to *An. gambiae* and it was decided to examine the responses of the mosquito to this chemical further. No further tests were made of the bacterial solutions as there was little clear evidence of an oviposition effect from any of the seven tested. Low numbers of replications for some bacteria tested may be responsible for

this, however power calculations suggest that the effect reported in Lindh *et al*. (2008a) should be observable even given the number of replicates used in Chapter 5.

Pile *et al.,* (1991) reported that a putative oviposition attractant for *Culex quinquefasciatus* induced behavioural differences in the mosquito, specifically upwind anemotaxis towards the odour source and an orthokinetic (a response of change in speed to a change in stimulus intensity) reduction of flight speed once in the proximity of the oviposition site. This supports the hypothesis that the responses of gravid females would differ in their behaviour to oviposition sites dosed with 4-methylphenol compared to untreated sites.

6.2.9 One-dish scenario

A large still air flight arena (Section 6.1), measuring 120 cm wide, 120 cm tall and 200 cm long was maintained at a temperature of 27°C±1°C and a relative humidity of 70%±10% (confirmed by a data logger during each experiment). The arena floor was evenly lit from below using enough white-light LEDs to simulate night lighting conditions and with infrared lights to illuminate the video cameras. One long side of the arena was also lit using indirect infrared illumination.

An oviposition target consisting of a large Pyrex petri dish, of 15 cm diameter and 1 cm depth (Fisher Scientific, UK), was positioned centrally on a 25 cm x 25 cm black, infrared transparent Perspex sheet, which in turn was positioned on a levelled platform. The platform was positioned with one edge on the infrared illuminated wall, towards the open end of the arena (to enable access for filling). The centre of the target was 50 cm from the open end of the arena.

Solutions tested were 0.9% sterile saline (control) and 0.9% sterile saline into which a known amount of 4-methylphenol (98% 4-methylphenol, 2% 3-methylphenol; Sigma-Aldrich, UK) had been dissolved (treatments). The doses of 4-methylphenol tested were: 0.001, 0.01, 0.1 and 1 mg/ml. Eight replicate (i.e. 8 nights x 20 fresh mosquitoes per night) were made of 1mg/ml 4-methylphenol dose (six replicates plus two additional replicates included from preliminary studies). Six replicates of each of the remaining doses were tested. Eight replicates of the control were tested (as for the 1mg/ml 4-methylphenol dose, six replicates plus two additional replicates included from preliminary studies). Treatments were presented in a random order, determined by randomised Latin square.

Sample size calculations were made to ensure a suitable predicted effect size could be obtained from this number of replicates for dose/response model GLMs using R as follows:

Total sample size = 34; Numerator df = 1; Denominator df = 32; Significance level = 0.05; Power = 0.8

Giving a predicted effects size of 0.25, classified as SMALL. The number of replicates used can therefore detect effects of a small size.

The solutions were made the morning of the experiment and the dish was filled immediately prior to experimentation. The arena open end was sealed using a fine, white nylon mesh net, affixed with Velcro adhesive strips and temporarily sealed with sheets of transparent plastic, which was secured with adhesive tape.

The platform was positioned so that the target would be aligned and in focus for two Samsung SHC-735P cameras (Samsung, Korea), each fitted with Samsung SLA-550D (Samsung, Korea) lenses. These f. 1.4 lenses are vari-focal, have an adjustable zoom and adjustable focal length of 5-50 mm. The cameras were powered using model 188-725 12 v adaptors (RS Components, UK) and connected to by BNC cable to a digital video recorder (DVR), model SRD-470D (Samsung, Korea). The DVR permitted the simultaneous viewing of the target while recording took place. Film was recorded on a DVR internal hard drive and backups were made after filming had taken place.

The cameras were fitted to mounts, held in place by a metal frame (Handy Angle, Link 51, UK). Camera 1 was positioned so that the centre of the image was along the axis of the meniscus of filled target dish, with a field of view wide approximately 15 x 11.5 cm at 80 cm from the lens (viewing angle = c. 11°). The camera lens was positioned 80 cm from the centre of the dish.

Camera 2 was positioned above the target, with the lens 80cm from the meniscus, with the image centred on the dish and a field of view approximately 16 x 12 cm at 81 cm (viewing angle c. 11.5°) from the lens (Figure 6.1). The cameras and DVR were powered up prior to preparation of target to ensure the dishes positioning and the focus was maintained.

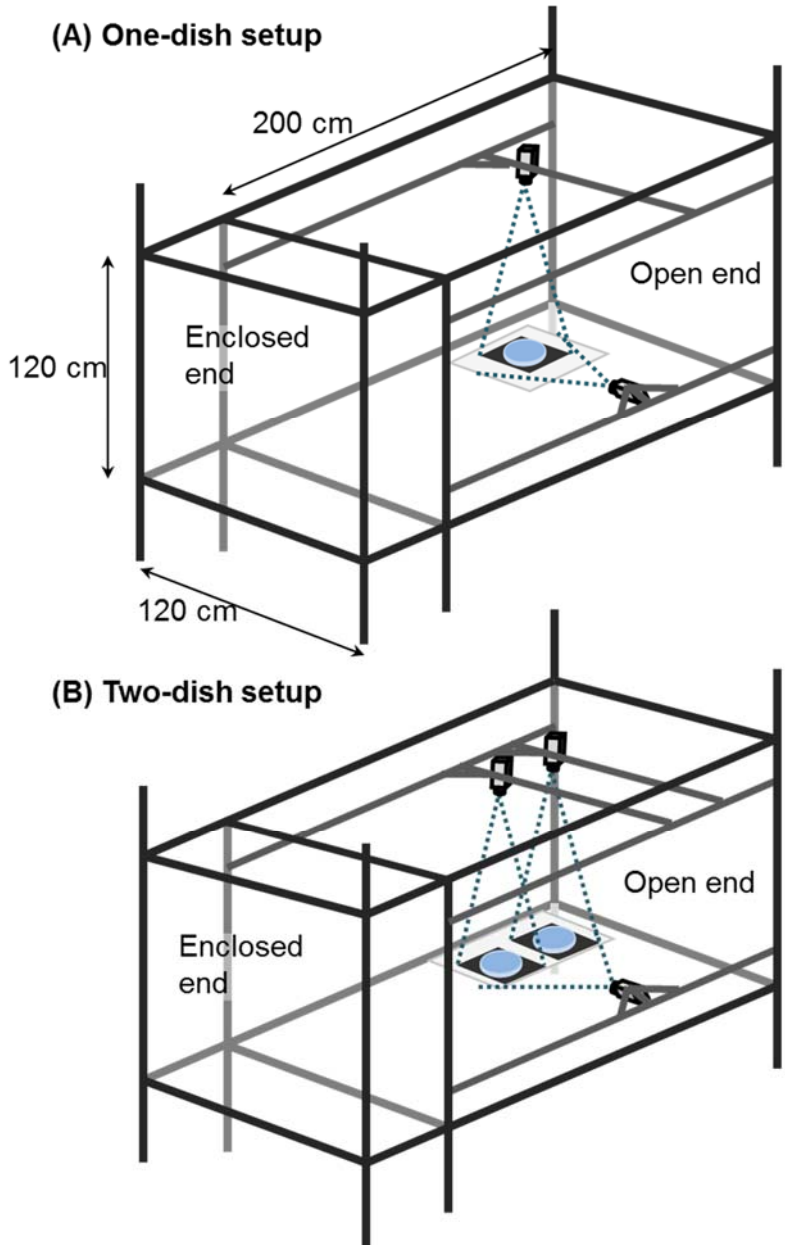


Figure 6.1 Arrangement of the cameras and oviposition targets in the arena. The one-dish setup utilises one side camera and one downwards facing camera observing a single target. B) The two-dish setup has one side camera observing two dishes, with two downwards facing cameras, each observing one of the targets.

For each run of the experiment an oviposition dish was prepared with the appropriate water treatment and placed in the flight arena. Twenty gravid mosquitoes, reared as per Chapter 2.2 and blood fed 48 h prior to experimentation, were selected based on the appearance of their abdomens

(Chapter 2.3) and transferred manually to a mesh covered container. The container was placed in the prepared arena chamber at the open end, through the sleeve of the mesh screen. At the beginning of the scotophase the DVR began recording, the mosquitoes were released into the flight arena and the emptied container was removed. The room lights were extinguished, leaving the arena lit only from beneath and by infrared light.

Recording took place for the first 4 hours of the scotophase. Once recording had finished, a backup copy was made, the room was illuminated and the water bath was deactivated and drained. The number of eggs in the dish was recorded, the contents of the dish drained and mosquitoes were removed and disposed of. The dish and the arena interior were then cleaned using ethanol and wiped down to remove any remaining odour. The arena was left open to dry overnight.

6.2.10 Two-dish scenario

For two-dish experiments the procedure differed from the one-dish experiments by the use of two targets and an additional camera. The interior of the arena was maintained at a temperature of 27°C±1°C and a relative humidity of 70%±10%, confirmed by a data logger during experiments. The larger platform was positioned and levelled at the open end and two infrared transparent black 25 cm^2 Perspex sheets were positioned side-by-side with a 5 cm gap between them. The dishes were positioned centrally on the black sheets. One was designated the control and filled with sterile 0.9% saline.

The second was designated the treated dish, and a paper marker was placed under the black backing (visible in recordings but not in ordinary light) to

differentiate between the two dishes. The treated dish was then filled with 0.9% sterile saline, as in Section 6.2.9, into which a known amount of 4-methylphenol had been dissolved. Four doses were tested (0.001, 0.01, 0.1 and 1 mg/ml) and four replicates were made of each dose vs. control. Four replicates were tested of control vs. control. Sample size calculations were made to ensure a suitable predicted effect size could be obtained from this number of replicates for dose/response model GLMs with binomial errors using R as follows:

Total sample size = 20; Numerator df = 1; Denominator df = 32; Significance level = 0.05; Power = 0.8

Giving a predicted effects size of 0.44, classified as MODERATE. Thus, even with sample sizes = 4, moderate effect sizes were detectable.

The position of the control and treatment dish was changed for each test, and the dishes, having been thoroughly cleaned prior to experimentation, were also selected by randomised Latin square. Order of treatments was also selected in this fashion.

Camera 1 was aligned to view both dishes, with the meniscus of both in the centre of view, giving a viewing angle of approximately 30°. Camera 2 was moved and positioned above the dish closest to the enclosed end of the arena, centred on the dish. Camera 3, identical to Camera 2, was fitted to a new bar above the dish nearest the open end of the arena (see Figure 6.1 for a comparison of the one-dish and two-dish setups). The mosquitoes, filming and cleaning procedures were the same as for the one-dish procedure.

6.2.11 Scoring the observed behaviour

The recordings were watched live to ensure no problems occurred during the experiment. The recordings were backed up onto portable media and then reviewed using VLC media player (version 1.1.9, VideoLAN project). For both scenarios most of the data was collected by reviewing the bird's-eye-view recordings, including; 1) clock time mosquito entered the camera view (i.e., 'beginning of a visit'), 2) clock time mosquito left view (end of a visit), and occurrence of the following behaviours; 3) egg laid, 4) type of egg laying (in flight or landed on the water), and 5) number of eggs laid per visit.

Additional qualitative information was gained from the side-view recordings, including the general posture of the mosquito and angle of the abdomen. In the case of the two-dish scenario, the bird's-eye-view of each dish was on a separate recording (as they were observed by separate cameras). These recordings were reviewed separately and care was taken to identify which dish (control or treated) was being reviewed.

The results of the reviewed observations were recorded in note form and then entered into a spreadsheet. From these raw data the following measures were calculated:

- Mean duration of visits per replicate (seconds a given mosquito was in view of the camera)
- Mean number of visits per replicate when at least one egg was laid
- Mean number of visits per replicate when at least one egg was laid in flight

- Total number of visits over all replicates when at least one egg was laid in flight
- Mean Number of visits per replicate when at least one egg was laid by a landed mosquito
- Total number of visits over all replicates when at least one egg was laid by a landed mosquito
- Mean number of in-flight eggs laid per visit
- Total number of in-flight eggs laid per treatment
- Mean number of landed eggs laid per visit
- Total number of landed eggs laid per treatment

These measures were then analysed statistically to determine differences between behaviours within treatments or against other doses/control tests. The measures were analysed using the statistical package R as per Chapter 2.6. Data was analysed by generalised linear model (GLM) with quasi-binomial errors to compensate for over-distribution. Chi-square tests for count data and analysis of covariance (ANCOVA) for dose responses were used to compare the degree of similarity of behaviours between scenarios.

6.3 Results

6.3.1 Comparison of illumination in arena and natural scenarios

Recordings were made of the radiance of the sky and several substrates in late summer at St Mary Hoo (north Kent, UK). Substrates measured were sand, sand submerged in 1 cm of water and short grass.

The radiance of the arena floor was approximately the same as the luminance from all three substrates at St Mary Hoo approximately 15 min post sunset (Figure 6.2) and is similar to the recorded light intensity of full-moonlight near the equator (Brady, 1987).

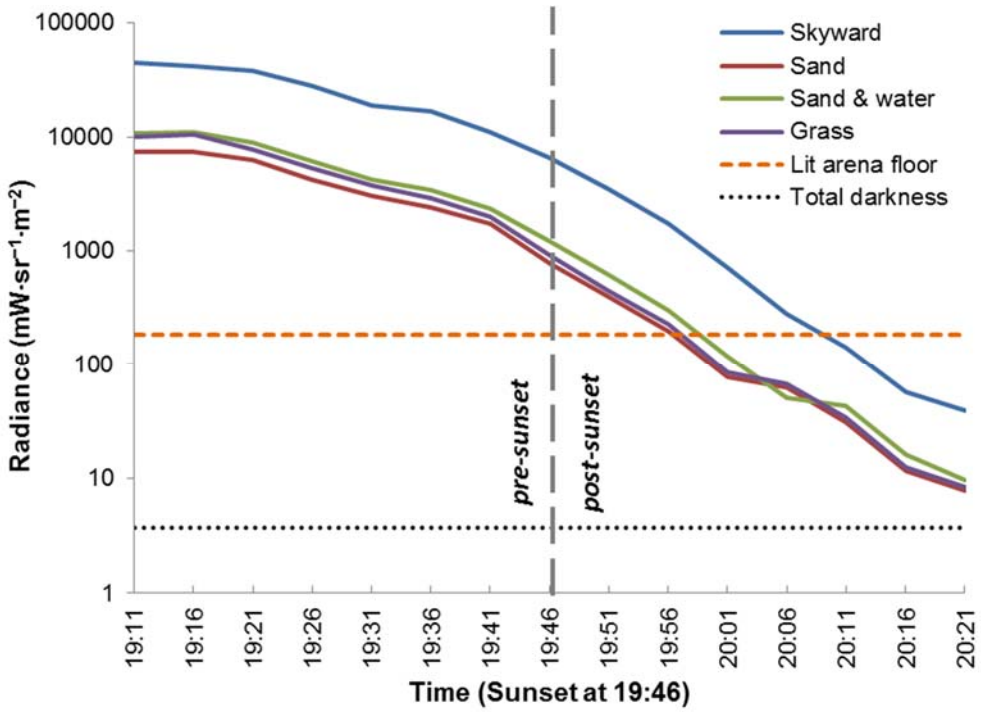


Figure 6.2 Measured radiance of different substrates. Radiance of different substrates on the ground from dusk to post sunset compared to the arena floor when illuminated and the arena in total darkness. Radiance levels are given in milliwatts per steradian per meter squared.

6.3.2 First preliminary experiment to investigate influence of mud in water on oviposition

After allowing mosquitoes to choose between a dish containing saline and a dish containing muddy water, the control dish was inspected and found to contain 46 eggs. The muddy dish was unfortunately disturbed when removing it from the wind tunnel, making it impossible to count the eggs laid. Instead the muddy dish was inspected each morning after the experiment for new larvae, which were counted and then removed. After three days a total of 375 larvae had hatched in the muddy dish, compared to the 46 eggs laid in the control dish.

Mud was chosen as mosquitoes are seen to oviposit into temporary pools formed in mud, such as those from which bacteria were isolated by Sumba *et al.* (2004) that were later reported to be oviposition attractants (Lindh *et al.,* 2008a). However, the mud around Mbita Point consists of tertiary sediments on volcanic rocks, whereas the mud used in this study was isolated from northern Kent and consisted of London clay (Sumbler, 1996), thus providing a poor analogue. This experiment was not repeated, primarily due to the difficulties in recording egg numbers, but also because of the author felt the mud used did not reflect that found in the natural environment of *An. gambiae*.

6.3.3 Second preliminary experiment to measure effect of background on oviposition

When mosquitoes were allowed to choose between two identical dishes containing saline, eggs were counted the next morning, and were found to have been deposited in both dishes but in low and nearly equal numbers in each dish; the numbers of eggs laid in each pair of dish for the three replicates were: 163:149; 71:69 and 185:163 (χ^2=0.24, df=2, p=0.89).

In the following experiment, significantly more eggs were laid in the dish placed on the black plastic square; black bottomed dish vs. clear bottomed dish for the three replicates: 493:28; 723:8 and 575:63, (χ^2=52.96, df=2, p<0.001), and significantly more eggs were laid in total than when no background was used in the first test (χ^2=116.14, df=2, p<0.001), suggesting the use of a dark cue beneath an oviposition target leads to a greater number of females ovipositing, as per Kennedy (1942).

6.3.4 Third preliminary experiment to determine timing of oviposition

An initial bout of oviposition activity occurred in the second hour after the beginning of the scotophase, as reported by Sumba *et al.* (2004b) and indicated by Fritz *et al*. (2008), but there was no sign of a second peak. Oviposition continued after the first peak, but at a reduced rate. Eggs were also laid during the photophase, when the room lights were on (Figure 6.3). An average of 574 eggs were laid per night, or 28.7±3.31 (mean ±SE) eggs per female.

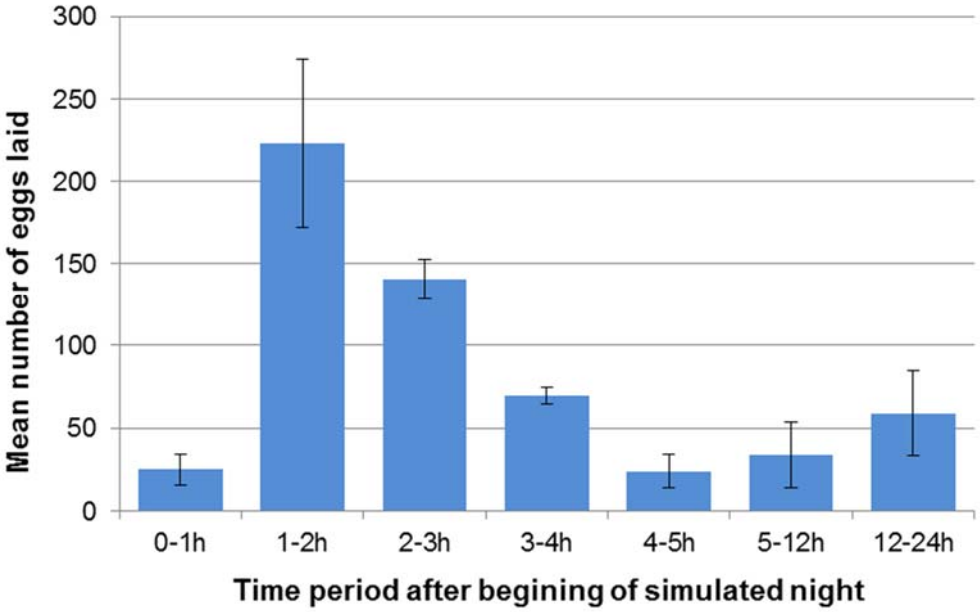


Figure 6.3 The timing of mosquito oviposition in the arena. Most eggs were laid during the second and third hours after the lighting in the area was dimmed to simulate night. Graph shows mean eggs counted/per hour, per replicate, for three replications ±1 SE, with 20 gravid mosquitoes/replicate in the flight arena.

Thus, it was decided that a filming period need only include the first four hours of the scotophase to capture most of the ovipositions that would occur over a 12h night.

6.3.5 Fourth preliminary experiment to determine optimum number of mosquitoes for experiments

Initial video recordings of oviposition of five mosquitoes resulted in very few flight tracks (i.e. 'visits') observed (12, 5 and 19 flight tracks in three replicates, respectively, averaging 2.4±1.14 (mean ±SE) visits per female), and no eggs laid. A greater number of visits was observed with ten mosquitoes per replicate (28, 52, 34 visits/replicate, 3.8±1.02 (mean ±SE) visits/female) and eggs laid during the same period (14, 18, 9 eggs/replicate, average 1.4±0.37 (mean ±SE) per female). Twenty mosquitoes gave a higher number of visits (58, 47, 51 visits/replicate, 2.6±0.23 (mean ±SE) visits/female) per replicate, and a higher number of eggs (74, 49, 63 eggs/replicate, average 3.1±0.51 (mean ±SE) eggs/female).

It seems likely that that not all females oviposit in the allotted time, but by increasing the number of females in the arena the chance of viewing a female oviposit increases. A GLM with poisson error distribution demonstrated that there is a reliable relationship between number of visits and number of eggs laid

above about 30 visits, in a group of 10 mosquitos. A group of 20 was chosen to reduce the chances of highly variable data (Figure 6.4).

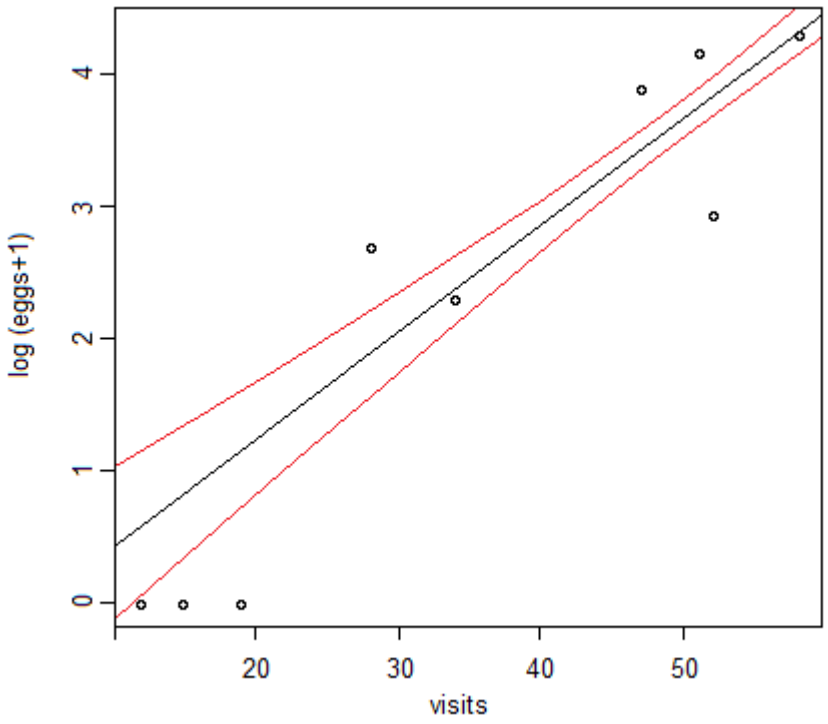


Figure 6.4 Predictions of the relationship between egg numbers per female and visits. Modelled on 10 females from a generalised linear model with poisson error distribution (red lines are 95% CIs for the regression line)

6.3.6 Effect of 4-methylphenol on oviposition in one dish scenario

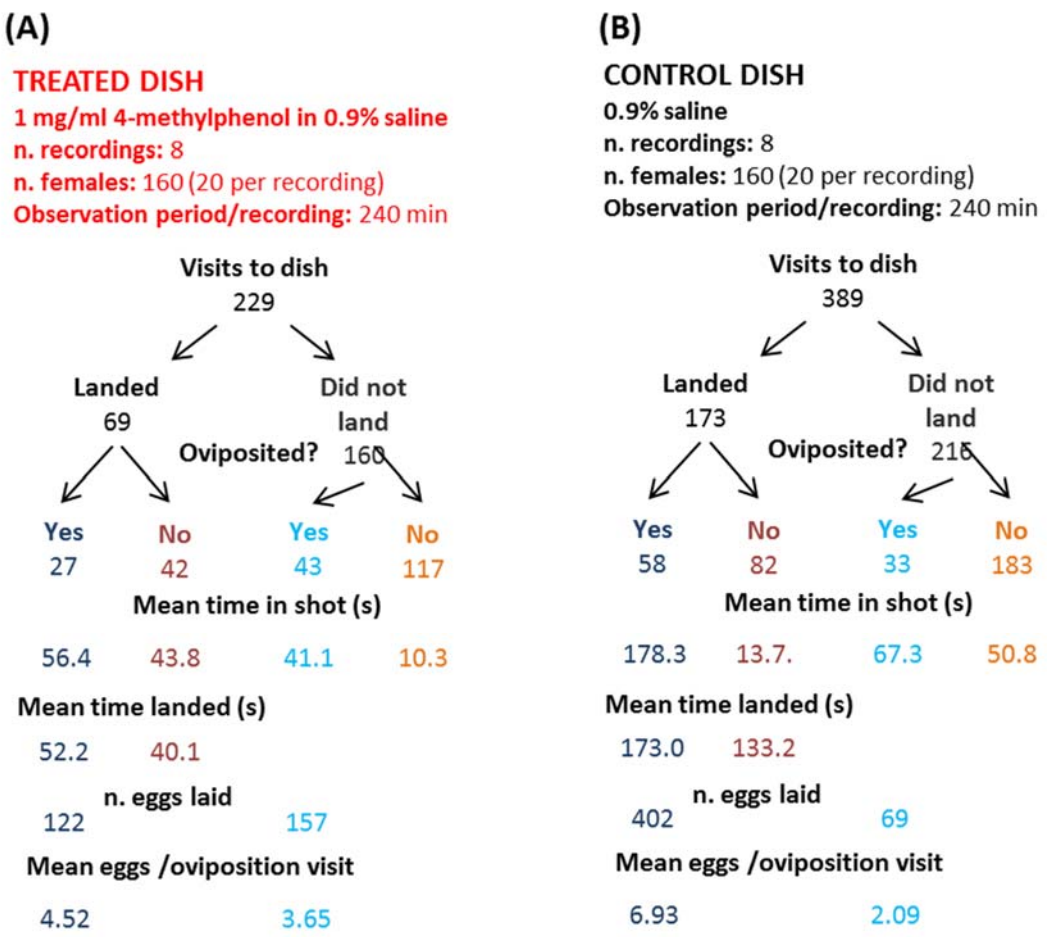
Video recordings were made of the oviposition responses of 20 gravid *An. gambiae* females released into in a large arena with simulated moonlight conditions to a target oviposition dish. Recordings consisted of the following: eight replicates of 1 mg/ml 4-methylphenol in 0.9% saline, six replicates each of 0.1 mg/ml, 0.01 mg/ml and 0.001 mg/ml and eight replicates of the control (0.9% saline).

Mosquitoes were seen to oviposit into the control water as expected, but also in the treated water containing the highest dose of 4-methylphenol (1 mg/ml),

which previous cage tests had shown to be repellent to ovipositing females in two-choice assays (Chapter 5). This suggests a level of plasticity may exist in the mosquito's responses to a given stimulus under different environmental conditions. The first analysis of the data was to compare the responses to the highest dose of 4-methylphenol and the control (Figure 6.5).

Mosquitoes made fewer visits to the dish treated with 1 mg/ml 4-methylphenol (229 total visits observed, averaging 28.6±6.63 visits (mean ±SE)) than to the control dish (389 total visits, mean (±SE) 48.7±2.44 per replicate; χ^2=25.09, df=7, p<0.001). Of these visits, a significantly lower proportion of visits to the treatment dish ended with landing (30%), compared to 44% of visit to the control dish (χ^2=31.88, df=7, p<0.001).

It is important to note, however, that as it was not possible to distinguish individual mosquitoes; a visit simply refers to any female seen on the recordings to enter camera shot, and therefore enter the vicinity of the dish. Therefore, 'visits' is considered to represent the relative amount of time mosquitoes spent near the target dish. It is not known how many, or if all the mosquitos released did or did not oviposit.



<u>Figure 6.5</u> Summary of behaviour in the presence of a single oviposition dish. A) Shows the combined totals from eight nights of recordings where the mosquitoes had only a treated dish present, at the highest dose tested (1 mg/ml 4-methylphenol), B) shows combined data from eight recordings where the control dish was present. New mosquitoes were used each night. Total numbers over all replicates are given, however for statistical purposes means were used for analysis. Note, means shown here are means of the replicate means, i.e. "mean eggs/oviposition visits" is the mean number of eggs per replicate per mean oviposition visits per replicate.

Females did not oviposit in every visit recorded, and those that did oviposit, laid a variable number of eggs. The mean number of eggs laid per oviposition was significantly different between the control (5.1±0.75 (mean ±SE)

eggs/oviposition visit) and the treated dish (3.9±0.58 (mean ±SE) eggs/oviposition visit; χ^2=32.96, df=7, p<0.001), which suggests that 4-methylphenol has an effect on both the number of visits to a dish as well as the mean eggs/oviposition visit.

Additionally, it appears that oviposition by *An. gambiae* has more than one mode; mosquitoes were observed to oviposit either while landed on the water or whilst in flight.

In the first mode, termed “landed oviposition” the female approached the oviposition dish and landed on the water’s surface. Once landed, the female rested on the water with the end of the abdomen raised slightly above parallel to the water’s surface (see Plate 6.4). It was not possible to see from the recordings if the females used their proboscis to sample the water.

Oviposition itself took place with the abdomen in the same upright position. The end of the abdomen could be observed to twitch (more obviously seen from above) as the egg was passed out, but the abdomen was not lowered into the water itself. The eggs sometimes simply fell into the water, or, alternatively, were seen to be manipulated by the hind legs of the female. The hind legs did not assist the egg in leaving the abdomen, but the eggs can be seen ‘running’ down their legs and the female then moved the egg away by slight movements of her hind legs. Several eggs were observed to be laid by individual females in a single egg-laying bout during this mode of oviposition.

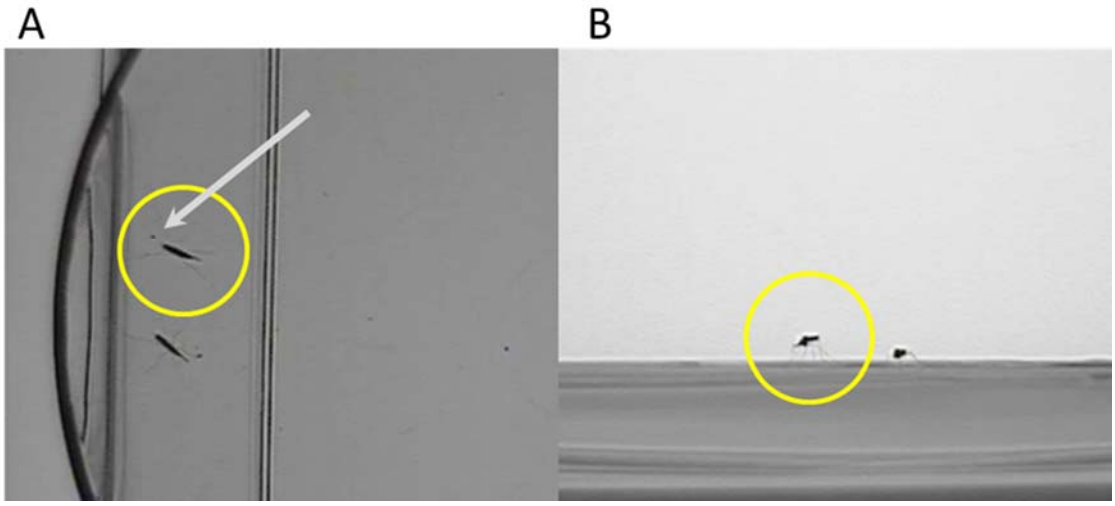


Plate 6.4 Female *Anopheles gambiae* ovipositing while resting on water surface. The ovipositing female is ringed in yellow. A) Bird's eye view; the recently oviposited egg can be seen (arrow). B) Horizontal view; the female's abdomen shown to be above the water's surface, as it remained throughout oviposition.

The second mode of oviposition was 'in flight'. Mean visit duration was shorter in these ovipositions than for landed ovipositions with a female approaching the dish before engaging in a rapid jabbing flight above the oviposition dish. The female continued to jab up and down above the target dish, often (seen from above) looping over the dish as it did so. The bouncing over the target dish became more rapid and confined. At the moment the egg was ejected the body of the ovipositing female was aligned so that the abdomen posterior was pointed downwards, with the legs trailing beneath (Figure 6.6 and Plate 6.5).

It is important to note, however, that as it was not possible to distinguish individual mosquitoes; a visit simply refers to any female seen on the recordings to enter camera shot, and therefore enter the vicinity of the dish. Therefore, 'visits' is considered to represent the relative amount of time mosquitoes spent near the target dish. It is not known how many, or if all the mosquitos released did or did not oviposit.

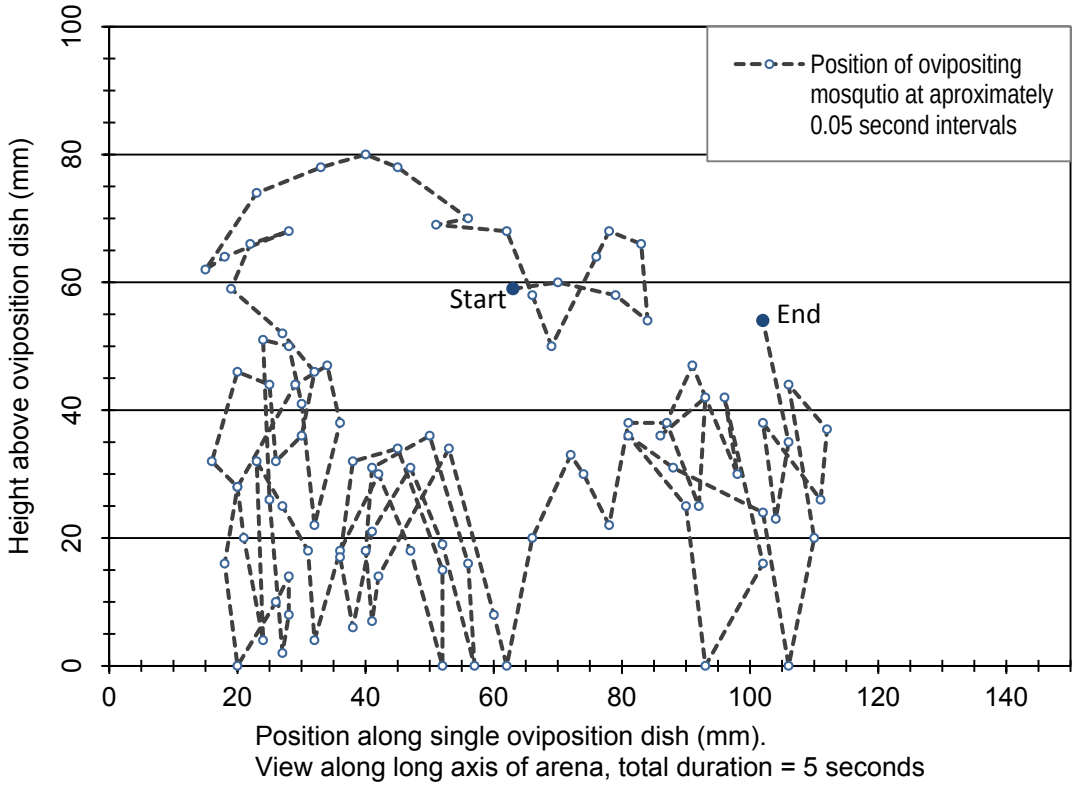


Figure 6.6 Track of jabbing oviposition flight. View from camera 1, i.e. looking from the side along long axis of arena. The oviposition dish was positioned between 0 and 150 mm on the X-axis. Units are approximately 1mm, taken from the video recordings, calibrated to the centre of the dish (positions are approximate as they are based on 2D information, and take no account of depth of field). Start and end points are shown.

This bouncing, dancing flight was observed to occur between the water's surface and ~5 cm above the surface of the oviposition dish and was sometimes so low that the tarsi may have contacted the water surface. Image quality was not clear enough to determine if the females did indeed make contact with the water, even using very slow playback and freeze frame. The video recordings do suggest that the hind tarsi probably came into contact with the water meniscus during at least some jabs towards the water.

Plate 6.5 The observed flight path of an ovipositing mosquito. Screen captures of flight video (14 frames), combined to show the attitude of the mosquito's body over the oviposition dish. Recordings made simultaneously from above show that an egg was released during the loop on the left of the image. This was not clearly visible from side on recordings. Width of view = 15 cm, time between shots = 20 ms.

These females oviposited in the dish during flight without ever landing on the water. Oviposition was not seen directly, as it was obscured from above by the female's body, but eggs were seen to have been laid immediately after a jab. It is also not clear when during the 'bounce' the egg was laid, but the abdomen did not appear to contact the water, suggesting they were released in mid-air and fell into the water. Once the eggs were released, the female's flight was not seen to change back to a less frantic mode, but they often left the shot still bouncing. Some females were seen to come to rest after ovipositing, landing either on the water's surface or the Perspex of the arena floor, however this was less common.

In the treated dish, 69 mosquito visits (out of 229 total visits) landed on the water, of which 39% oviposited (Figure 6.5). In the control dish 173 mosquito visits landed (389 total visits), of which 33% oviposited. The proportion of ovipositions was not significantly different (χ^2=14.07, df=7, p=0.18, n/s). Of the 160 visits from mosquitoes that did not land on the treated dish, significantly more oviposited in-flight (27 %), compared to the control dish (15% from 216 visits; χ^2=15.99, df=7, p<0.05).

In the treated dish, females that oviposited while landed were in situ for 52.2±21.19 (mean ±SE) s, and those that oviposited from flight were in view for significantly less time (40.5±21.17 s (mean ±SE), χ^2=55.0, df=7, p<0.005).

The duration landed ovipositions were in situ in control dishes (160.9±155.84 s (mean ±SE)) was significantly greater than those that oviposited in flight (51.3±17.36 s (mean ±SE), χ^2=371.95, df=7, p<0.001). Landed ovipositors were also in situ significantly longer in the control dish than in the treated dish (χ^2=66.8, df=7, p<0.005). A significant difference was also observed between the time in view of in-flight ovipositors in the control and treated dishes (χ^2=24.16, df=7, p<0.005).

Landed ovipositions occurred at a variable time after landing. The mean time to first oviposition after landing was 14.4± 4.47 s (mean ±SE, n=27 landings) in the treated dish (range: 6 - 21 s) and 20.3 ± 9.90 s (mean ±SE, n= 58, range 5 – 57 s) in the control dish. The reason for this is not clear, but it may show that 4-methylphenol acts to induce oviposition more rapidly than saline water. It is not clear what benefit this would convey if 4-methylphenol is a repellent. Landing was usually immediate once in shot and 97.6% of all landed

mosquitoes (97.2% in treated dish and 98.1% in control) left shot within 10 s of taking off.

Of the 618 recorded visits to the treated dish and the control dish combined, 242 (39%) landed. Of these, a significantly smaller percent of females in the treated dish experiment landed (69 landings from 229 visits: 30%) than in the control dish experiment (179 landings from 389 visits: 44%; χ^2=22.05, df=7, p<0.01). Total landing duration was significantly shorter in the treated dish (52.2±27.45 s (mean ±SE)), than in the control dish (173±79.7 s (mean ±SE); χ^2=518.39, df=7, p<0.001) experiments.

Significantly fewer eggs were laid in the treated dish (mean 34.9±9.24 (mean ±SE) per replicate, 279 total) than in the control dish (58.8±14.49 (mean ±SE) per replicate, 471 total; χ^2=49.15, df=7, p<0.001).

Landed females in the treated dishes did not lay significantly more eggs (4.5±1.01 (mean ±SE) eggs/oviposition visit) than those that oviposited in flight (3.7±0.46 eggs/oviposition visit; χ^2=3.39, df=7, p=0.85, n/s), whereas a significant difference was observed in the control dish (landed: 6.9±1.13 (mean ±SE) eggs/oviposition visit, in flight: 2.1±0.72 eggs/oviposition visit; χ^2=23.16, df=7, p<0.001). However, there was no overall difference in eggs/oviposition visit between the treated dishes (3.9±0.58) and the control (5.1±0.75 (mean ±SE); χ^2=2.84, df=7, p=0.90, n/s).

Overall, these observations suggest that mosquitoes exposed to the treatment dish, compared to the control dish, had:

- shorter duration of visits (with or without egg laying)

- shorter bouts of activity
- fewer number of visits
- fewer number of oviposition visits
- fewer total number of eggs laid
- shorter time to first oviposition
- higher proportion of all ovipositions occurring in flight than landed

However, overall there was no difference in the number of eggs laid per oviposition visit between the treated and the control dishes.

In the absence of an alternative oviposition site, 4-methylphenol reduced the time a gravid female stayed near the potential oviposition site, thereby reducing the number of oviposition events. Females were not completely deterred from egg-laying.

6.3.7 Effect of an oviposition repellent when a treated and control dish are present (two dish scenario)

In this experiment 20 gravid mosquitoes were offered a choice between a treated dish and a control dish positioned on separate IR transparent black plastic backgrounds 15 cm apart to oviposit into (Plate 6.6). The protocol was otherwise the same as in the one dish scenario (Section 6.3.2).

A total of 2,442 individual visits to the dishes were recorded, of which 1,122 included oviposition bouts, of which 215 (19.1%) were by females landed on the water surface, A total of 4,540 eggs were observed being laid, of which 899 (19.8%) were laid by females that had landed on the water surface, and the rest were laid in mid-flight.

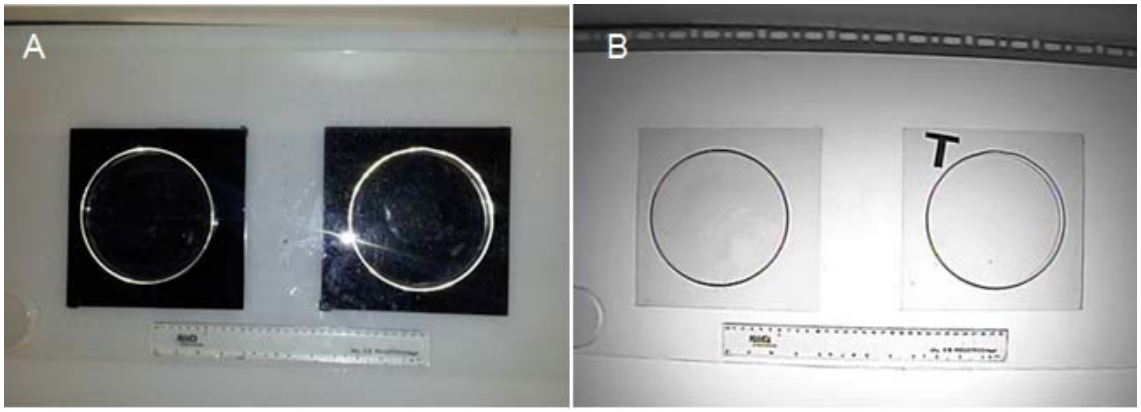


Plate 6.6 The arrangement of the two-dish oviposition dishes. A) shows dishes illuminated with visible light, as mosquitoes would see them. B) shows the same view, but as the cameras, illuminated by infrared light, would record the dishes. Note the black plastic becomes transparent when viewed in infrared, revealing a paper marker indicating the position of the treatment dishes. The ruler shown is for scale and measures 30 cm.

Saline vs. saline

No significant differences were found in the number of visits or number of eggs laid in each of the two dishes in the control experiment (i.e., both dishes contained only 0.9% saline, but one dish was nominally the 'treatment' dish); of the 500 visits observed, 244 (48.8%) were over the 'treatment' dish and 256 were over the 'control' dish (χ^2=1.93, df=3, p=0.59, n/s), and a total of 390 eggs were laid in the treatment dish compared to 436 in the control dish (χ^2=1.32, df=3, p=0.27, n/s, Figure 6.7A).

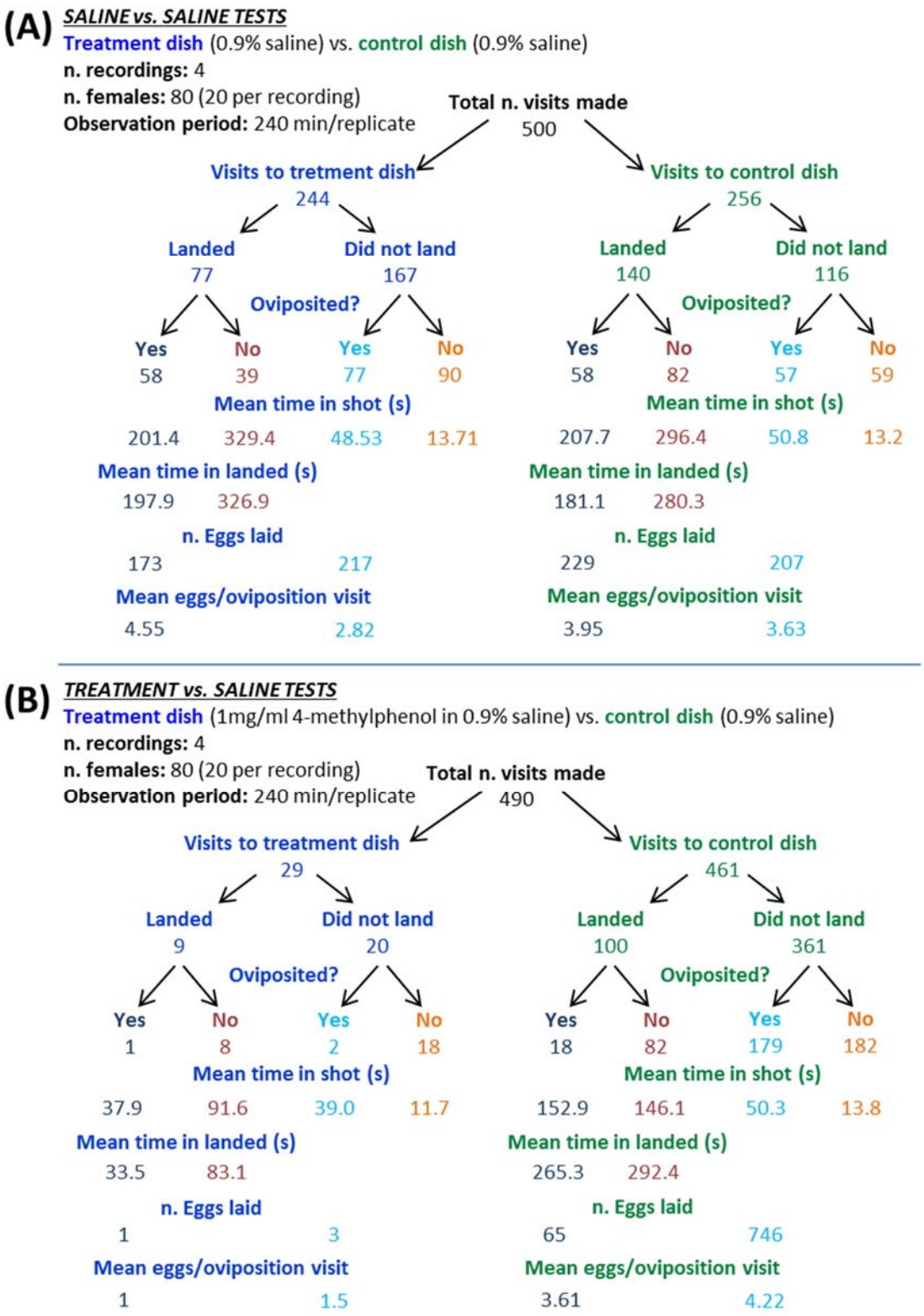


Figure 6.7 Summary of behaviours observed towards two control oviposition dishes. A) Control experiment, with two dishes of 0.9% saline and B) Treatment experiment with one dish containing 1 mg/ml 4-methylphenol and the other 0.9% saline.

Saline vs. treatment

The difference in visits to the dishes increased as dose of 4-methylphenol increased. When 4-methylphenol was present in the control dish there was a significant difference at all levels, with a greater number of visits to the control dish (Table 6.1). This pattern was repeated in the number of ovipositions, with the level of oviposition decreasing in the treatment dish compared to the control as the 4-methylphenol dose increased (Table 6.2). When the treatment dose was 1 mg/ml 4-methylphenol the numbers of ovipositions in the treated dish were so low as to preclude analysing the details of the associated behaviour any further (Figure 6.7B).

Table 6.1 Summary of visits in two-dish tests. The visits to the treatment and the control dish are shown with significance.

Dose 4-methylphenol in control dish (mg/ml)	Visits to treatment dish	Visits to control dish	χ^2	df	p
0	244	256	1.93	3	0.41
0.001	399	164	99.79	3	<0.001
0.01	347	58	210.13	3	<0.001
0.1	412	72	246.45	3	<0.001
1	461	29	385.07	3	<0.001

Table 6.2 Summary of oviposition in two-dish tests. The visits to the treatment and the control dish are shown with significance.

Dose 4-methylphenol in control dish (mg/ml)	Ovipositions into treatment dish	Ovipositions into control dish	χ^2	df	p
0	115	114	1.3222	3	0.27
0.001	72	208	73.486	3	<0.001
0.01	22	146	96.571	3	<0.001
0.1	30	214	150.62	3	<0.001
1	3	197	192.49	3	<0.001

The levels of oviposition activity associated with the treated dish were so very much lower than in the one dish scenario, there is practically no need for statistics to confirm consistency with the conclusions of the one dish scenario. The number of visits between saline vs saline tests and treatment (1 mg/ml 4-methylphenol) vs saline tests (A vs B in Fig. 6.7) is not significant (χ^2=1.933, df=3, p=0.59), nor is the difference between visits in saline vs saline tests (A In Fig. 6.7, χ^2=6.67, df=3, p=0.09). The number of visits between dishes in treatment (1 mg/ml 4-methylphenol) vs saline tests was significantly different (χ^2=385.096, df=3, p<0.99).

Similarly numbers of oviposition where at least 1 egg was laid observed in saline vs saline tests and treatment (1 mg/ml 4-methylphenol) vs saline tests (A vs B in Fig. 6.7), i.e. total number of ovipositions observed, is not significant (χ^2=4.54, df=3, p=0.21). There was no difference in total number of ovipositions in saline vs. saline tests (A in Fig. 6.7, χ^2=1.323, df=3, p=0.73), but this was highly different in treatment vs saline tests (B in Fig. 6.7, χ^2=35.096, df=3, p<0.99).

Overall, the activity of mosquitoes near the treated dish was reduced by nearly all measures (Figure 6.7): they had shorter bouts of most activities (duration of all visits, duration of landing) and they did less of each activity (fewer visit overall, fewer landings, fewer eggs laid), and laid fewer eggs/female than the controls. In the presence of an alternative oviposition site, 4-methylphenol reduced the time a gravid female stayed near the potential oviposition site, and almost completely deterred her from laying eggs. This suggests that 4-methylphenol acts to reduce the amount of time a female will stay in proximity/direct contact with the solution, perhaps explaining why the oviposition occurs more quickly at higher 4-methylphenol doses.

6.3.8 Comparison of oviposition behaviour in single or two dish scenarios

From the analysis of the video recordings, the observed behaviours of the mosquitoes in the one and two dish scenarios were compared. The data for all replicates of all four doses of 4-methylphenol were analysed as per Chapter 2.6 and plotted to assess dose responses and trends fitted using a GLM, and differences in behaviour were identified using analysis of covariance (ANCOVA).

Duration of all visits

Dose of 4-methylphenol was seen to have a significant effect, reducing visit duration with increasing dose (GLM; df=1, F=75.07, $p<0.001$) for the treated dish in both the scenarios, as well as in the control dish in the two dish scenario (Figure 6.8). In the two-dish scenario there is no difference in visit duration where 4-methylphenol is present at all doses (i.e. excluding the control vs. control, $\chi^2=7.5$, df=9, p=58 n/s). The zero dose (i.e. the control vs. control) is significantly different to the treated doses ($\chi^2=217.3$, df=3, $p<0.001$), showing the presence of 4-methylphenol to consistently reduce visits to the treatment dish at all doses.

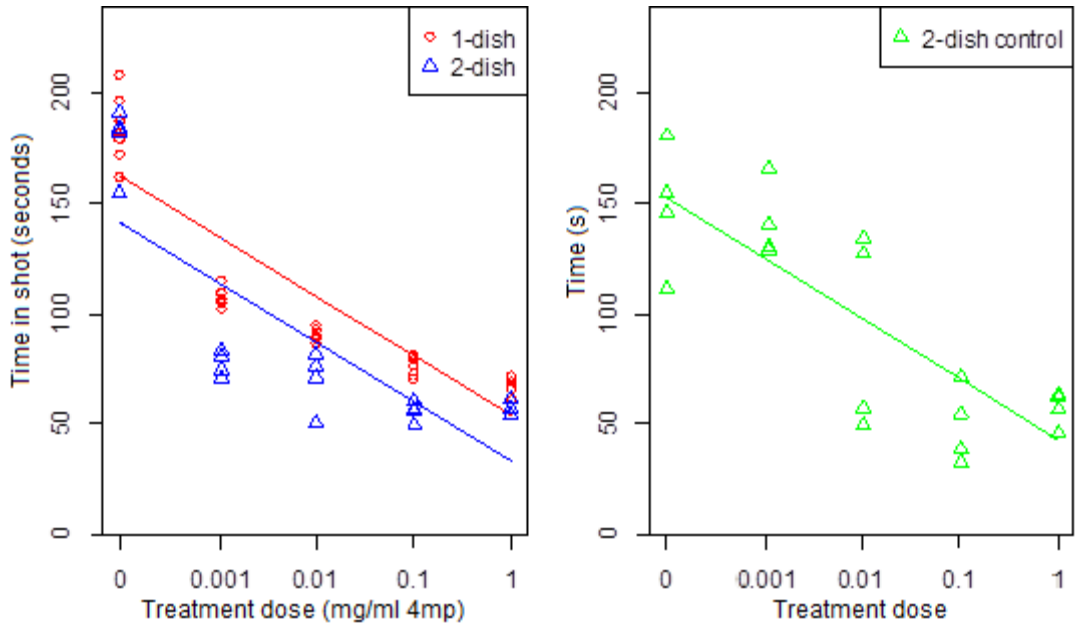


Figure 6.8 The average duration of visits in the one and two-dish scenarios. The average visit duration (time in shot) per replicate for visiting mosquitoes in one-dish (red) and two-dish (blue = treatment, green = control) scenarios. Regression lines are shown to indicate dose-response.

In the two-dish scenario an effect is also seen, apparently as an artefact of proximity of the treatment dish, at doses of 0.1 mg/ml and 1 mg/ml of 4-methylphenol, with the duration of the visits reduced compared to 0 m/ml and 0.001 mg/ml (χ^2=100.4, df=7, p<0.001). The threshold of this difference appears to be around 0.01 mg/ml 4-methylphenol as the duration of visits to the control at this level is greatly mixed.

The number of landings on dishes

A significant effect of dose on the numbers of landings per dish per replicate was also observed for the treated dishes in both scenarios (ANCOVA: F=97.85, df=1,4, p<0.001). No significant difference was observed in the number of landings in the two-dish control at any dose (χ^2=10.8, df=12, p=0.29, n/s),

suggesting that the number of landings was not affected by the treatment dish (Figure 6.9).

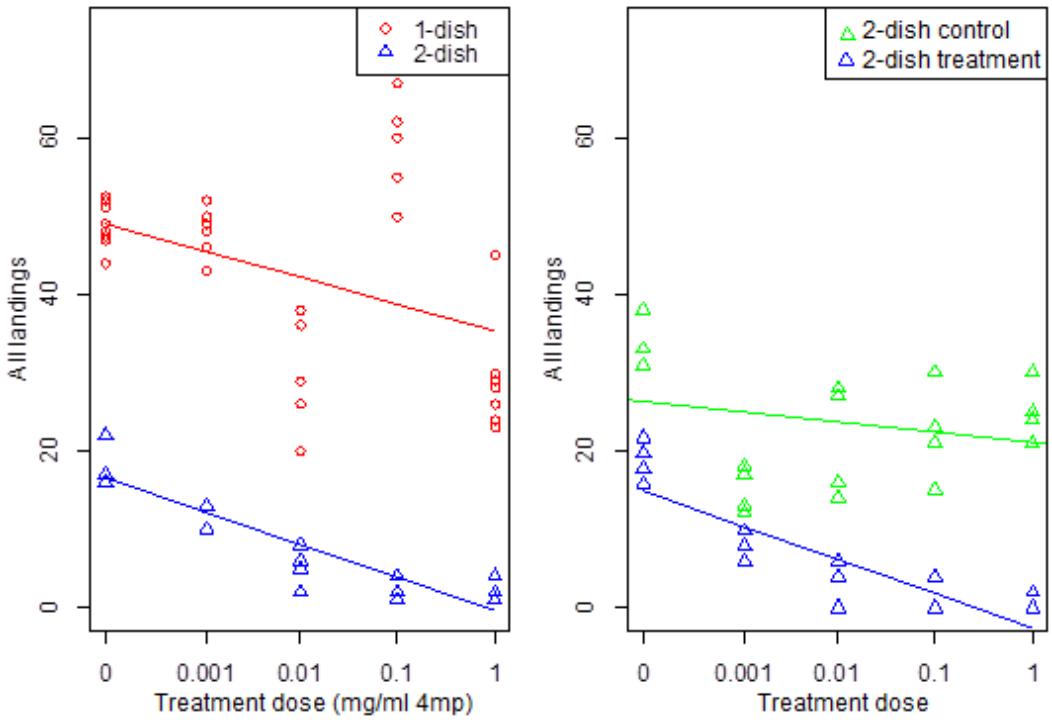


Figure 6.9 The number of landings vs. dose in one dish and two dish scenarios. The left panel shows the number of landings per night (recorded for 4 h/night) by 20 mosquitoes for the one-dish scenario (red, left panel) and in the treatment dish (blue, left panel) for the two-dish scenario. The right panel shows the landings observed in the control dish (green, right panel) and treatment dish (blue, right panel) for the two-dish scenario.

The mean numbers of landings per dish per replicate were significantly higher in the one-dish scenario than the treated dishes of the two-dish scenario (χ^2=99.3, df=3, p<0.001).

The number of landings per night can be seen to fluctuate in the one-dish scenario, but follows an overall trend of reducing as dose increases. The combined number of landings in the two dish scenario appears to follow a dose response, reducing as dose increases, but again, when the zero dose is

removed no overall significant difference can be seen between doses (χ^2=11.2, df=9, p=0.62, n/s, Figure 6.10)

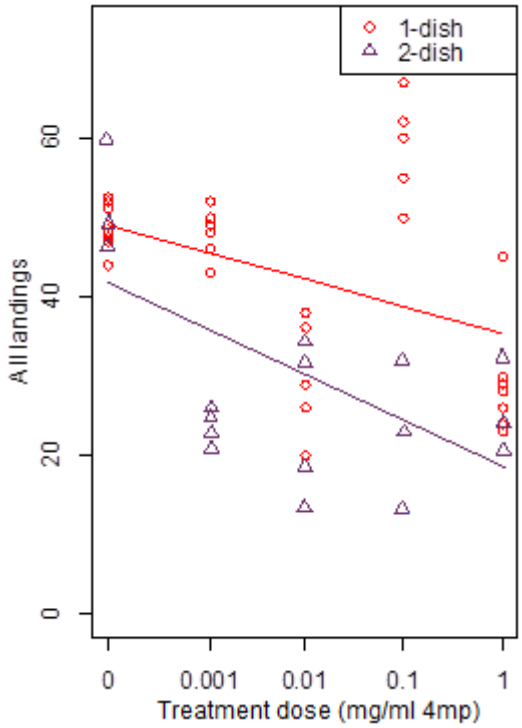


Figure 6.10 Total number of landings in one-dish and two-dish scenarios. Red circles indicate the landings in the one-dish scenario, purple triangles show landings in the two-dish scenario, in both treatment and control dishes.

In the one-dish scenario at 1mg/ml 4-methylphenol dose 389 visits to the control dish were observed (48.8±2.44 (mean ±SE) visits per replicate) and 229 visits to the treatment dish were observed (28.7±6.63 (mean ±SE) visits per replicate). In the two-dish scenario, with the treated dish containing the same dose of 4-methylphenol, from a total of 490 visits (122.5±9.75 (mean ±SE) per replicate), there were significantly fewer visits (29 in total, 7.3±2.77 (mean ±SE) per replicate) to the treatment dish and 461 to the control (115.3±7.25 (mean ±SE) per replicate, ANCOVA: F=19.7, df=1,1, p<0.001). The highest absolute

number of visits observed in the two-dish scenario was observed at 0.001mg/ml vs. control, with a total of 563 visits (140.8±3.34 (mean ±SE) visits per replicate), and, overall, there was a decline in the number of visits as dose increased; 405 visits (101.3±9.60 (mean ±SE) visits per replicate) observed at 0.01mg/ml vs. control and 484 visits (121±9.03 (mean ±SE) visits per replicate) at 0.1mg/ml vs. control (ANCOVA: $F=5.12$, $df=1,1$, $p<0.05$).

In both the one-dish and the two-dish scenarios the number of landings was significantly reduced as dose of 4-methylphenol increased (ANOVA: $F=44.5$, $df=1,1$, $p=<0.001$). In the two-dish scenario, the untreated dish (i.e. the control) received a greater number of visits than the treated dish ($\chi^2=153.4$, $df=4$, $p<0.001$). This was unaffected by dose, although a reduction of landings may have occurred at 0.001 mg/ml 4-methylphenol, which was not seen at other doses. The effect of dose was not significantly difference between the one-dish and two-dish scenarios (ANCOVA: $F=49.42$, $df=1,4$, $p<0.01$).

In the two-dish scenario the total number of landings observed (i.e. the sum of the landings in the control and treatment dishes) was lower than the total number of landings in the one-dish scenario ($\chi^2=106.4$, $df=4$, $p<0.001$, Figure 6.11). While the majority of mosquitoes in the two-dish scenario oviposited in the untreated dish, the total level of landings was less than in the one dish, suggesting that having two dishes affects the mosquito's behaviour.

Thus, a single dish may be better for assessing the effect of a repellent on landings, as this appears to force the mosquitoes to make more landings per observation period, while giving the same dose response slope as in the two-dish scenario.

Landing and ovipositing

A significant effect of dose on the mean number of mosquitoes landing and ovipositing per replicate was seen, with higher 4-methylphenol doses reducing the number of females that landed and oviposited (ANOVA: $F=76.6$, $df=1,1$, $p<0.001$, Figure 6.11).

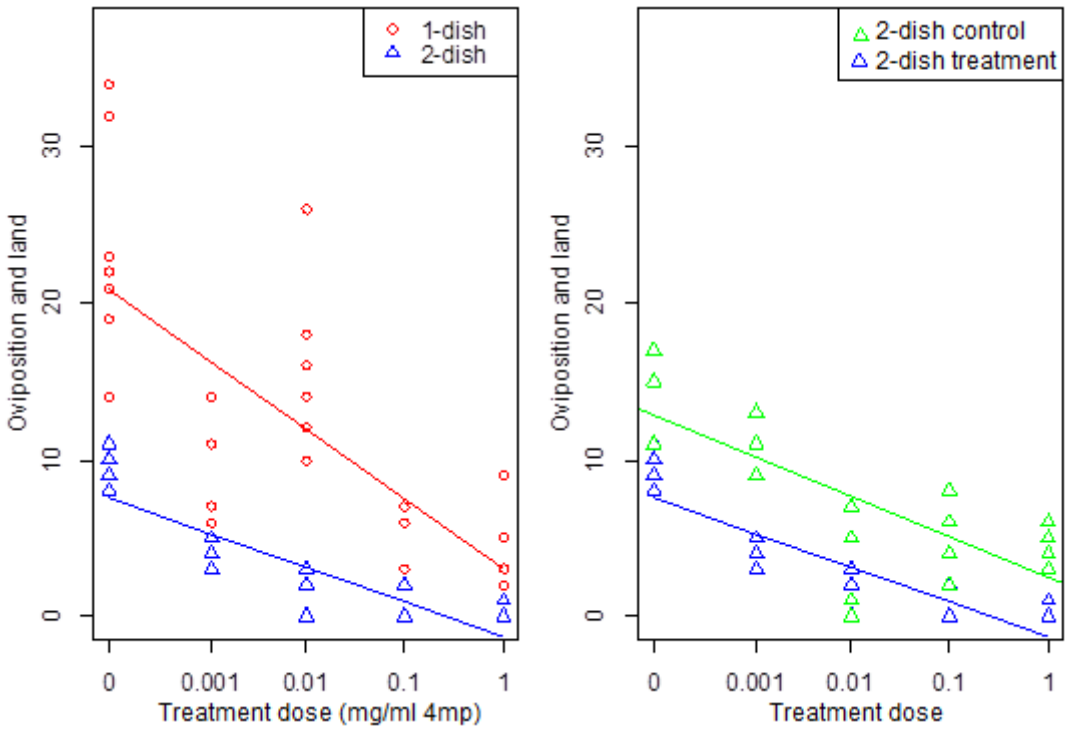


Figure 6.11 **Number of mosquitoes that landed and oviposited.** The left panel shows the number of mosquitoes that both landed and oviposited per night (recorded for 4 h/night) by 20 mosquitoes for the one-dish scenario (red, left panel) and in the treatment dish (blue, left panel) for the two-dish scenario. The right panel shows those observed in the control dish (green, right panel) and treatment dish (blue, right panel) for the two-dish scenario.

However, the one-dish and two-dish treatment dose responses are significantly different from one another (GLM 1df, F=6.43, p<0.05), suggesting the behaviour is altered by the presence of a second dish. When summed, the number of landing and ovipositing females is similar in both scenarios, but shows a reduction in observations at 0.001 mg/ml doses in the one-dish scenario compared to the two-dish, and, conversely a reduction at 0.01 mg/ml doses in the two-dish scenario compared to the one-dish (Figure 6.12).

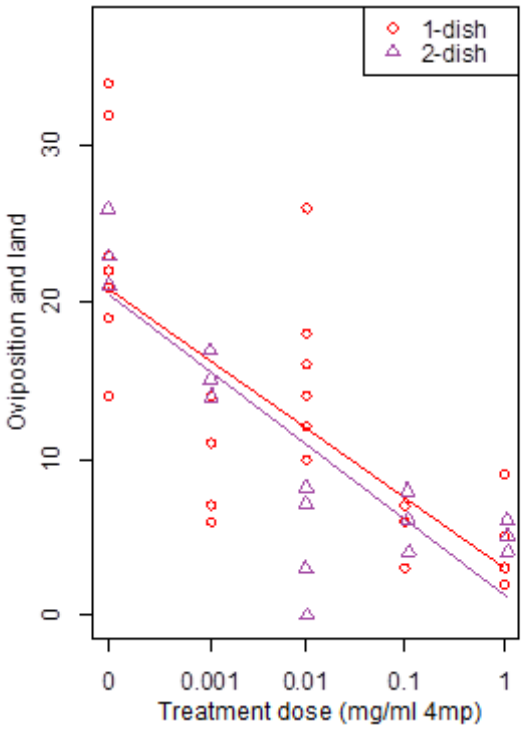


Figure 6.12 The total numbers of females observed to land and oviposit. Red circles indicate the number of mosquitoes which landed and oviposited in the one-dish scenario, purple triangles show the sum of all landings in two-dish scenario, in both treatment and control dishes.

Despite this, the similar trends for both scenarios suggest that, overall, having two dishes does not have an effect on the total number of observed occurrences

of females that land and oviposit. Instead the mosquitoes are seen to exhibit the same level of behaviour in both scenarios, but distribute the behaviour, favouring the control dish, in the two-dish scenario.

The numbers of eggs laid per female that lands and then oviposits was not significantly different between dishes (ANOVA: $F=2.412$, $df=1,2$, $p=0.13$). Bouts of in-flight oviposition appear to result in fewer eggs/female in the two-dish treated dish per bout (ANOVA: $F=5.726$, $df=1,1$, $p<0.05$, Table 6.3). The mean number of eggs/female that oviposited while landed per oviposition bout was not significantly different to the mean number of eggs/female that oviposited in-flight per oviposition bout ($\chi^2=0.72$, $df=4$, $p=0.99$, n/s).

Table 6.3 The mean number of eggs laid per observed oviposition per female at different doses of 4-methylphenol (4-mp). Listed by scenario and flight oviposition type.

4-mp dose (mg/ml)	Mean eggs laid per observed oviposition in:					
	One-dish		Two-dish treated dish		Two-dish untreated dish (control)	
	Landed	In flight	Landed	In flight	Landed	In flight
0	3.97	4.54	4.55	2.82	3.95	3.7
0.001	4.06	3.47	4	2.79	4.41	4.61
0.01	4.36	4.02	3.67	3.53	4.46	4.19
0.1	4.66	3.87	0	3.61	4.65	4.27
1	3.81	3.69	3	0.5	3.61	4.22

Comparison of oviposition with and without landing

Treatment dose was seen to have a significant effect on the visit duration of females that oviposit into the one-dish or two-dish treatment and the two-dish control. This applies to both females that oviposited while landed (GLM 1 df, $F=146.04$, $p<0.001$) or in flight (GLM 1 df, $F=9.39$, $p<0.01$) in these dishes. No dose response effect was seen in females ovipositing into the two-dish control while in flight without landing (ANOVA: $F=0.467$, $df=1,4$, $p=0.76$). All other ovipositing females are seen to reduce the duration of visits as dose increases, regardless of oviposition mode (Figure 6.13), suggesting that the in-flight ovipositors are affected by a factor that acts to reduce their latency to the oviposition dish when in close proximity to the treated dish. This suggests the alteration is olfaction based.

The duration of visits for ovipositing mosquitoes that landed and did not land was significantly different ($\chi^2=17.6$, $df=4$, $p<0.01$). The overall number of both landed and in-flight ovipositing females is greater in the two-dish tests at all doses.

The effect of dose is seen in landing ovipositing females in the two-dish control as well as two-dish treatment. This finding suggests that the decision making process for ovipositing while landed on the water was based upon a global stimulus prior to landing – e.g. a volatile odour source causing a response in all of the mosquitoes, not just those that had landed in the treatment dish.

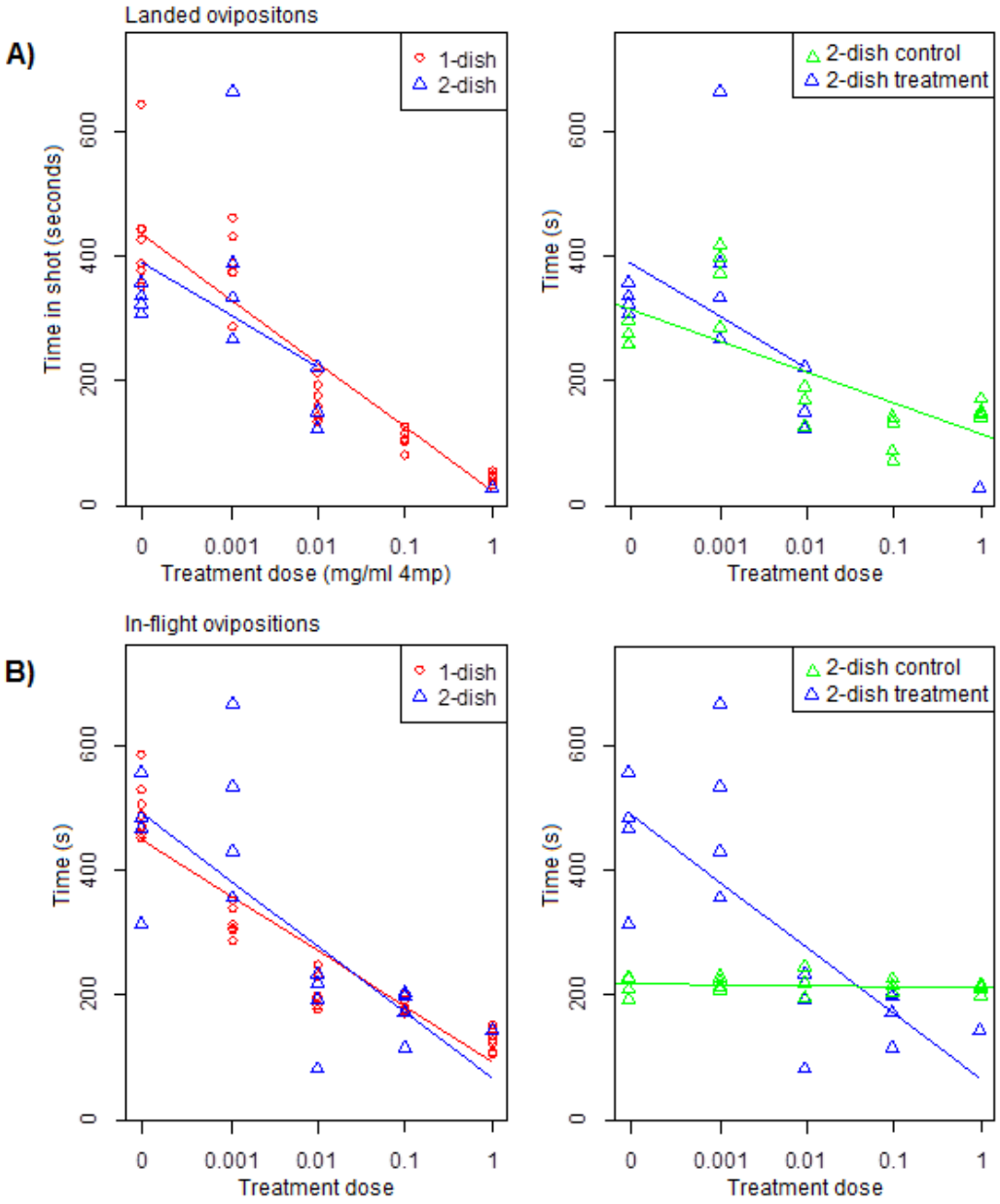


Figure 6.13 The average visit duration of all ovipositing mosquitoes. The average duration of visits of mosquitoes ovipositing. A) Shows mosquitoes that land and oviposit. The left panel shows the mosquitoes that both landed and oviposited per night (recorded for 4 h/night) by 20 mosquitoes for the one-dish scenario (red, left panel) and in the treatment dish (blue, left panel) for the two-dish scenario. B) Shows the same for in-flight ovipositing mosquitoes.

6.4 Discussion

6.4.1 The effect of a repellent on mosquito oviposition

Previous attempts at video recording *An. gambiae* have explored mating behaviour through swarm analysis (Butail *et al*., 2012) or mosquito-host interactions (Hawkes *et al*., 2012). In this study successful video recordings were made of *An. gambiae* S molecular form (*Anopheles gambiae* s.s. Giles, as per Coetzee *et al.* (2013)) ovipositing.

Oviposition was seen to have two modes: “in-flight oviposition” and by females resting on the water’s surface – “landed oviposition”. Caged *An. gambiae sensu lato* have been seen to oviposit when landed and from flight in small cages (McCrae, 1984), and the cage size had little effect on this. McCrae observed that females which landed tended to oviposit, but that was not observed in this study, with more of the females which landed not ovipositing at all concentrations.

Observations of landed oviposition show the female’s abdomen does not touch the water, remaining parallel to the water’s surface. However, it is not clear if she touches the water with her proboscis or ingests water while at rest on the surface, although the observations do not preclude this, making it difficult to determine if the mosquito samples the water by taste.

The flying mode of oviposition was also recorded by McCrae, which he termed “dancing oviposition”, and concluded that it was used in optimal conditions and over dark based targets and that sub-optimal targets elicited a greater level of landed oviposition. McCrae’s findings and the observations reported here were not able to determine whether the female touches the water when dipping

towards the water. The hind legs fall below the flying female while dipping, and since chemosensilla are located on the tarsi, these may be used to sample the water on contact (Clements, 1999).

The proboscis tip, antennae and tarsi of mosquitoes all detect stimulatory substances (Roth, 1951). Bently & Day (1987) demonstrated that oviposition of *Cx. pipiens fatigens* can be stimulated upon contact with septic water by the tarsi and proboscis (under conditions of forced oviposition), suggesting sampling may play an important role in oviposition site selection, or even initiation of oviposition itself. The tarsi of *An. gambiae* have gustatory receptors (Pitts *et al.*, 2004) and it has been shown that *Ae. aegypti* can discriminate against food sources based on tarsal detection (Ignell *et al.,* 2010). It is therefore likely that the tarsal detection of oviposition semiochemicals, does play a role in *An. gambiae*, and that the dipping motion seen in in-flight oviposition is likely a behaviour that encourages contact with the oviposition media as a means of sampling.

It is clear that while filming *An. gambiae* in nocturnal conditions has a number of advantages, the use of a better level of magnification, higher definition would help improve our understanding of *An. gambiae* oviposition behaviour by allowing more accurate analysis of the mosquito in flight prior to and post oviposition. Additionally a higher rate of data capture (such as recording at a higher frame rate) might permit determination of when eggs are released by in-flight ovipositing females.

The video work supports the hypothesis that *An. gambiae* oviposition can be affected by volatile chemicals; in particular a chemical associated with biological

processes such a wood rotting or the presence of livestock, such as in cattle urine. In this case, the dose response from the cage tests (Chapter 5) and the results of the two-choice experiments here suggest that 4-methylphenol is an oviposition repellent to *An. gambiae*. However, it has been reported that 4-methylphenol attracts *An. gambiae* larvae (Xia *et al*., 2008), as well as pre gravid *An*. *arabiensis* (Torr *et al*., 2008) and is an oviposition attractant for *Aedes triseriatus* (Bentley & Day, 1989). 4-methylphenol is a human sweat compound, and has been shown to elicit large electrophysiological responses in *An. gambiae* both prior to host feeding (Cork & Park, 1996) and after (Qui *et al*., 2006; also see Chapter 5).

It was also observed that *An. gambiae* behaviour near a potential oviposition site was affected by the presence or absence of alternative sites. In the one-dish scenario *An. gambiae* was seen to oviposit into treated dishes containing 4-methylphenol at a range of doses. Egg counts, duration of visits, time landed and number of landings were all significantly affected by dose, but oviposition was not completely stopped.

In the two-dish scenario oviposition was almost completely biased away from the treated dish at 1 mg/ml of 4-methylphenol treatment. Duration of visits, time landed, numbers of visits, number of landings and number of ovipositions observed were all significantly negatively correlated with dose.

The findings suggest that mosquitoes are affected by the 4-methylphenol in the treatment dish. They also suggest that a level of discrimination is taking place, which must be due to sampling by the mosquito. Visits, oviposition and the number of eggs laid all appear to be affected by dose. In the two-dish scenario,

at high doses the total level of behaviours is maintained but diverted to the control dish, whereas in the one dish scenario behaviours are slightly suppressed at high doses but not prevented.

This suggests that despite finding a sub-optimal oviposition site, at least some females lay their eggs if there is no alternative site.

The egg laying behaviour of *An. gambiae* is a key life history event. Natural selection must have acted to optimise oviposition, which in turn will act to optimise the fitness of the female. However, the results presented here suggest that a female will not necessarily oviposit many eggs into a suitable habitat; in these experiments the number of eggs laid per female was surprisingly low. In the one-dish experiments the high dose of 1mg/ml 4-methylphenol yielded just 3.49 eggs per female overall, while the clean water control yielded 5.89 eggs per female. The two-dish experiments yielded a higher average egg count per female, at 10.31 for the high dose and 10.33 in the control. Efforts were made to ensure humidity remained constant between the 1-dish and 2-dish experiments, and monitoring of humidity rules this out as a factor behind the higher egg numbers in the 2-dish experiment.

Typical *An. gambiae* egg count numbers are difficult to obtain from other studies, which vary widely in terms of numbers of mosquitoes used, uncertain identity of strains (or even species), whether or not the study was lab or field based and the nature of the study itself (Table 6.4).

Table 6.4 **Observed *Anopheles gambiae* egg numbers from various studies.**

Place of study	Year	n. ♀ in study	Duration of oviposition	Eggs laid/ female		Species	Colony type	Sources
				Mean	Min/ max			
Gambia	1993	?	n/a	100	20/180	*An. gambiae* s.s.	Lab.	1
Tanzania	?	?	1 night	12.6	?	*An. gambiae* s.s.	Lab.	2
Tanzania	?	?	n/a	108.6	?	*An. gambiae* s.s.	Lab.	2
Tanzania	1991	?	>1 night	150	66/290	*An. gambiae* s.l.	Lab.	3
Tanzania	1991	?	1 night	111	48/178	*An. gambiae* s.l.	Lab.	3
Nigeria	2009	c. 150	1 night	78.33	?	*An. gambiae* s.s.	Lab.	4
Israel	2010	540	1 night	11.9	?	*An. gambiae* s.s.	Lab.	5
Kenya	1982	334	>1 night	72.3	?	*An. gambiae* s.l.	Field	6
Sweden	2008	588	1 night	13.01	0/63	*An. gambiae* s.s.	Lab.	7

Sources: 1. Hogg *et al.* (1996); 2. Takken *et al.* (1998); 3. Lyimo & Takken (1993); 4. Olayemia & Andeb (2009); 5. Warburg *et al.* (2011); 6. McCrae (1983); 7. Lindh *et al*. (2008a). Note, n/a in duration column indicates eggs were counted through dissection, not oviposition.

From the cage experiments presented in Chapter 5 an average of 24.6±16.24 (mean ±SE) eggs were laid per female in a 19 h night (n. 13 replicates), although this assumes all females oviposit during that night and that they do so evenly. The timing experiments (as shown in Section 6.3.3) in the large arena give a similar number of 28.7±11.15 (mean ±SE) eggs/female over 24 h (n. 3 replicates). Personal experience of rearing *An. gambiae* shows that a cage of nulliparous mosquitoes, once fed will produce eggs over more than one night. As such probably neither of these represents the true total egg yield of gravid *An. gambiae*, but they do give an indication of what it might be.

The preliminary timing experiments also showed evidence that oviposition activity peaked in the first 4 h of the scotophase, with 80.0±0.07% (mean ±SE)

of eggs laid recorded in this period, and in these 4 h 22.9±2.13 (mean ±SE) eggs were laid per female released. Again, this assumes that each female laid, but if this was not the case, the average number of eggs per female must then be even higher. Assuming the same proportion of eggs was laid in the first 4 h in the cage experiments, then an average of 19.7±12.99 (mean ±SE) eggs/female was observed. Both the cage tests and the preliminary timing experiment suggest that the numbers of eggs/female observed in the video recordings are low.

The data obtained from video observations are also not helpful for explaining the observed low egg count per female, as it is not possible to say if more than one, or a small subset of the females in the arena that were observed to oviposit at the same time, laid eggs. Each individual visit was recorded, but it is not known how many times each mosquito was observed. This could be overcome by releasing just one gravid mosquito per night and filming her over the course of a whole night, but the huge amount of time required of this would make this difficult to achieve.

The results shown here represent 56 hours of recording time and several times that analysing the recordings, in addition to many hours of preliminary work and setting up the arena. The preliminary work also suggested that fewer than 20 mosquitoes would yield too few observations over the recording period to be useful. Using 20 mosquitoes provided ample observations, but only in the area the cameras were focused on. Without individually marking the mosquitoes, which would be difficult to do without damaging them and probably impossible to see in recordings (as the mosquitoes were effectively in silhouette), it is not possible to say which mosquito is which from the video recordings.

The low egg yield per female may be due to either each individual laying fewer eggs, or a difference in the proportion of females in each experiment ovipositing. Oviposition timing has a large number of factors. Blood feeding occurred as early in the scotophase as practically possible, but was delayed by an hour or two, resulting in less time for digestion and egg development. This may not be important however, as the peak oviposition time of *An. gambiae* is largely regulated by the light-dark cycle and not the time of feeding (Sumba *et al*., 2004b; Dieter *et al*., 2012).

However, nulliparous (i.e. in their first cycle of ovulation) females often require a second meal before their eggs develop, (Clements, 1999; Yang, 2008). All of the mosquitoes used in these experiments had only been offered a single blood meal and were nulliparous, a potentially key reason for low egg numbers.

The characteristics of the oviposition site do not affect timing either, but do play a role in egg numbers (McCrae, 1983) with laboratory experiments suggesting darker sites yield more eggs per female (Dieter *et al*., 2012).

Fritz *et al.* (2008) reported that a single female will oviposit in a continuous 2-4 hour bout, but their observations of groups of mosquitoes show two oviposition pulses. These pulses are not due to a female spreading her egg-laying over two distinct periods, but are due to some members of the group deferring oviposition until later. Hyper-gravidity does not affect this pulse. Oviposition occurs predominantly in two pulses, but can occur at any time, including during daylight. The data obtained in this study did not show a second oviposition pulse, but both preliminary data (Section 6.3.4, Figure 6.3) and Fritz *et al.* (2008)

suggest this would begin 8 – 10 h after the scotophase commences, several hours after the recording finished.

In spite of the preliminary observation, the majority of ovipositions may not have taken place during the filming period; Fritz *et al.* (2008) showed that less than 40% of eggs are laid in the first four hours of the scotophase, and even 15% may be laid prior to this. First-hand experience of rearing *An. gambiae* also demonstrates that oviposition may occur three or even four nights after a blood feed in nulliparous females and a single female can lay eggs over at least 2 nights.

Oviposition site deprivation (or inability to find it) can prevent a female from ovipositing (Clements, 1999; Yang, 2008). In these cases, another blood feed may be required before oviposition can occur (Dieter *et al*., 2012). The presence of deterrents may cause a mosquito to delay egg-laying to search for a suitable oviposition site (Warburg *et al*., 2011). The observations made here suggest a deterrent or repellent will cause a mosquito to seek alternatives. If this is the case, then it may result in higher failure to oviposit, perhaps due to the high daily mortality rate of adult mosquitoes (Silver, 2008). This cannot be supported by the findings made here though, as the total number of visits (regardless of the dish visited) in the two-dish scenario did not differ greatly, suggesting no difference in the numbers of females that did oviposit. Mortality was found to be low during experiments, with all mosquitoes accounted for alive at the end of most experiments.

What can be said is that since more oviposition bouts were seen than there were females in the arena, it is clear that here at least some of the females engaged

in oviposition more than once over the 4 hour recording period. This suggests that females may distribute their eggs across more than one location in order to maximise their chance of successful development.

From the observations reported here, it appears that both the one-dish and the two-dish assays are useful in determining mosquito behaviour. Observations of landings suggest that the one-dish assay is a better indicator of behaviour to determine repellence. The single dish appears to force mosquitoes to make more landings per observation period, giving greater data for statistical analysis, while displaying a similar dose-response slope to the two-dish scenario.

The numbers of mosquitoes that land and oviposit also displays this effect, and created an even steeper dose response curve in the one-dish scenario.

However, taken together with number of eggs laid, the duration of visits show that landed and in-flight females lay fewer eggs and do it more quickly at higher doses of 4-methylphenol. For control dishes in the two dish scenario, the landed females were affected by the nearby treated dish, but the females that laid eggs in-flight were not affected by treated dishes. This effect is not observed in the one-dish scenario.

Thus, while one-dish experiments are useful in determining repellence, the two-dish scenario is required to give a fuller picture of the effect of the repellent on the mosquito's behaviour.

6.4.2 The use of a neutral control

The methods outlined in this chapter use a dish containing isotonic deionised water as the control. That is, this water is assumed to be a suitable oviposition site that is neither repellent nor attractive

As shown in Sumba *et al.* (2004a), as well as being suggested for other mosquito species (Hazard *et al*., 1967; Hasselschwert & Rockett, 1988; Burket-Cadena & Mullen, 2007), the natural oviposition sites of *An. gambiae* are not pristine, but are often muddy, containing organic compounds and organisms. They are also dynamic and diverse to such an extent that it can be argued that there is no archetypal *An. gambiae* oviposition site (Fillinger *et al*., 2009).

What is clear though is that the type of water in the control dish does not exist in nature – there is no such thing as a neutral site, and the water in the dish could be said to offer not a control, but rather an absence of stimuli (other than perhaps the humidity of the water itself) – it is not a "control", to which we can expect a standard and normal behaviour, but rather a "nothing", to which there may be no behaviour (as there is no stimulus other than H_2O).

Using "nothing" as the control to test the effect of treatments may not be considered a fair test (Brady, 1975; Brady *et al*., 1989). Given the role that the organic contents may play in the development of larvae (Rozeboom, 1935; Wooton *et al*., 1997) and the attractiveness of soil infusions over plain water (Sumba *et al*., 2004a), it seems that the saline control water may in fact offer a poor breeding site to mosquitoes. It has been shown in this chapter that *An. gambiae* will oviposit in water containing a repellent if given no choice. Perhaps the saline control is simply a less poor choice rather than one it would normally

choose in nature. This may also be a factor in the difficulties in finding data on how many eggs a female will typically lay. It is also possible that mosquitoes from lab colonies are pre-conditioned to oviposit into the medium offered – here the 0.9% saline. Offering different waters (i.e. distilled, filtered tap water, 0.9% saline) as a control and the water into which the treatment was dissolved would help answer this, but time did not permit this line of enquiry.

Having analysed the response of *An. gambiae* to dishes containing 4-methylphenol it seems that this repellent has a number of effects on oviposition. Where no choice is offered, saline containing 1 mg/ml dose of 4-methylpenol will:

- Not completely deter oviposition
- Reduce the time to first oviposition compared to the control
- Reduce the number of visits to the dish compared to the control
- Cause a smaller proportion to land than in the control
- Reduce the duration of the visit compared to the control
- Show no difference in the number of eggs laid per female ovipositing compared with the control

Where a choice is offered between the same treatment and the control:

- The proportion of visits to the treatment is greatly reduced
- The total number of visits to the treatment is reduced
- The duration of the visits to the treatment is reduced
- The number of eggs/female ovipositing is reduced
- A clear repellence is seen, with females preferentially ovipositing in the control.

While some behaviours differ in the presence of the treatment dish in both the one dish and two dish scenario, some, such as the time to first oviposition, are affected in only one scenario. These differences in responses to treatments are shown in Table 6.3.

Table 6.5 Observed differences in oviposition behaviours between the one dish and two dish scenarios.

	One dish	Two dish
Number of visits to treatment dish	Reduced in treatment dish	Reduced in treatment dish (proportionally & in total)
Eggs laid per female	No difference	Reduced in treatment
Time to first oviposition	Reduced in treatment dish	No effect seen
Landings	Smaller proportion land compared to control	Reduced number of landings in treatment dish
Duration of visit	Reduced in treatment dish	Reduced over the treatment dish
Overall repellence	Oviposition reduced but not completely deterred in treatment dish	Oviposition nearly completely deterred in treatment

Because of the unsatisfactory nature of the control water, it may be useful in future tests to use breeding site water that is known to be either repellent or attractive and provides stimuli, rather than their absence. 4-methylphenol may, therefore, be useful as a standard repellent for the comparison of other semiochemicals, for example 4-methylphenol can be used as a standard, against which the behaviour of mosquitoes in response to putative attractants and repellents can be tested to determine if a particular chemical is more or less repellent. However, if the mosquitoes are presented with water containing natural stimuli, then the effects being investigated may be less potent, or at least conflicted, than if a (supposed) neutral control is used.

The capacity shown by *An. gambiae* in its oviposition behaviour to lay eggs into sub-prime or even normally repellent substrates shows an unexpected level of plasticity, but also shows that the control water used here is only preferable to 4-methylphenol, not a more or less natural site. Using a saline control cannot be a fair test to attempt to discover what occurs in nature; given this is such an unnatural choice. Instead comparing a repellent to a novel chemical may offer a more useful and robust tool for identifying the natural behaviours of *An. gambiae*.

6.4.3 Areas for potential future investigations

It still remains to be seen if mosquitoes sample the water while ovipositing in flight, as it was not possible to determine if the tarsi contacted the water, or if this played any role in oviposition site selection. Future experiments may investigate this by filming with higher resolution and frame rates to determine if the hind legs make contact with the water. It may also be possible to use sticky film to trap the mosquitoes to determine contact, but the behaviour towards the sticky substrate could be different to that towards water dishes, which have a moisture component that sticky traps would lack.

It is also unknown if the females that oviposit while landed on the water surface are sampling the water. Clearly they are in contact with the water when landed, so perhaps investigations using mosquitoes with their hind tarsi removed or the chemosensilla disabled will help determine if the ability to directly sample the water has an effect on oviposition or dish choice. This may also be useful in

determine the role of the hind legs if they do contact the water when ovipositing from flight.

Landed females may also sample the water by ingesting it, and it may be possible using dyed or otherwise doped water in the dishes and dissection of females to determine if ingestion took place. Tests would need to be carried out, however, to confirm the altered water itself had no effect on oviposition behaviour.

It would also be interesting, now that the behaviour towards a known repellent has been categorised, to explore the responses of gravid mosquitoes to other semiochemicals, or perhaps even to identify the response of *An. gambiae* or other mosquitoes to water from natural breeding sites.

In summary Objective 5, the characterisation of oviposition flight behaviour, and determination of differences between scenarios where no-choice and a choice of oviposition targets were offered from video-recordings of gravid females of *An. gambiae* was met.

7 THE EFFECT OF BREEDING SITE ODOUR ON THE OVIPOSITION BEHAVIOUR OF *ANOPHELES GAMBIAE*

This chapter addresses Objectives 6 and 7 (as described in Chapter 1.5.3). Objective 6 was addressed by characterising the effect of presenting the behaviour modifying repellent 4-methylphenol either in water or in the air above the water. Objective 7 was addressed by determining whether the effect of 4-methylphenol on oviposition by gravid females of *Anopheles gambiae* is due to the insect's detection of the volatile compound in the air or contact with the compound in solution. In order to meet these objectives the use of partially permeable sachets to deliver the compound is investigated.

7.1 Background

The oviposition behaviour of *Anopheles gambiae* likely involves the detection and motor response to volatile chemicals emanating from water bodies (Takken & Knolls, 1999), either attracting (Blackwell & Johnson, 2000) or repelling them (Omolo *et al.,* 2004). One source of these volatiles is thought to be metabolites of bacteria present in such water bodies (Sumba *et al.,* 2004a; Lindh *et al*., 2008a; Rinker *et al*., 2013). In Chapter 5 it was shown that a chemical associated with some potential breeding sites (as a biological metabolite and component of cattle urine; Bursell *et al*., 1988), 4-methylphenol, elicits a negative oviposition response in *An. gambiae* (i.e. oviposition repellent). Oviposition behaviours associated with water containing 4-methylphenol were examined in Chapters 5 & 6.

Odour dispersal in still air

The assays described in Chapters 5 & 6 used still air, through which odour molecules disperse by simple diffusion (Elkington & Cardé, 1984). The rate of dispersal is a product of the diffusion coefficient (a property of the molecules in a gas based on weight and intermolecular forces) and the concentration gradient (Bossert & Wilson, 1963). Lighter, unreactive or non-polar molecules will disperse faster and further than a heavy molecule which interacts with its surroundings in a given time.

The concentration of odour molecules is inversely proportional to the distance from the source. In the vicinity of the odour source exists an "active space"; a region where the concentration of odour molecules is above the threshold required to produce a behavioural response (Mankin *et al.,* 1980; Baker & Roelofs, 1981). In still air, diffusion should take place evenly, resulting in a sphere of odour centring on the odour source. Over time this sphere will expand, increasing the size of the active space (Bossert & Wilson, 1963). In an enclosed space, such as the cages used in Chapter 5, this might lead to the entire arena becoming saturated with an odour.

Determining the active space or even the behaviour threshold can prove complicated: odours may elicit different responses at different concentrations (Baker & Cardé, 1979), or effects may be due to a specific blend of chemicals, where subtle alterations to the ratio of the constituents can give rise to different responses (Roelofs, 1978). Additionally, odour concentrations that are too small to elicit a response can eventually lead to a behavioural response after sustained exposure (Cardé & Hagaman, 1979). Temperature can alter the

responsiveness of organisms (Mankin *et al.*, 1980; Cardé & Hagaman, 1983), and alter the size of the active space (Baker & Roelofs, 1981).

Thus, while it may be difficult to determine how large the active space will be, a general model can be imagined, where the diffusion gradient of odour molecules creates a boundary to the active space, beyond which an insect is not responsive, but within which the insect will alter its behaviour in response to the volatile (Figure 7.1).

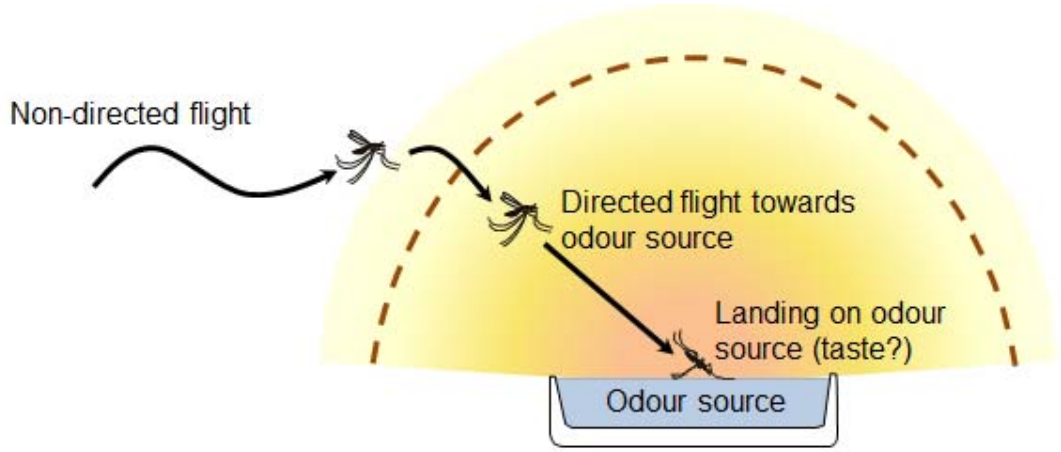


Figure 7.1 A model of the mosquito's approach to an attractant target in still air. The odour diffuses from the source in still air, decreasing in concentration in the air as distance increases. The mosquito flies randomly until contact with the active space (dashed line) surrounding the odour source. It then flies towards the odour source by following the concentration gradient, orientating itself with visual cues (Kennedy, 1940; Bossert & Wilson, 1963; Elkington & Cardé, 1984).

The use of artificial semiochemicals

Anopheles gambiae is strongly attracted to human odours (Costantini *et al*., 1998) and field collections have been made, at first using carbon dioxide (Gillies & Snow, 1967) and later other human odours (Njiru *et al.,* 2006; Torr *et al*., 2008). Research into the development of a synthetic human odour (Gibson *et*

al., 1997) has resulted in lures more attractive than humans (Okumu *et al*., 2010). *Culicoides* midges (Ceratopogonidae; Ventner *et al*., 2011) and tsetse flies (Glossinidae; Vale, 1974; 1980; Torr, 1989; 1994; Gibson *et al*., 1991), are now routinely trapped in the field by using synthetic odour baited attractants based on host odours. The efficacy of mosquito trapping with baited sachets has also been shown (e.g. Costantini *et al.*, 1993; 1996; Xue *et al.,* 2008; Roiz *et al.,* 2012).

Tsetse flies *Glossina pallidipes* Austen and *G. morsitans* Westwood, vectors of trypanosomaiasis in Africa, for example often feed on cattle, and are attracted strongly by ox-breath (Vale, 1974; 1977). Carbon dioxide attracts tsetse and mosquitoes (Vale, 1980; Costantini *et al*., 1996) extremely well, but field application is limited due to high cost and complexities of delivery. Other attractive components of ox breath to tsetse are: acetone, butanone and 1-octen-3-ol (hereafter referred to as octenol) (Vale & Hall, 1985a, 1985b) and phenols (Hassanali *et al.,* 1986; Vale *et al.,* 1988), e.g. 4-methylphenol (Bursell *et al.,* 1988; Green, 1994; Kappmeier & Nevil, 1999).

A 1:4:8 blend of 3-*n*-propylphenol, octenol and 4-methylphenol has proved particularly successful in attracting tsetse while remaining cost effective (Torr *et al*., 1997). This can be dispensed effectively and cheaply in the field using polythene sachets (Hargrove & Langley, 1990; Vale, 1991) which release their contents at a continuous rate until the sachet contents are exhausted (Torr *et al.,* 1997) and effectively attract a variety of haematophagous insects (Torr *et al*., 2007; Torr *et al*., 2008 ; Venter *et al*., 2011).

Oviposition cues have been successfully used to trap mosquitoes by mimicking the odour of oviposition sites (Hazard *et al.,* 1967; Bentley *et al.,* 1979; Millar *et al.,* 1992). While potential oviposition attractants of *An. gambiae* have been identified (Sumba *et al.,* 2004a; Huang *et al.,* 2006; Knols *et al*., 2004; Lindh *et al.,* 2008a), there have been no successful attempts to use baited oviposition trapping in the field (Himeidan *et al*., 2013).

In 2015 Lindh *et al*. (2015) identified cedrol ((1S,2R,5S,7R,8R)-2,6,6,8-tetramethyltricyclo-undecan-8-ol) as improving gravid trap catches and have suggested that this could be the first *An. gambiae* oviposition attractant to be discovered.

4-methylphenol repellence

The results of Chapters 5 and 6 suggest that 4-methylphenol can mediate the oviposition behaviour of mosquitoes, albeit as a repellent, but did not show how the chemical was detected by the insect. It may be by contact, either in the aqueous phase by their tarsi or sampling the water and detecting the chemical by gustatory means, i.e. 'tasting' it in the water, or by olfaction in the gaseous phase by their antennae. The results of Chapter 6 show landing was reduced when a choice was offered, indicating that the repellence might not be due to contact.

Chapter 4 demonstrated that the mosquito is capable of detecting 4-methylphenol in the gaseous phase, but it was not known if the behaviours observed were a response to the chemical in the air or if other sampling was

required. Video analysis (Chapter 6) did not show how many (or if any) mosquitoes were directly sampling the solution, either ingesting it or by contact with their tarsi.

Because this study did not encompass any neurological investigations it was decided to use behavioural experiments to determine which phase, the gaseous or the aqueous, was responsible for the observed repellency. This required the effective separation of the gaseous and aquatic phases, leaving a clean water source for oviposition to take place in and an odour source, removed from the water.

Therefore, permeable sachets of 4-methylphenol, calibrated to give a similar release rate as the solutions used in Chapters 5 & 6, were placed next to, but not in contact with a standard dish of water used for oviposition (0.9% saline, Chapters 5 & 6) to observe the response of mosquitoes to air-borne gaseous 4-methylphenol. By using sachets to deliver 4-methylphenol into the air, it was possible to repeat the video assay, but separating the behavioural components of contact with an aqueous solution from contact with an air-borne source of the test compound. The oviposition dishes contained only saline and not the chemical being tested. Comparisons could then be made of the mosquito's observed behaviours in the presence of 4-methlypheol in the air and in the water (Chapter 6) versus the chemical in the air only.

Anticipated behaviours

Based on the results of chapter 6, it was hypothesised that 4-methylphenol has a long range effect as a repellent, given the reduction in numbers of visits to dishes containing high 4-methylphenol doses. If the repellent effect is due to

volatiles detected in the air, then the repulsion should still be observed when the 4-methylphenol is separated from the water. If 4-methylphenol detected in the air is repellent to gravid *An. gambiae* it is expected that:

- The number of visits to the control dish will reduce;
- The proportion of visits to the control dish will reduce;
- The time in shot of all mosquitoes visiting the treatment dish will be reduced compared to visitors to the control dish;
- Additionally, if the active space around the sachets does not completely cover the treatment dish, there may some difference in the direction from which mosquitoes approach the treatment dish.

If 4-methylphenol detected by contact with a water-borne solution is repellent to gravid *An. gambiae* it is expected that the behaviour of mosquitoes that land on the 'treatment' and 'control' water dishes will not differ, because neither contained the test chemical. Therefore, no differences would be expected in the following behaviours that were affected when the treatment water contained 4-methylphenol (Chapter 6):

- No difference in the number of landings between control and treatment dishes;
- No difference in the duration of visits;
- No difference in the duration of landing times on the treatment dish;
- No difference in the number of eggs per landing female that oviposits.

These measures were scored, analysed for significant difference and then qualitatively compared to the results of Chapter 6 to further understand the behavioural effects of 4-methylphenol on *An. gambiae* oviposition.

7.2 Methods and materials

7.2.1 Preparation of sachets

Sachets were made from heavy duty 440 gauge (0.12 mm thickness) polythene layflat tubing (5 cm wide; www.tranpack.co.uk, part number PT5002) as used in Vale *et al.,* (2012) and contained an adsorbent made of cotton dental roll (www.coltene.com, product: Luna Dental Rolls, size 1). Neat 4-methylphenol (1ml; 98% 4-methylphenol, 2% 3-methylphenol; Sigma-Aldrich, UK) was added to the adsorbent to make treatment sachets.

An unaltered adsorbent was used in 'blank' sachets, i.e. for use in control experiments. Once the adsorbent was added the sachets were closed by heat-sealing. "Standard" sachets had a length of 5 cm to give a total surface area of 50 cm^2. Half-size sachets (2.5 cm x 5 cm; surface area 25 cm^2) were also prepared. The excess was trimmed at one end, with approximately 5cm left at the opposite end to permit handling and labelling (Plate 7.1).

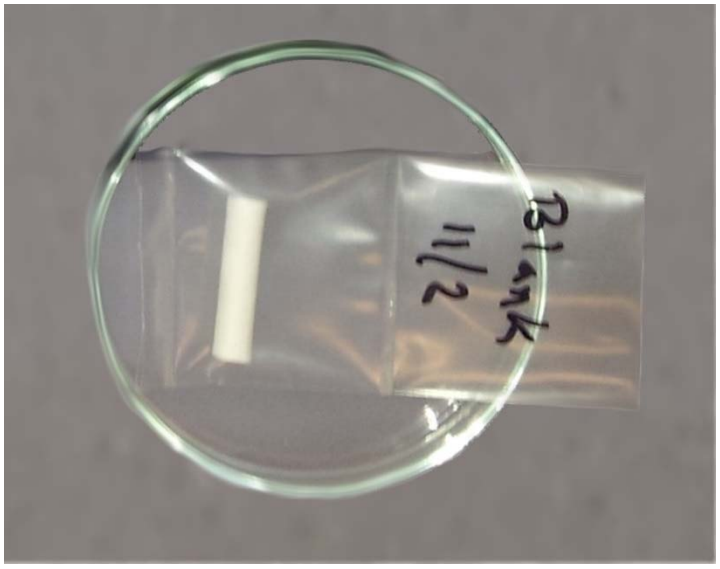


Plate 7.1 A blank sachet. The adsorbent can clearly be seen within the sachet enclosure. Blank sachets were identical to treated sachets in all respects other than the presence of 4-methylphenol. The glass dish measures 9.5cm in diameter and is identical to those used in the arena assays (see Section 7.2.2)

7.2.2 Odour release and delivery rates

Volatile sampling

Pyrex dishes (15 cm diameter, 1 cm internal depth) were prepared as for an assay; they were cleaned with ethanol spray and left over night in a fume cupboard to dry. Each dish was placed in a 10 L glass beaker and the dish was filled to the brim with a known concentration of 4-methylphenol in 0.9% saline. Four concentrations of 4-methylphenol solution were tested; 0.001, 0.01, 0.1 and 1 mg/ml 4-methylphenol. The beaker was covered with aluminium foil to enclose the headspace above the dish.

Volatiles from the headspace were collected using a field entrainment kit (Barry Pye, Rothamsted) onto Porapak Q adsorbent cartridges (200 mg; 50/80 mesh;

Waters Associates Inc., Milford, MA, USA) held in a Pasteur pipette (4 mm i.d.) with silanized glass wool plugs.

Dynamic sampling (rather than a static sampling technique, such as SPME) allowed a qualitative and quantitative measurement of the release rate of 4-methylphenol in the headspace. Dynamic sampling is a well-known and validated technique and is effective for sampling extremely low volatile concentrations. SPME provides more qualitative analyses and there are reports of problems reproducing results from extractions (Snow, 2002).

The Porapak cartridge was inserted into the headspace through the foil. Sampled air was replaced by clean charcoal-filtered air at the same rate as it was extracted (1 litre/min). Cartridges were removed and replaced at 1 h intervals with fresh filters. Each solution was sampled for 4 h (i.e. four 1 h samples). Temperature was maintained at 27±2ºC throughout the sampling period. Two samples were run simultaneously under identical conditions.

Sampling of the release rate from sachets was done in the same manner, with a sachet containing 1 ml 4-methylphenol suspended over a dish containing 0.9% saline, mimicking the arrangement of the sachets and dishes in the arena experiments (Figure 7.2). Two sizes of sachet (25 cm^2 surface area and 50 cm^2 surface area) were tested to determine the appropriate size for use in the arena.

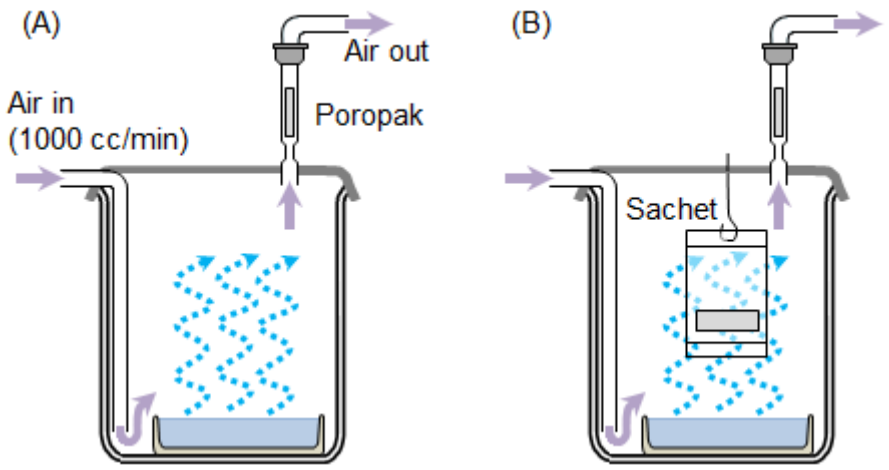


Figure 7.2 Arrangement of the samples for headspace volatile entrainment. Sampling of volatiles from (A) treatment dish. The headspace above a 15cm dia glass dish containing 0.9% saline and a known concentration of 4-methylphenol is sampled from within a 10 litre beaker, sealed with aluminium foil. Sampling volatiles (B) from sachet containing a known amount of 4-methylphenol, suspended above a dish containing only 0.9% saline.

Gas Chromatography (GC)

Volatiles collected were eluted from the exposed cartridges using dichloromethane (1 ml per filter; Pesticide Grade, Fisher Scientific, UK). In addition, decyl acetate (5 µg) was added as an internal standard. The samples were analysed using a HP6850 GC (Agilent, UK) on a column (30 m x 0.32 mm internal diameter) coated with polar DB Wax (0.25 µm film; Supelco, UK) with helium carrier gas (2.4 ml/min) splitless injection (200°C), and flame ionization detection (FID) (250°C). Oven temperature was programmed from 50°C for 2 min, then at 10°C/min to 250°C and held for 5 min.

The presence of 4-methylphenol was confirmed by comparison with authentic 4-methylphenol solution (2% 3-methylphenol; Sigma-Aldrich, UK).

7.2.3 Arena assay

Using a flight arena, measuring 120 cm wide, 120 cm tall and 200 cm long (Section 6.1), video recordings were made of the oviposition responses of 20 gravid *An. gambiae* females (released in the flight arena at the same time) to two target dishes, each containing 0.9% saline, the 'control' and the 'treatment' dish (next to which sachets containing an adsorbent wetted with 4-methylphenol).

The arena was maintained at 27°C±1°C and a relative humidity of 70%±10% (measured each night). The arena was illuminated as in Chapter 6 from below by diffuse visible light approximately replicating nocturnal conditions of a moonlit light. Infrared illumination from beneath and the side of the arena permitted filming using three Samsung SHC-735P analogue, high resolution, wide dynamic range video cameras (Samsung, Korea).

Camera 1 was fixed 15 cm above the floor of the arena, looking horizontally at the infra-red illuminated wall, positioned to view the two dishes from the side. The axis of the camera lens was directly along the surface of the meniscus of the filled dishes so that the height of a mosquito above the water's surface could be determined. The field of view was approximately 40 by 30 cm, at 80 cm where the dishes were placed, with a viewing angle of 30º.

Camera 2 was positioned 80 cm above the arena floor, facing down and centred above the position of dish A (Figure 7.2A). The field of view was approximately 30 by 23 cm at 80 cm where the dish was placed, with a viewing angle of 11º. Camera 3 was positioned as for Camera 2, but dish B.

Camera lenses and recording equipment were as described in Chapter 6.

A small platform (clear 0.5 cm thick Perspex® measuring 70 cm wide and by 30 cm deep) was placed adjacent to the illuminated wall and levelled off to provide a flat base for the oviposition dishes. The platform was lightly marked in pen to indicate the intended position of the target dishes.

Oviposition targets consisted of 15 cm diameter, 1 cm deep glass dishes, positioned on top of 25 cm^2 black infrared-transparent sheets (Figure 7.2B). The sheets were 5 cm apart and placed on the levelled platform. The dishes were positioned centrally on the black sheets and filled until the meniscus was level with the top of dish with sterile 0.9% saline. Dishes were filled approximately 10-15 min prior to the start of scotophase in order to allow the relative humidity to return to the desired level.

It was initially planned to suspend sachets above the water to duplicate the active space of the oviposition dishes containing 4-methylphenol used in Chapter 6, but due to the arrangement of video cameras, the sachets could not be placed directly above the water or they would obscure the view of the flight paths of the mosquitoes (Figure 7.3).

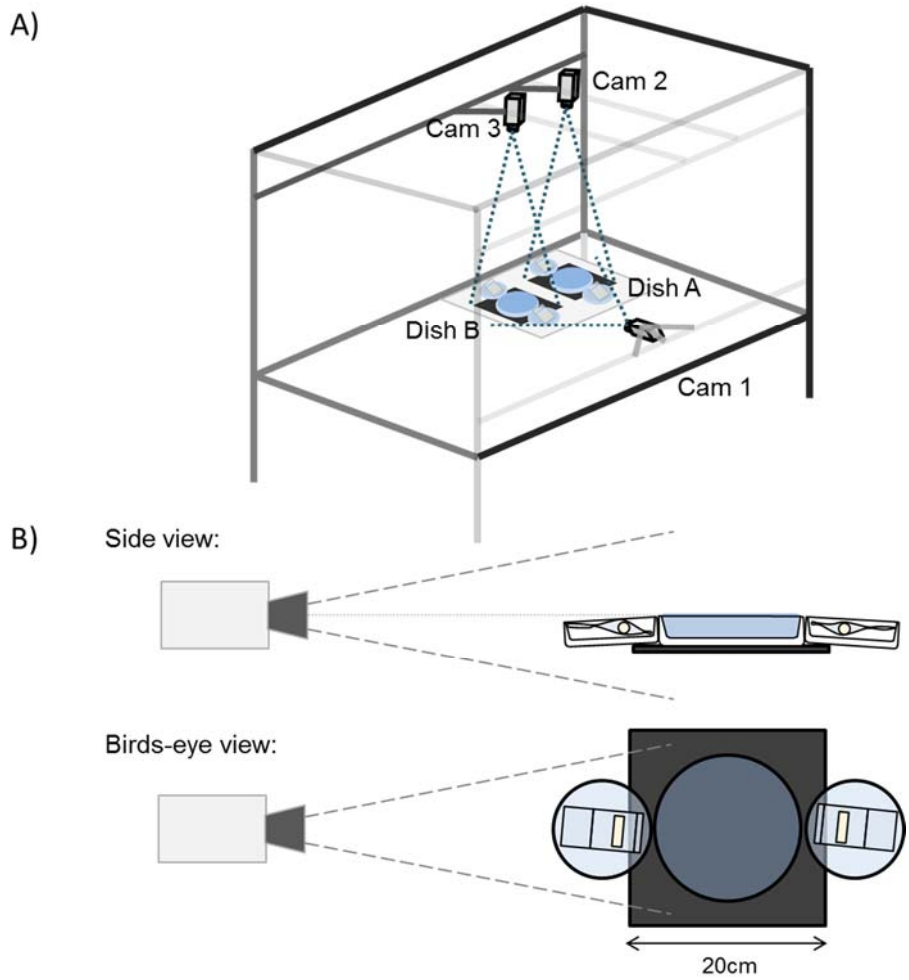


Figure 7.3 The arrangement of the cameras and oviposition dishes in the arena. Camera filming positions in the arena: horizontal (Cam 1), and birds-eye view (Cam 2 & 3). B) Oviposition dishes (15cm dia.) were filled to brim with 0.9% saline and positioned on top of black infrared transparent sheet. Two sachets are positioned next to the oviposition dish in smaller (9.5cm dia.) dishes, slightly overlapping the black sheet. Camera in B is positioned so that the horizontal axis is aligned to the meniscus of the water in the dish. Camera position in B is representative and not drawn to scale.

Instead two smaller glass dishes (9.5 cm diameter, 0.75 cm depth) were positioned adjacent to each larger dish and a sachet was placed into each smaller dish, approximately level with the surface of the water as placing them higher would obscure the horizontal facing camera (Figure 7.3B). Two blank sachets (i.e. with no 4-methylphenol added) were placed in the small dishes

adjacent to the control dish, and two treatment sachets were placed in the dishes adjacent to the treatment dish (Plate 7.2).

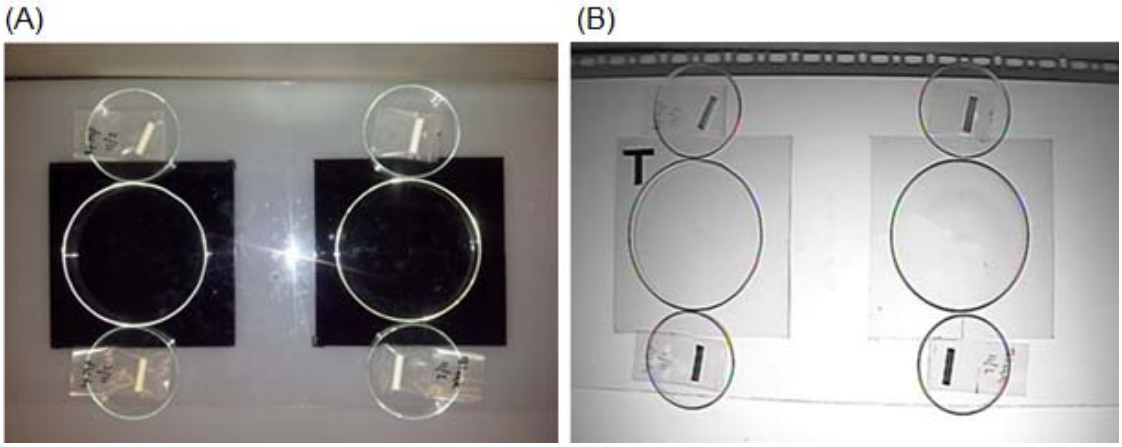


Plate 7.2 Arrangement of dishes in the large arena. The sachets are positioned in small glass dishes at "12 o'clock" and "6 o'clock". The plates are shown in visible light (A) and as seen under infrared illumination (B). The treatment dish is indicated by the 'T', as seen in B, placed under the black sheet and visible under infrared illumination.

Recordings took place from the start of the scotophase. Recordings lasted 4 h and all observed mosquitoes were scored for behaviour and the direction of approach to the dishes. Mosquitoes were prepared as per Chapter 2.3, (i.e. gravid females, given a single blood meal 48h prior to the experiment commencing).

After each recording the mosquitoes and sachets were removed from the arena and disposed of. The numbers of eggs laid in the large dishes was recorded and the contents drained and disposed of. All of the dishes, targets and arena surfaces were then wiped down with ethanol to remove any odour and the arena was left open overnight to dry.

Pairs of sachets either containing 1 ml of 4-methylphenol (treatment) or without 4-methylphenol (blank) were each tested 4 times against a pair of control

sachets (identical to blank sachets). One experiment was performed per night experiments took place. A total of eight experiments were conducted over approximately five weeks, according to availability of suitably aged mosquitoes. A power calculation shows that 20 mosquitoes allows detection of moderate sized effects (neumerator df=1, denominator df=18, significance level =0.05, power =0.8 gives predicted effects size of 0.44, moderate), meaning that 4 replicates should be sufficient to determine moderate effects.

Scoring the observed behaviours

Recordings were watched live to monitor the progress of the experiment, then backed up onto portable media and reviewed using VLC media player (version 1.1.9, VideoLAN project). Observations of the three video feeds for each experiment (two birds-eye-view of dishes, one side-on view) were made and behaviours scored as per Chapter 6, including: clock time mosquitoes entered the camera view (beginning of visit) and left it (end of visit); if any eggs were laid (yes or no); how the eggs were laid (landed or in flight) and number of eggs laid per visit.

The observed behaviours were recorded in note form and then used to compile a spreadsheet detailing the behaviour of all visits. From the spreadsheet the following measures were calculated:

- Mean visit duration per replicate (number of seconds a mosquito was in view of the camera)
- Mean number of visits per replicate where at least one egg was laid
- Mean number of visits per replicate when at least one egg was laid in flight

- Total number of visits over all replicates when at least one egg was laid in flight
- Mean number of visits per replicate when at least one egg was laid by a landed mosquito
- Total number of visits over all replicates when at least one egg was laid by a landed mosquito
- Mean number of in-flight eggs laid per visit
- Total number of in-flight eggs laid per treatment
- Mean number of landed eggs laid per visit
- Total number of landed eggs laid per treatment

These measurements were analysed as per Chapter 2.6, fitting data using a generalised linear model (GLM) with quasi-binomial errors to compensate for over-distribution and then analysed by Chi-square test (χ^2) if count data or analysis of variance (ANOVA) if measurement data to determine the degree of similarity of behaviours between treatments.

Scoring the direction of approach

The direction from which a mosquito approached any given target dish was determined from the video recordings. Each dish was divided into 12 30º sectors, by overlaying a 12 segmented circle with the apex of each sector extended to the edge of the frame over the recording. When an insect appeared in shot the dish approached and the sector it approached the dish from was recorded and entered into a spreadsheet. The direction of approaches to the treated dish and the control dish was then compared using Chi-square test.

7.3 Results

7.3.1 Odour delivery rates

GC analysis of the volatiles extracted from the headspace above a 15 cm dish containing 4-methylphenol in 0.9% saline solutions shows a linear effect of dose on release rate from the 4-methylphenol solutions (Figure 7.4).

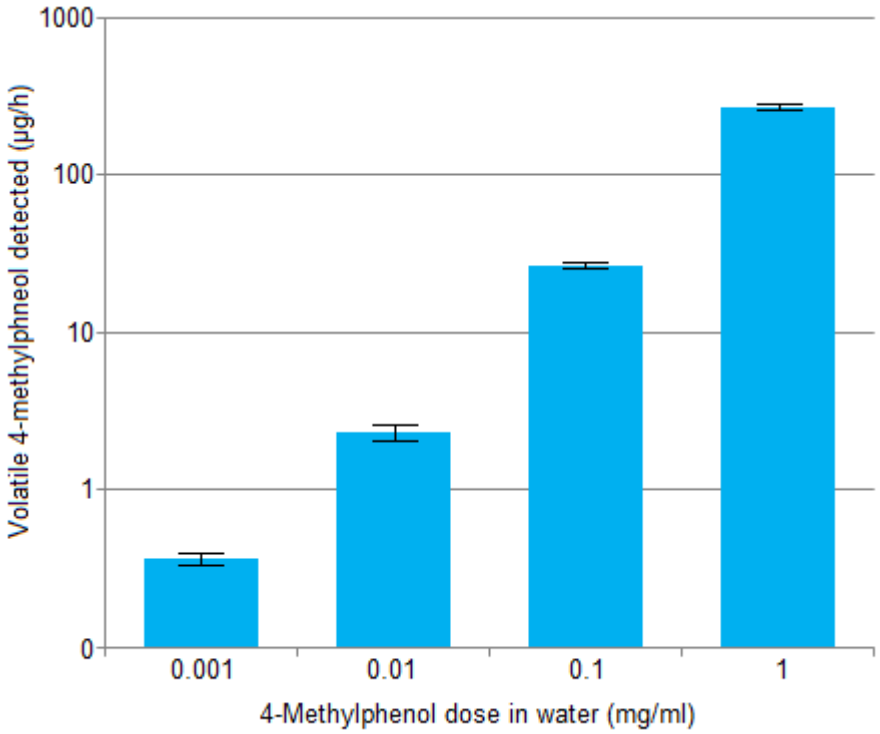


Figure 7.4 The release rate of 4-methylphenol from water solutions. Dishes contain solutions of 0.9% saline and 4-methylphenol at specified dose. Release rates sampled in moving air (1000 cc/min) at 27ºC. Overall means for replicates measured each hour over 4 h, ±SEM.

Release rates were measured from duplicate sachets each hour for four hours, the period used in subsequent behavioural experiments. Release rates at each dose were remarkably constant during this period, and results in Figure 7.4 are overall means with standard error (N = 8). For each increase in the amount of 4-methylphenol in solution, e.g. a tenfold increase, the rate of release is

increased by approximately the same increment, e.g. 0.268±0.02 (mean ±SE) µg/h volatile 4-methylphenol detected at 0.001 mg/ml solution dose, 2.32±0.22 (mean ±SE) µg/h detected at 0.01 mg/ml, 26.1±0.99 (mean ±SE) µg/h detected at 0.1 mg/ml and 268.9±14.4 (mean ±SE) µg/h detected at 1 mg/ml.

Release rates of 4-methylphenol from the sachets were also highly uniform over the 4 h measurement period. The release rate from the smaller sachets (25 cm^2 total surface area) was found to be 80.36±3.55 (mean ±SE) µg/h. The larger sachets (50 cm^2 total surface area) showed a release rate of 120.65±4.56 (mean ±SE) µg/h, approximately half the release rate of a 1 mg/ml aqueous solution. The sachet release rate suggested that using two larger sachets for each treatment would be a suitable substitute for 1 mg/ml of 4-methylphenol in solution.

Comparison with standard 4-methylphenol solutions confirmed that the volatile released from both the solutions and the sachets was 4-methylphenol.

7.3.2 Arena assay

Visit duration and oviposition

Video recordings were made of the oviposition responses of 20 gravid *An. gambiae* females to oviposition targets with adjacent 4-methylphenol containing sachets in the large arena. Four replicates each were made of the mosquitoes' responses to the treatment sachets (1 ml 4-methylphenol) and the blank sachets. Mosquitoes were seen to oviposit into the 'treatment' dishes (saline with treatment sachets nearby) as well as control (Figure 7.5).

Blank sachets vs. blank sachets

In blank sachets vs. blank sachets tests there were no significant differences between the dishes in the number of visits (χ^2=2.01, df=3, p=0.22, n/s, critical value for p=0.05 is 7.82 at df=3, for two tailed test), the duration of visits that included landing (χ^2=3.01, df=3, p=0.40, n/s) or the number of visits that did not include landing (χ^2=0.492, df=3, p=0.99, n/s). There were also no significant differences between the number of eggs laid per ovipositing female when landed (χ^2=2.01, df=3, p=0.33, n/s), in flight (χ^2=2.01, df=3, p=0.33, n/s) or overall (χ^2=5.66, df=3, p=0.70, n/s) (Figure 7.5).

Blank sachets vs. treatment sachets

In blank sachets vs treatment sachets (containing 1 ml 4-methylphenol), a significant reduction in the number of visits to the treatment dish was observed (χ^2=42.32, df=3, p<0.001; Figure 7.5B).

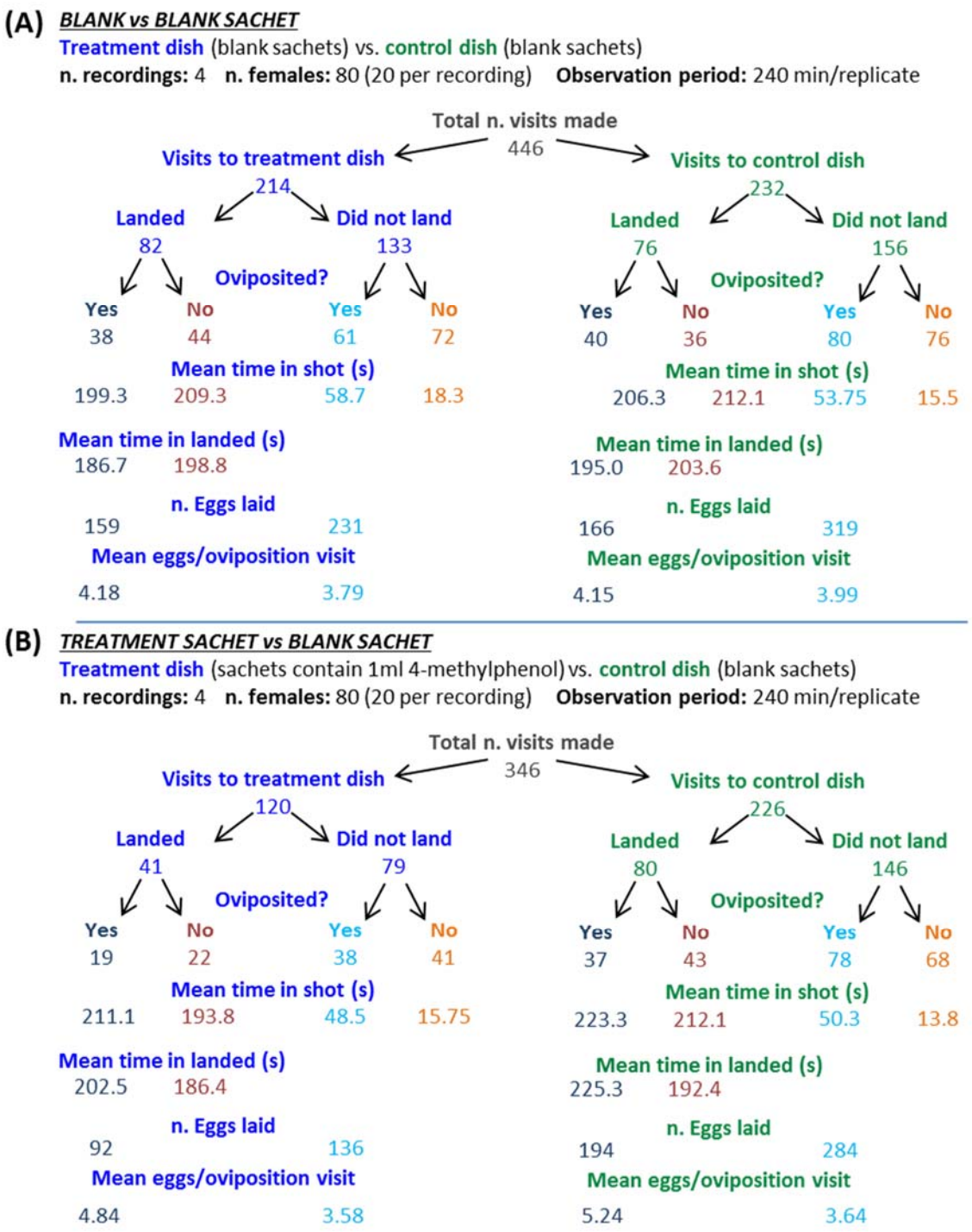


Figure 7.5 Summary of behaviours observed towards two oviposition dishes. Control experiment, with two dishes of 0.9% saline flanked by blank sachets, (containing no 4-methylphenol) and B) Treatment experiment with one dish flanked by blank sachets and the other flanked by treatment sachets containing 1ml 4-methylphenol. All dishes contained 0.9% saline only.

There were no significant differences in the mean duration of visits (i.e. time in shot) between mosquitoes that visited the treatment or blank dish in either the treatment vs. blank or the blank vs. blank (control) tests (χ^2=6.32, df=3, p=0.81, n/s) There was also no difference in the duration of visits where mosquitoes landed (χ^2=5.72, df=3, p=0.70, n/s) or the time in shot of non-landers (χ^2=2.87, df=3, p=0.33, n/s), although visits where the mosquito landed were shorter than visits where the mosquito did not land (χ^2=87.46, df=15, p<0.001; Figure 7.6).

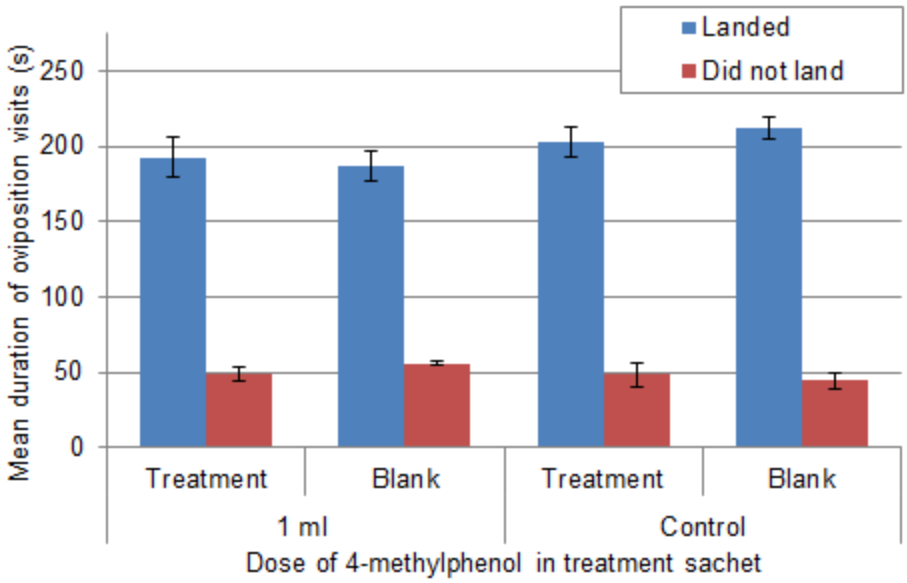


Figure 7.6 Duration of visits with and without landing. Mean duration of all visits observed for each replicate ±SEM. Dishes contain 0.9% saline, with two sachets next the dish. N= 160 mosquitoes making 792 visits, of which 279 visits included landing.

There was no effect seen on the duration of visit of mosquitoes which landed and oviposited, with visits of a similar duration to all dishes (χ^2=9.45, df=7, p=0.74, n/s; Figure 7.7). There was also no significant difference in the duration of ovipositing mosquitoes which did not land (χ^2=5.62, df=7, p=0.22, n/s), but, their visits were shorter than those that landed and oviposited (χ^2=56.37, df=15, p<0.001).

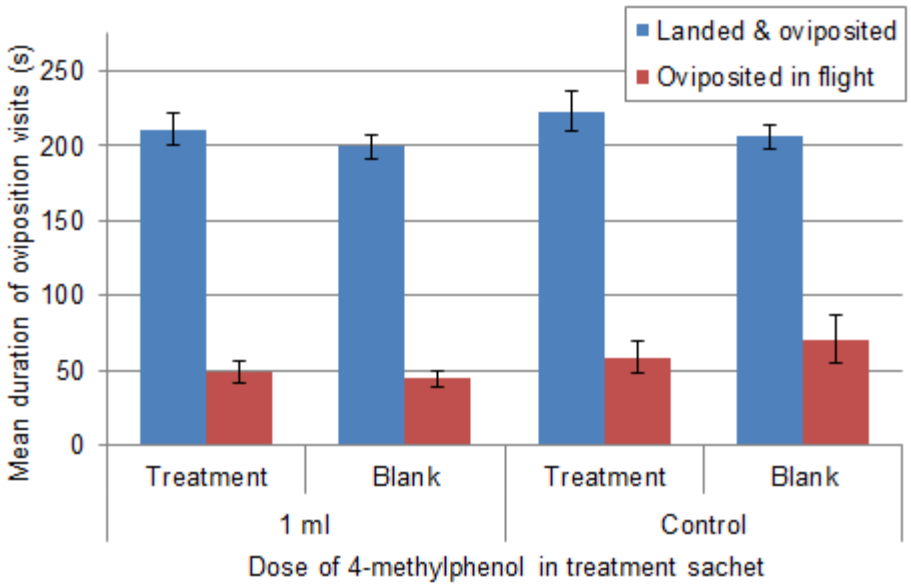


Figure 7.7 The duration of visits when female oviposits. Mean duration of all visits observed in seconds for each replicate when at least one egg was laid are shown for mosquitoes that oviposited while landed (blue columns) and those that oviposited while in flight (red columns) ±SEM. N= 160 mosquitoes observed, making 391 visits, of which 134 visits included landing.

No difference was seen between the proportion of mosquitoes that visit and land in the treatment dish or the control dish (χ^2=0.45, df=3, p=0.99, n/s). There were also no differences in the proportion that oviposited after landing (χ^2=0.80, df=3, p=0.99, n/s) or that oviposited during flight (χ^2=0.62, df=3, p=0.99, n/s) between the treatment and control dishes.

No differences were seen in the numbers of eggs laid per female (χ^2=0.716, df=3, p=0.99, n/s) between treatment and control dishes.

Direction of approach

A significant difference in the direction of approach was seen between the control and treatment dish when the treatment sachet contained 1 ml 4-methylphenol (χ^2=37.57, df=11, p<0.001). No difference was seen in the direction of approach where a blank treatment was offered, i.e. control vs. control (χ^2=0.729, df=11, p=0.99; figure 7.8).

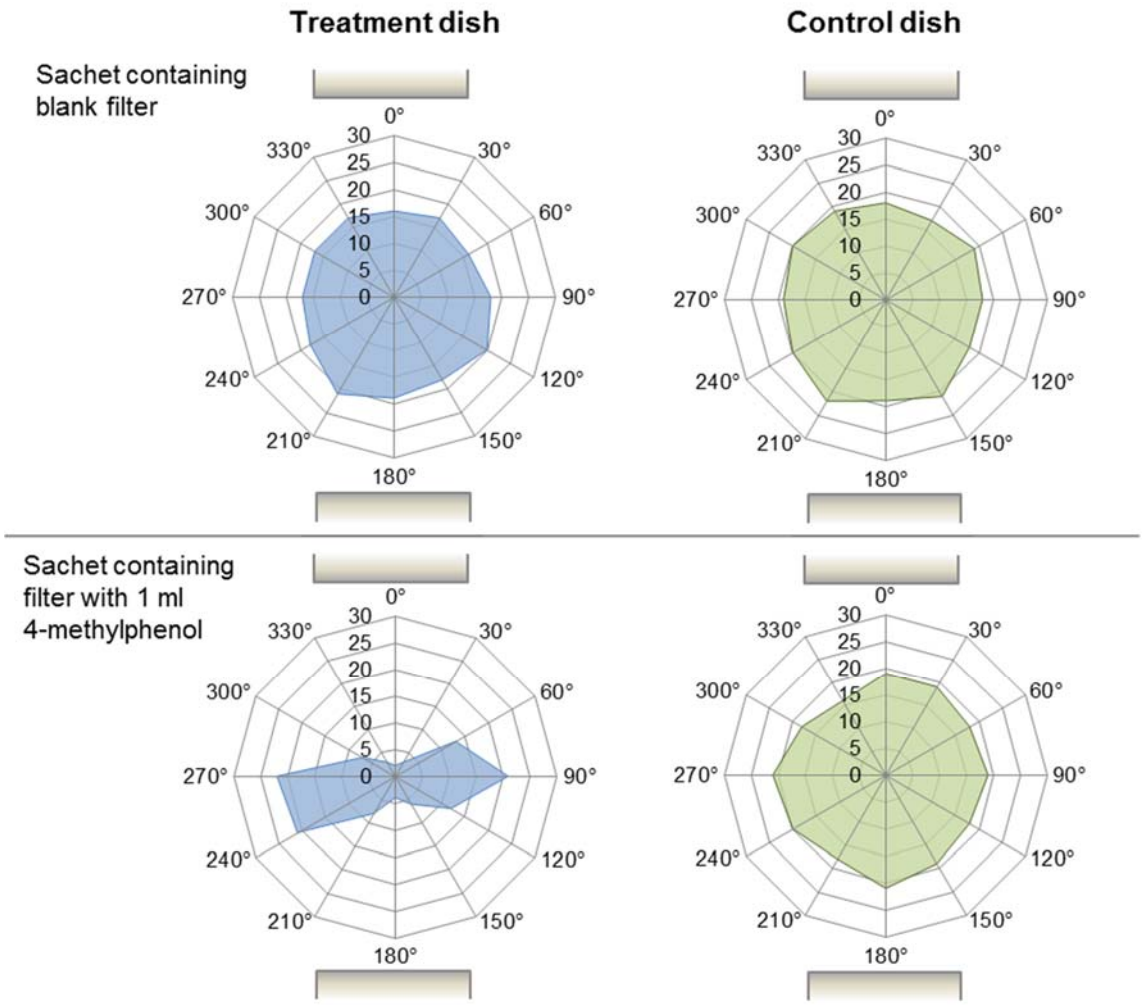


Figure 7.8 Approach vectors of mosquitoes visiting dishes. The sachets were positioned next to the oviposition dishes, covering the angles 330° - 30° and 150° - 210° (as indicated by the silhouettes above and below each diagram). Coloured areas indicate numbers of mosquitoes approaching the oviposition dish along the respective vector. Circular axis is numbers of mosquitoes that approached within each 30° sector (i.e. the angle shown ±15°). Control dishes had blank sachets positioned adjacently.

With 4-methylphenol present the majority of mosquitoes that enter the field of view were seen to appear and approach the oviposition dishes from between 30°-150° and 210°-330°. Mosquitoes avoided approaching the dishes in the sectors that corresponded with the position of the treatment sachets, suggesting that they were repelled from the area of the sachets. No such 'channelling' effect was seen around the control dishes.

7.4 Discussion

7.4.1 4-Methylphenol repellence

As shown in Chapters 5 and 6, 4-methylphenol acts as an oviposition repellent in *An. gambiae* when added to water in potential oviposition sites. When given a choice between dishes containing either 1 mg/ml 4-methylphenol saline solutions or pure saline solutions, treated dishes are strongly rejected. This repulsion is not limited to the treated target, but also affects nearby untreated targets, resulting in fewer visits, shorter visits and a reduction in the rate of oviposition. The active space of the repellent apparently extends beyond the range of the treated dish.

Here it is shown that the same chemical, when presented as only an air-borne chemical contained in a sachet, causes repellence, resulting in significantly fewer visits. However, once a mosquito arrives at a treatment dish there is no discernible difference in behaviour compared to a mosquito that arrives at a control dish.

When the 4-methylphenol is detected in the water (as in Chapter 6), overall fewer mosquitoes land in the treatment dish, mosquitoes that do land remain landed for shorter time and total oviposition is reduced compared to the untreated dish. In the sachet tests none of these effects were seen. This suggests that the mosquitoes can sample the 4-methylphenol in the aqueous phase, through contact (either with palps, antenna or tarsi) or ingestion, although it is still unclear which.

Future work might be able to determine the mechanism of sampling employed. This could be done behaviourally (e.g. comparing the oviposition of mosquitoes with damaged, excised or inactivated sensory apparatus, i.e. with tarsi removed, to these results), determining which sense organs can detect 4-methylphenol using genetic techniques, or by a combination of these and other approaches.

Receptors on the labellum of *An. gambiae* can detect 4-methylphenol (Kwon *et al*., 2006), suggesting that gustatory sampling may be used to discriminate between clean and treated water bodies. Gustatory receptors are also found on the maxillary palps and antennae of *An. gambiae* (Pitts *et al*., 2011) and *Drosophila melanogaster* (Shiraiwa, 2008; Syed *et al.,* 2011). The tarsal receptors of *An. gambiae* share remarkable similarities with the gustatory receptors of *Ae. aegypti* and *An. gambiae* (Kent *et al*., 2007) and, like odourant receptors, are conserved within the group Diptera (Hill *et al*., 2002), but there is no evidence as to whether or not the tarsi of *An. gambiae* are sensitive to 4-methylphenol.

The major effect of 4-methylphenol when delivered by sachet is one of repulsion from the treated dish, before contact has been made with the water. This effect is presumably olfactory.

Natural *An. gambiae* breeding sites are normally characterised as being 'clean' water, i.e. low in decaying matter content. Since 4-methylphenol is strongly associated with organic metabolic by-products (high levels are found in urine; Bursell *et al*., 1988), it would not be surprising if this chemical served as a reliable indicator of unsuitable breeding sites for this species.

On the other hand, 4-methylphenol is thought to be an attractant to host seeking *An. gambiae* (Cork & Park, 1996; Meijerink *et al*., 2001; Zweibel & Takken, 2004; Wang *et al*., 2010), but once blood-fed the mosquito appears to use it to determine unsuitable oviposition sites. It may be worth testing if unbloodfed mosquitoes still approach the target, to examine if the presence of the host attractant 4-methylphenol makes the targets appear to be hosts. This could also build on the work of Hawkes (2013), which investigated the response of *An. gambiae* to host odours with and without visual stimuli. Thus, the effect of the chemical on *An. gambiae* depends on its physiological state. As the mosquito blood-feeds between each oviposition cycle, it is likely that its response to 4-methylphenol will alternate accordingly throughout repeated oviposition cycles.

7.4.2 The role of the active space

Through evaporation and then diffusion a volatile is released from a solution in an open dish evenly over the space above and around the dish, producing an evenly distributed 'cloud' of volatiles above the dish, assuming still air conditions. As distance increases, the level of volatile decreases, resulting in a

boundary of detectability determined by the sensitivity of the mosquito's sensory systems to that chemical, and this boundary is effectively the distance at which the mosquito's behaviour will be affected by that volatile.

The sachet tests suggest that the active space over the sachets is different to that associated with the open dishes. The respective surface areas of the sachets and dishes (exposed area of each sachets = 2 x 25 cm^2 and the surface area of the oviposition dishes = 47.12 cm^2) and the release rates of 4-methylphenol are roughly equal. Thus, in the experimental arrangements used here, with either two 4-methylphenol sachets (one either side of the clean water oviposition dish) or a single 4-methylphenol -treated dish, the level of 4-methylphenol in the air around each dish should have been equal in both cases. As the level of volatiles emitted was the same, the null hypothesis would be that the effect would be the same, but this did not take account of the fact that the distribution of the active space around one dish might not resemble the active space created by two sachets on either side of a dish of untreated water, regardless of release rates.

The single dish should produce a single active space above and around the dish. The sachets appear to have produced two active spaces next to the dish (Figure 7.9), resulting in a corridor between them of non-repellent air, through which the mosquitoes may pass without entering an active space. This results in the observed channelling effect, where the mosquitoes visiting the treatment dish approach along this corridor.

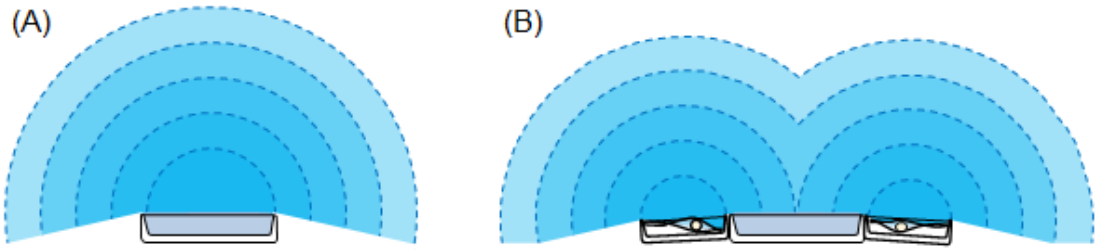


Figure 7.9 The diffusion of odours from a dish or sachets. The diffusion of a volatile by evaporation from an open dish (A) will create an even active space over the dish in still air. Delivering the odour from separate sources to the water body, such as by sachets (B) will result in two active spaces, spreading out from the odour and overlapping, creating a very different volatile profile around the target dish.

Polythene sachets have been shown to be an ideal way of delivering semiochemicals to control insect pests, such as pheromone lures for agricultural pests or tsetse host-odour attractant lures (Green *et al*., 1993; 1994; Torr *et al*., 1997; Mukabana *et al*., 2012). Having demonstrated that 4-methlyphenol to be highly repellent to gravid *An. gambiae*, the next step was to determine whether the repellent chemical could be delivered in sachets, still preventing malarial mosquitoes from laying eggs but without contaminating water bodies with 4-methylphenol. If 4-methylphenol, or other similar repellents, were used in this way for mosquito control, this would also reduce the cost of continuously replacing 4-methylphenol that would be washed away more quickly if poured directly into water ways, or lost through evaporation of water bodies.

The data obtained from the experiments reported here show that even though similar amounts of 4-methylphenol were released into the air by the two sachets as by an open dish of a 4-methylphenol saline solution, the two sachets appear to be less effective as ‘repellents’ than the single dish in preventing oviposition in the dish. It would seem likely that the difference in degree of repellency could

be related to the difference in distribution of active spaces between these two delivery methods. It appears, therefore, that the coverage of the active space over an oviposition target plays a crucial role in oviposition repellence. It is not clear if distribution of attractants would also suffer the same limitations.

In order to further examine the role of the active space, future work could examine if an alternative means to deliver the odour might replicate the active area of a simple dish containing a volatile repellent in solution. One method might be to release the odour at the surface of the water via tubes (Figure 7.10).

This arrangement would give a clean water body and may mimic the distribution of the active area above the target. It would, however, also clutter the environment, alter the water tensions on the meniscus and produce a heterogeneous surface, which could deter the mosquito from ovipositing.

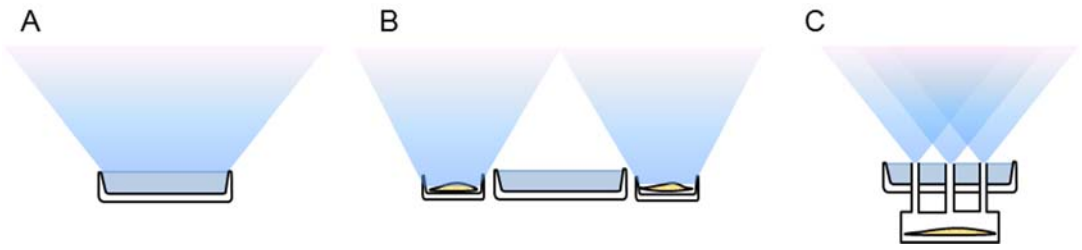


Figure 7.10 Three methods of odour delivery. A) Volatile diffusion from a baited open water source, B) using a secondary odour source, such as sachets, adjacent to a clean water source, and C) using a secondary volatile source separate from the clean water but with passages through which the odour is released at the surface of the water.

The release rate would also need to be higher to compensate for the reduced surface area of these tubes. Such a system might prove useful for preventing oviposition using a repellent, but would probably prove less practical than using

a physical barrier. Alternatively, this could be used to deliver an attractant, but this assumes the physical changes to the environment would not deter oviposition. This may not be so given that water acts as a pre-oviposition attractant itself (Okal *et al*., 2013).

Delivery pipes would also need to be smaller than the mosquito, as gravid *Culex* show great persistence in finding oviposition sites in inaccessible places (Harbison *et al*., 2007). Once the attractant has drawn a mosquito toward a water body, the presence of water itself will act as a sufficient attractant to the gravid mosquito to prevent it attempting to reach the odourant attractant. Given these concerns the practicality and usefulness of such a system may be questionable.

Despite the differences in active areas, the use of sachets to deliver 4-methylphenol has been successfully demonstrated, showing that the long range repellency of this chemical is olfactory based and a separate process from any close range repellency. The lack of close range repellency once the mosquitoes have approached the oviposition dishes and the sachet suggests that the presence of 4-methylphenol in the water is detectable, probably by direct or close sampling. Thus, there are a number of steps involved in the determination of individual water body suitability for oviposition, and these steps begin while the insect is some way from the water body. As with finding a blood meal, the mosquito appears to use its suite of sensory apparatus, including visual, olfactory and gustatory cues to determine where it will lay its eggs.

In contrast to the findings of this study, it may be interesting in future work to repeat these experiments using hungry (i.e. non-blood fed) females to see if

they approach the target. If they do so this would suggest that 4-methylphenol does have a key role in host target finding. See also Chapters 5 and 6 of Frances Hawkes PhD thesis for further discussion on host seeking flights of *An. gambiae* in the presence and absence of odour and the responses mediated by visual stimuli (Hawkes, 2013).

The usefulness of repellence is questionable given the vast size of areas that malaria affects. Given that repellence is initially olfactory, it may be possible, it is likely that any oviposition attraction, should an oviposition attractant effective in the field be found (such as suggested by Lindh *et al*., 2015), would act in a similar fashion. This could be employed to attract gravid *An. gambiae*, as long as the method of odour delivery does not contaminate the water. Trapping of gravid mosquitoes is a technique used currently for control and population monitoring, and the effective use of gravid traps outdoors for *An. gambiae* is now under serious investigation (Harris *et al.,* 2011; Dugassa *et al*., 2013). Interest in this has increased following a shift in behaviour observed following the reduction of mosquitoes indoors (Bayoh *et al*., 2010), due to the success of programmes that have targeted host-seeking mosquitoes (Tirados *et al.,* 2006; Russell *et al.,* 2011; Reddy *et al*., 2011) – mosquitoes are now more likely to feed outdoors, where insecticide treated bednets, for example, are not effective.

7.4.3 Monitoring and control applications

There are two main methods for collecting *An. gambiae;* when resting, e.g. by pit traps (Odiere, 2007), or when host-seeking, e.g. using humans or synthetic odours as bait (Dia *et al*., 2005; Qui *et al*., 2007). Harris *et al.* (2011) have demonstrated the use of a gravid trap for *An. gambiae* which uses a sticky sheet that floats on the surface of an oviposition site. The Harris trap is effective in

areas of high mosquito density and collects *An. gambiae* as well as other Anophelines and Culicines. The trap appears to be effective as it is not discernible from the oviposition site.

It has been shown, however, that other gravid traps, such as used for *Culex* monitoring may be repellent if they have a cluttered visual appearance (Irish *et al*., 2013). An effective *An. gambiae* trap needs to take into account the fact that the process of laying eggs in a particular place relies on both visual and chemical cues (Bentley & Day, 1989).

Grid traps or ovitraps have been commonly used to monitor and control *Ae. albopictus* and *Ae. aegypti* for over 40 years in a number of countries affected by diseases vectored by these mosquito species (Jakob & Bevier, 1969; Reiter *et al*., 1995). These traps are not complicated; Baak-Baak *et al.* (2011) as demonstrated by the effective use of small, home-made ovitraps baited with artificial odour lures for *Ae. aegypti*. *Culex quinquefasciatus* is effectively caught using baited CDC traps, which also catch other non-target species attracted to the bait (Irish *et al*., 2013). Most gravid traps, such as the CDC gravid trap, consist of an artificial water body, baited to attract gravid females, above which an electric fan is used to trap the approaching mosquitoes in a net or other container. While effective, the trap is limited by the life of the battery and the water body which may become less attractive or repellent over time or simply due to evaporation. Traps may also damage the catches, making identification difficult.

Despite there being no such thing as a typical oviposition site (Fillinger *et al*., 2009) *An. gambiae* tends to oviposit in open, sunny water bodies (Munga *et al*.,

2005; Gilles & DeMelion, 1968) that lack dense vegetation or other disruptions (Minakawa *et al*., 1999). Thus, a successful oviposition trap for this mosquito would seek to replicate this, such as the 'OviART' trap described in Dugassa *et al.* (2013), or to blend into existing sites, such as Harris *et al.* (2011). The OviArt trap mimics the physical characteristic of a successful oviposition site, but lacks any long range attraction.

Passive traps, such as the Harris sticky trap (Harris *et al*., 2011) share one drawback - their passive nature dictates that in order to trap a mosquito, the trap needs to be positioned where a mosquito will oviposit, something which at present cannot be predicted with certainty.

It is now apparent that 4-methylphenol repels gravid female *An. gambiae* when the mosquito has a choice of oviposition sites, suggesting the chemical is incompatible for use with gravid traps. However, it has been shown that it is possible to mediate the oviposition behaviour from one target. Thus, it might be possible to force a mosquito to oviposit into a particular target, so long as this target is less undesirable than surrounding targets, which could be dosed with repellent to increase their unattractive qualities in contrast to the target. In this way oviposition might be a behaviour that can be controlled and exploited as control technique for *An. gambiae*. While field testing would obviously be required to confirm this theory, it may well be that it is not a practical solution to *An. gambiae* larval control. But, if it was shown to not reduce oviposition significantly, this might be useful as a monitoring tool, given the effect will likely not travel far.

It is also apparent that if they contain 4-methylphenol, human (or animal) odour baited traps (either natural or synthetic) will likely be repellent to gravid mosquitoes. Care should be taken to not position traps for monitoring host-seeking or gravid *An. gambiae* in too close proximity to one another. Despite this, the directional approach assay demonstrates that *An. gambiae* will not approach an oviposition site if it has to pass through the active space of a repellent, suggesting that it may be possible to create odourant 'walls' around potential oviposition sites. Sachets could provide a convenient method of dispensing airborne repellent in the field for such applications.

Alternatively, it seems that sachets containing host-seeking attractants might be useful in both drawing in non-gravid mosquitoes (Torr *et al.,* 2008). Should these attractants, like 4-methylphenol, also be oviposition repellents, it may be possible to also deter gravid females of *An. gambiae* from approaching a potential oviposition site by masking it with host odours. This added benefit to studies using host odour might be an interesting area of future study.

While it is possible that 4-methylphenol may have a role to play in the control of gravid *An. gambiae* it is, as a repellent, unlikely to have a useful role in monitoring this insect. Sachets of 4-methylphenol do not appear to be useful for mosquito repellent, requiring the deployment of too many to be practical in real-life situations. However, what is clear is that an effective oviposition attractant or a strong volatile repellent which acts over a wide area could effectively be dispensed by sachet. Should either of these be found, future research should consider the use of sachets as a means dispense these chemicals in laboratory or field trials.

In summary, Objective 6 was met by demonstrating behavioural differences between the responses of gravid females of *An. gambiae* to 4-methylphenol presented dissolved in water, or in sachets above the water. By doing so, Objective 7 was also met, demonstrating that 4-methylphenol has an airborne repellent effect, suggesting an olfactory repellence, but also having an effect on those mosquitoes that contact the compound in solution, deterring oviposition and reducing time the mosquito remains in situ.

8 GENERAL DISCUSSION

The research presented in this thesis was undertaken to investigate the oviposition behaviour of the mosquito *Anopheles gambiae* by examining the chemical ecology of potential oviposition cues and the physiological and behavioural responses of the insect to semiochemicals of practical importance.

8.1 Critical analysis of methodologies

8.1.1 Mosquitoes

In the following chapters the presence or absence of eggs, as well as the numbers laid were used as indicators of oviposition preference. Testing the oviposition responses of *An. gambiae* requires that they be mated, blood fed and then gravid. The rearing techniques used make a number of assumptions, the first of which is that mating occurred.

Males tended to eclose from pupae the day prior to females, and during this first day the eighth to tenth segments of the male abdomen undergo a process termed “inversion”, which is necessary to permit successful copulation (Clements, 1999). However, not all mosquitoes pupate and then emerge at the same time, resulting in cages with mosquitoes of slightly different ages. Mosquitoes are blood-fed in the cages, when they are 3-5 days post emergence and it is assumed that most of the males in the cage are sexually mature and have mated with at least some of the females.

Females are blood fed only once prior to experiments, and despite great care, visual inspection can be difficult, which may result in misidentification of gravid and non-gravid mosquitoes.

It was assumed that the mosquitoes would be gravid 48 h after a blood feed, and that they would oviposit on the following night. Accordingly, it is not possible to make assumptions when an experiment returns no eggs because it is not clear if a choice to 'not oviposit' was made or if some blood fed females had not mated. Direct observations made during rearing suggest that after a single blood meal has been offered eggs can be found not just following the second night after feeding, but also after successive nights as well. It is not clear why this happens, nor is it clear if the eggs are laid by females that oviposited on previous nights or not. It is known that supplemental blood meals may be required by some female *An. gambiae*, especially those previously fed on sugar, prior to their first oviposition (Briegel & Hörler, 1993; Clements, 1999; Manda *et al*., 2007).

Ideally, each experiment would use a single gravid female; with many replicates, the observations of single females, unimpeded by other gravid females competing for space to oviposit or even deterred from oviposition by previous oviposition by other females, would give an excellent (if perhaps an idealised) baseline for mosquito oviposition behaviour. However this is impractical due to restrictions on both rearing and experimental cages, and also physical space (Chapter 5). Thus an experiment must use more than one mosquito in order to reliably guarantee oviposition will occur each time.

Because it is impossible to tell which eggs were laid by which female, it is also not possible to determine how many females had oviposited. Dissection of every female after experiments could be used to determine if mating had occurred and examine the ovarioles for signs of oviposition, but this was not

employed due to time constraints and difficulties in recapturing mosquitoes from the large arena (Chapters 6 & 7).

Thus, it is never certain how many of the mosquitoes in an experiment did not oviposit, and if this was in any way due to the treatment or simply because they were not ready to oviposit.

8.1.2 Bacteria

The use of bacterial solutions to simulate natural oviposition sites was based on the work described in Lindh *et al*. (2008a), in an attempt to replicate the findings reported. As such, it was decided that the solutions made for this body of work should follow Lindh *et al.* (2008a) methodologies. Additional information was obtained directly from Lindh, who also provided samples of the bacteria and commented that the growth medium was removed from the solutions as it had been found to act as an oviposition repellent in preliminary cage tests (personal communication).

In order to produce the solutions, the growth medium was therefore removed (although a minute amount remained despite attempts to remove it in all solutions), resulting in a mixture almost entirely consisting of 0.9% saline and live bacteria. In order to confirm the bacteria had not been destroyed, an extra bottle of saline was prepared, left in the laboratory overnight and a sample plated out onto agar. Colonies that visually resembled the bacteria being tested were produced from all bacteria, with no signs of contamination.

Removing the growth media and suspending the bacteria in a solution containing no nutrients did not kill the bacteria, but it most likely halted their growth, and probably had an effect on their metabolism. As a consequence the

volatiles emitted may be different from bacteria in agar growth media and from bacteria in natural mosquito breeding sites.

The intention was that the solutions should replicate the conditions of a natural *An. gambiae* oviposition site; clean water containing a low level of bacteria found in the surrounding soil. The bacteria originate from collections by Sumba *et al.* (2004a) of soil from around sites where *An. gambiae* oviposition had occurred. Sumba *et al.* (2004a) also showed that the soil in solution was an oviposition attractant in laboratory assays, as used in Chapter 5. Bacteria were identified from these soil solutions by Lindh *et al*. (2008a) and suspensions of some of these were found to be oviposition attractants in the same laboratory assays as Sumba *et al.* (2004a). Thus, the bioassays of the bacterial solutions used in the project reported in this thesis should reproduce this attractance, as reported in Lindh *et al*. (2008a). Crucially, the bacteria in solutions should be in the same metabolic state as in nature because the aim was to identify oviposition attractant volatiles that occur in nature, which are produced as a result of the metabolic activity of the bacteria.

Some doubt remains as to whether this aim was achieved or not in this study, and critically, it is not clear if the bacteria solutions tested reflect those found in nature. Only by testing the bacteria in the field (or at least semi-field conditions) would we know if they are oviposition attractants to wild *An. gambiae*. Breeding sites do clearly contain some organic content (i.e. the bacteria) as well as matter from the substrata of the water body, from which the nutrients that sustain the bacteria no doubt come (such as the solutions used by Sumba *et al.* (2008a)), and it is likely that any bacteria in a water body would not be in a metabolic state identical to that of the prepared solutions, and may therefore produce different

volatile profiles. It may even be that the attractive volatile producing bacteria are not in the water but in the surrounding mud, in which case they almost certainly will be in a natural growth media.

Thus, it is by no means certain that the oviposition tests using bacterial solutions in Chapter 5 (made as per Section 2.4 above) were replicating natural oviposition sites at all. However, the bacteria used are the same as in natural sites, and may be in a similar metabolic state in both the laboratory solutions and in natural water bodies, but unless this is tested it cannot be known. What is known is that because the methodologies used in the studies reported in this thesis were the same as those used in the work reported by Lindh *et al*. (2008a), the results should be similar. There may be many possible reasons for any differences found; bacteria are organisms, not stock chemicals, and can vary due to any number of environmental conditions in our respective laboratories.

8.2 The role of volatiles in the attraction of *An. gambiae* to oviposition sites

Mosquito oviposition is mediated by a complex of factors, including external stimuli, circadian rhythms and the physiological state of the insect (Muirhead-Thompson, 1945; Clements, 1999). However, the choice of oviposition site by *An. gambiae* is still little understood, partly due to the variety of typical natural breeding sites (Fillinger *et al*., 2009). Water bodies offer a number of cues, such as visual stimuli (Kennedy, 1942) and the presence of water is itself an *An. gambiae* attractant (Kennedy, 1942; Bentley & Day, 1989; Bernáth *et al*., 2012; Okal *et al*., 2013), but not all water bodies are utilised by *An. gambiae*, suggesting there must be factors they use to discriminate between water bodies.

Sumba *et al.* (2004a) reported that *An. gambiae* oviposit preferentially into soil infusions from natural oviposition sites, but this attraction was eliminated by sterilisation of the infusion, suggesting volatile semiochemicals originating from the bacteria in the soil might provide oviposition cues (Bentley & Day, 1989; Rejmankova *et al*., 2005). Oviposition attractants have been found for several mosquito species (e.g. Hazzard *et al.,* 1967; Bentley *et al.,* 1979; Millar *et al.,* 1992; Blackwell *et al*., 1993), and although potential oviposition attractants for *An. gambiae* have been identified (Huang *et al.,* 2006; Knols *et al*., 2004; Lindh *et al.,* 2008a), none have proved successful at attracting large numbers of *An. gambiae* in the field (Himeidan *et al*., 2013).

In Chapter 3 the chemical constituents of volatiles released by a range of bacteria species isolated by J. Lindh and her co-authors from water bodies where *An. gambiae* were found to be breeding were re-examined. Unfortunately, the results of Lindh et al. (2008a) could not be reproduced exactly; fewer compounds were identified in the volatiles and none of the bacteria appeared to be oviposition attractants in a cage bioassay. Why the chemical profiles differed between these two studies so greatly is not known, but the bacteria also failed to produce the oviposition responses reported by Lindh et al. (2008a) in bioassay cage tests (Chapter 5), possibly as a result of the different volatile profiles and the reasons discussed in Section 2.6 concerning the standard operating procedures for handling bacterial solutions, which may explain some of the discrepancies between the results reported here and by Lindh et al. (2008a). As none of the bacteria were found to be attractive (Chapter 5), it was not possible to determine which, if any, volatiles might be the cause of the previously reported attraction, and so no potential attractant

oviposition semiochemicals could be identified from the data presented in this thesis. This has cast doubt on the findings of Lindh *et al.* (2008a), which may need to be thoroughly reviewed reassessed.

It was established that 4-methylphenol, a human sweat odour also found in contaminated natural water bodies (Bursell *et al*., 1988) elicited significant oviposition repellence. Kweka *et al.* (2011) suggest 4-methylphenol may be an *An. gambiae s.l.* oviposition attractant when fresh, but it may be that the attractive features of the artificial oviposition sites of Kweka *et al.* (2011) overrode the repellent effects of 4-methylphenol, or that the dose of 4-methylphenol was too low to be repellent. There may have been an unidentified synergistic effect with another compound in the urine creating the observed attraction, although Kweka *et al.* (2011) do not suggest this. As a host attractant and an indicator of undesirable oviposition sites, it is sensible to hypothesise that a gravid mosquito might be repelled by 4-methylphenol when a choice of oviposition targets is offered, as observed in Chapters 5-7 despite, being attracted to it when host-seeking.

8.3 The role of *Anopheles gambiae* olfaction in oviposition

Electroanntenographic studies to determine the responses of *An. gambiae* to host odours have previously measured the antennal response to a volatile 'puffed' over it from a substrate (such as a filter paper; e.g. Cork & Park, 1996; Blackwell & Johnson, 2000). This method can be used to determine if a response occurs, but is not fully quantitative as it is not known how much of the compound is delivered to the insect with each 'puff'. In Chapter 4 results are

presented of an EAG study of *An. gambiae* responses to these chemicals using gas-chromatography linked to electroantennography (GC-EAG), which delivers a known dose of volatile compound to the insect antenna.

Of the compounds selected to test, 4-methylphenol (*p*-cresol) had previously been tested found to be active in gravid female mosquitoes by Blackwell & Johnson (2000) and, therefore, was used as a positive control, against which the strength of responses to other compounds could be compared. Compounds typically thought to be human host attractants, such as octenol and 3-methylbutanoic acid were found to be detected infrequently and weakly, which may not be surprising, as we tested gravid female mosquitoes.

Olfaction is ultimately regulated by of the activity of odour binding proteins in the sensilla, which are the functional units that bind to odourants. The physiological changes that occur post-blood feeding in *An. gambiae* include altering the level of expression of certain odour binding proteins (Fox et al., 2001, Rinker et al., 2013), suggesting that the ability to detect certain volatiles may vary with time of day, physiological state or age of a mosquito, contrary to traditional notions that if an insect has the capacity to detect a particular odour, that odour will be detected regardless of physiological state. It is likely that different sets of cues attract mosquitoes to their blood hosts or oviposition sites – it would be metabolically advantageous to 'switch off', or at least reduce the receptors that are not needed in favour of those that are for a given stage in the mosquitoes life cycle. This change is an important aspect of *An. gambiae* olfaction, and highlights the fact that mosquitoes that are host seeking and those that are gravid and searching for an oviposition site are not likely to behave in the same way and that attracting them requires different cues.

The results of Chapter 4 demonstrate that, in the absence of visual cues, *An. gambiae* are still able to discriminate between two oviposition targets which are identical except for the addition of a repellent or an attractant to one dish, as reported in Sumba *et al.* (2004a) and Lindh et al. (2008a).

In order to determine if the observed repellence was in the aquatic or gaseous phase the repellent odour was separated from the water by using sachets containing 4-methylphenol placed next to dishes containing 0.9% saline in 2 choice tests (Chapter 7). The sachets did not reduce oviposition in the same way as the 4-methylphenol in solution did. However, a volatile effect was observed; the presence of 4-methylphenol in sachets altered the direction of approach by flight to the oviposition dishes by mosquitoes. This suggested that 4-methylphenol acts as a long range repellent, affecting the behaviour of the mosquito as it approaches a potential oviposition site. 4-Methylphenol in the volatile phase alone does not act to alter oviposition behaviours of mosquitoes which do approach the dish (Chapter 7): if the long range repellent effect is overcome or insufficient than the mosquito can approach the dish and more closely investigate the water.

Odour concentration is also important (Baker & Cardé, 1979), suggesting there is a threshold, below which 4-methylphenol is not repellent. Attraction to 4-methylphenol was not observed in any experiment reported here, in contrast to the results of Kweka *et al.* (2001).

8.4 The behaviour of ovipositing *Anopheles gambiae*

Examining the oviposition responses of mosquitoes in small cages is a technique that has been used in a number of studies over many years, e.g. Kennedy (1942), Sumba *et al.* (2004a) and Lindh et al. (2008a). Cages, however, offer a very different environment to that of wild mosquitoes, and the fitness requirements likely differ, too (Lecomte *et al*., 1998). The use of a larger arena did not completely mitigate these issues, especially as the mosquitoes were cage-reared, but the flight arena better simulated a natural environment by offering more space, more visual cues and natural lighting conditions instead of the unnatural total darkness experienced by mosquitoes in cage tests. Flight arenas also permitted the study to be laboratory-based, rather than field-based, which reduces complexities of filming and is preferable for investigating the behaviour of nocturnal insects in free-flight (Cardé & Gibson, 2010).Videoing the oviposition responses of *An. gambiae* to a single dish of water and to two dishes of water in the arena allowed a fuller description of oviposition to be made than by relying on cages tests alone. Mosquitoes were seen to oviposit in two modes; landed on the water, and in flight, consistent with the observations of McCrae (1984). It is not clear why the mosquitoes exhibited two types of oviposition behaviour. These may have arisen to achieve oviposition in different ecological conditions, such as different habitats, but that does not explain why both were seen under the same conditions, where there was no difference (aside from the treatment of 4-methylphenol) in either target, or even when there was only one target.

Females that oviposit while landed on the water's surface expel eggs with their abdomens raised above the water, letting the eggs fall briefly through the air

and then onto the water, occasionally further manipulating them with their legs (presumably to prevent the insect carrying them off upon departure).

Those that laid eggs in flight were seen to oviposit more frequently and laid the majority of eggs. They underwent a frenzied flight over the water, rapidly jabbing over the dish and releasing their eggs in flight. The jabbing flight typically occurred 0-5 cm above the water's surface. It remains unclear if the females sampled the water directly, before ovipositing, although the findings of Chapter 7 suggest that this does occur to some extent. The exact reason for the dipping flight during oviposition is not clear. Kennedy (1940) demonstrated that *Ae. aegypti* use visual cues to orient themselves in relation to wind direction, and it may be that after each dip down it must then re-orientate itself using visual cues, by returning to a particular height. Alternatively, the mosquito may be moving through the active space above the dish, sampling it and using this to orientate itself (Gillett, 1979), or even to sample the water. Alternatively, it may be that the mosquito approaches the water so the egg does not fall too far once released. It may be that the behaviour is a modification of normal flight, where a stimulus (such as the water, an odour, the visual cues or a combination of these and perhaps others) initiates the dipping behaviour, much like the 'programmed' pattern of turning by moths (Kennedy, 1983). The insect then returns to the 'default' flight position before the stimuli trigger the next dip.

When given no choice but a dish containing a strong repellent, oviposition was not completely deterred, but the mosquitoes were less inclined to approach the treated dish than a control dish. The number of eggs laid per visiting female was not reduced, suggesting that if a female begins to lay then she will lay the same number of eggs regardless of perceived site suitability. When given a

choice, a dish containing a strong repellent will be rejected in favour of a dish with no repellent, as in the cage experiments. The proportion of visits to the treated dish and their duration were also reduced. These behaviours contrast sharply with those of *Cx. quinquefasciatus* towards known oviposition attractants, where the presence or absence of an attractant does not affect the duration of visits to a bowl, regardless of whether or not the female oviposits (Pile *et al*., 1991; 1993). Thus, attractants might act at longer ranges (drawing the insect near to the oviposition site) but not affect the insect at a close range to the oviposition dish, as repellents such as 4-methylphenol appears to do.

The number of observed visits to oviposition dishes in the arena where mosquitoes oviposited was greater than the number of mosquitoes in the arena, suggesting that some mosquitoes oviposited on more than one occasion during the observed 4 hours. Despite this, the number of eggs per female (7.13+0.21 eggs/female) was lower in the arena than in cages (25.2±0.49 (mean ±SE) eggs/female), which may simply be due to the fact that tightly packed mosquitoes in a 0.027m^3 cage find oviposition dishes more readily than those in an arena with a volume of. 2.88m^3. Despite releasing more mosquitoes into the arena the density (6.9 mosquitoes/m^3) is far lower than in the cages (440.4 mosquitoes/m^3).

Mosquitoes were fed 48 h prior to experiments, and by appearance were gravid. Early experiments indicated that oviposition should peak in the first 4 hours after darkness, which is when video recording took place. However, oviposition does not always occur 2 nights after blood feeding, as expected, but can occur prior to the scotophase, or even in the third or fourth night after blood feeding. Wild females may also benefit from multiple blood meals, which may help explain

why caged mosquitoes with a single blood feed can oviposit at times other than when expected.

These experiments have further highlighted that *An. gambiae* will oviposit in a range of sites, and may oviposit in a less than ideal site if there is no other choice. It seems that the reason there is no idealised description of *An. gambiae* oviposition sites may be because such a thing does not exist. *Anopheles gambiae* shows this by the vast multitude of sites that larvae are found in, with apparently little or nothing in common between these sites. By adapting its behaviour to the environment it is presented with, a gravid female demonstrates plasticity which defies simple description.

Further plasticity is seen in the host seeking and biting behaviour of *An. gambiae s.s.*, which has shifted more towards biting outdoors (Reddy *et al*., 2011; Russell *et al*., 2011) and (as well as *An. arabiensis*) biting earlier in the evening (Yohannes & Boelee, 2012). Ironically, these changes are probably due to the success of indoor spraying and LLIN use, but further highlight the adaptability of this vector, which is such an important part of its success. Coluzzi (1984) suggests that the success of control techniques is reduced as the diversity within a vectoral system increases, and while this refers to the complexity within the *An. gambiae* complex, the idea can equally be applied to behavioural adaptations such as these. If current control techniques are rendered unsuccessful by changes in *An. gambiae s.l.* behaviour then the development and use of other methods, such as gravid trapping using attractants, becomes more pressing.

8.5 An integrated solution for a complex problem

When investigating behaviour it is easy to come to conclusions from what may seem like specific data, but is in fact quite general. For example, egg count data may seem to indicate an oviposition preference, but in fact tells little other than the fact there is an egg that has been laid. Kennedy (1978) argues that it may be a falsehood to group together one behaviour or a series of behaviours into broad categories based on responses to a particular stimuli. It does not follow that because we can observe where an egg has been laid that we can draw *a priori* conclusions about the behaviour of the insect prior to oviposition based on *a posteriori* evidence. Each individual piece of information is a snapshot in time of part of a larger sequence, which may involve many events prior to the one being measured, each of which is likely to rely on different cues. To make more broad deductions, a more broad approach is required and in the case of oviposition we will only truly understand the full complexities of the process through direct observations, rather than deducting the entire process from data gathered in a piecemeal fashion.

A simplistic view of oviposition is that that each potential oviposition site gives the insect a series of cues; be they attractants or repellents, which either draw in or push away the insect. However, the terms ‘attraction’ and ‘repulsion’ are often used in a crude and subjective way, explaining little in themselves (Hardie, 2012). By looking for an attractant using simple methods, such as egg counts after two choice assays for example, it is easy to miss the important behaviours that proceed, or even follow the point at which the metric is recorded (Kennedy, 1978). More eggs in one dish might suggest an attraction, but does not guarantee this.

This thesis has contributed to our technical understanding of *An. gambiae* behaviours by providing data and analysis of oviposition choices. However it also contributes to increasing our conceptual understanding of behaviour by suggesting that it is necessary to take a more holistic approach to the subject and that by observing the mosquitoes and drawing together a number of different approaches we can more fully understand the suite of behaviours encompassed by the term 'oviposition'. This process has been similar to that undertaken in the development of an effective integrated control program to control tsetse flies; a logical progression of attaining knowledge, whereby each question answered drives us towards the next question, is required until we can finally determine a solution to the whole problem (Torr, 1994).

Dekker and Cardé (2012) consider odour mediated changes in *Ae. aegypti* flight to be part of a 'motor programme', much as Kennedy (1983) related the turning in moths being the result of a single input that triggered a programed pattern. If we think of each behaviour in a sequence as being part of the insect's 'programming', it is clear that we must look at each input, how it enters the system, what effect the input has on the programme and how the programme responds. As Kennedy (1979) suggests, attraction and repulsion are crude terms. Mosquitoes do not act without a reason, but rather respond to changes in the stimuli they receive from the world around them and also internal cues. Oviposition is much more complex than a single behaviour such as a binary choice; it is a complex suite of behaviours that follow a logical sequence. We need to understand the sequence of behaviours in order to understand the question more fully – we need to know more about the 'programming' that drives oviposition. Similarly too narrow a focus on one control method, such as IRS,

LLIN or even larviciding to control *An. gambiae* would be simplistic and unlikely to work. This idea is the basis of integrated control, where a suite of techniques work together to reduce vector populations. For this approach to succeed, however, we first need to first understand the suite of behaviours involved to target these different aspects of the insect's life cycle successfully as part of an integrated strategy. Thus, understanding the insect's behaviour as broadly as possible is essential to controlling the disease.

8.6 Future research areas

The behaviours examined in this thesis have suggested two primary areas which may merit further future concentrated research; the practical use of both oviposition repellents and attractants in trapping, monitoring and controlling *An. gambiae* within the field.

Given the lack of agreement between the results presented here and the results of Lindh et al. (2008a) about the effect of bacterial volatiles on oviposition behaviour a reassessment of the work of Lindh *et al*. (2008a) and Sumba et al. (2004a) may be useful in confirming if the oviposition attractant effects can be repeated in a laboratory and, more importantly, to determine if the reported effects occur in the wild, i.e. does the presence of bacteria in water make potential oviposition sites more attractive than others?

Anopheles gambiae oviposits into a wide range of habitats (Fillinger *et al.,* 2004; Fillinger & Lindsay, 2011), and it is largely accepted that the gravid female of *An. gambiae* does 'chose' where she oviposits (Kennedy, 1942; Muirhead-Thomson, 1945; McCrae, 1984), likely due to volatile semiochemicals of

bacterial origin (Sumba *et* al., 2004a; Huang, 2006; Lindh *et al.*, 2008a). The results of Herrera-Varela *et al*. (2014) demonstrate that *An. gambiae* will oviposit in a wide variety of sites, and that in the absence of 'better' alternatives will accept many sites of seemingly lower quality, especially in urban environments. As shown in this thesis, the gravid female of *An. gambiae* will preferentially oviposit in a neutral pool where a repellent is offered as the alternative. It may be that in this species there are no attractants, but rather what is observed in the wild is a rejection of unsuitable, repellent or unstimulating oviposition sites. It is clear that visual cues are important (Kennedy, 1940; Bossert & Wilson, 1963; Elkington & Cardé, 1984), and it is shown in this thesis that the visual appearance of an oviposition target can alter the oviposition behaviour of *An. gambiae* (Chapter 6.3.1 & 6.3.2).

Even if an attractant-based oviposition trap was to be developed, it would need to compete with the multitude of natural and man-made oviposition sites if it were to be successful. Traps could be concentrated in inhabited areas, but this may not completely remove biting females from the area, and it is likely that immigration from outside the treated area will refresh numbers continually, which would not be targeted by gravid traps. However gravid traps may still be of some use in reducing populations of *An. gambiae* locally in the long term. The effectiveness of such traps would depend on the number of traps, their effective range and the migratory range of mosquitoes into the area. In order to create a boarder around inhabited areas, future research would be needed to determine how wide this boarder needs to be.

In the case of several other species of mosquitoes, gravid traps are used in monitoring or control. These require an effective attractant, such as rotting

matter (i.e. hay infusions (Hazard *et al.*, 1967)), which have been shown to effectively attract mosquitoes such as *Aedes albopictus* (Trexler *et al*., 2003), *Culex pipiens* (Jackson *et al.*, 2005), *Culex quiniquefasciatus* (Millar *et al*., 1992).

Specific compounds that attract gravid female *Culex* mosquitoes have been identified (Millar *et al.*, 1992; Du and Millar, 1999; Leal *et al.*, 2008), and commercially available baits are available for gravid mosquito traps designed to catch *Culex quinquefasciatus* (Irish *et al*., 2010; Irish *et al*., 2013). In other cases simple hay infusions are used as bait (Reiter, 1983). These are effective for catching gravid females of species such as *Ae. aegypti* (Baak-Baak *et al.,* 2011). However, there are still no effective baited gravid traps for *An. gambiae* in operational use, although water itself may be attractive to gravid females of this species (Okal *et al*., 2013), and Lindh *et al*. (2015) have recently suggested cedrol as an *An. gambiae* oviposition attractant. Future studies should therefore focus on whether an effective attractant, other than the presence of water, exists for *An. gambiae* in the wild.

From the data in this study it is not clear if oviposition repellents offer any advantage over attractants, other than the fact that a repellent was proven effective in deterring oviposition when a choice was given, whereas reported oviposition attractants did not prove effective at all. Xue *et al*. (2001) demonstrate that an insect repellent can deter oviposition in *Ae. albopictus*, suggesting that even applications at low doses may be effective. This has not, however, been used operationally as a practical oviposition deterrent in any major control applications or study.

Given that this thesis demonstrates that *An. gambiae* will oviposit into a less repellent oviposition target when given a choice it may be useful to examine if repellent treatments, such as topical sprays or targeted sachet deployment, might deter oviposition in *An. gambiae*. Although topical biting repellents have been shown to be effective in deterring human-mosquito contact (Maia *et al*., 2012, Lupi *et al*., 2013), there are doubts as to their effectiveness in malaria control, especially in lower socio-economic groups (Chen-Hussey *et al*. 2013, Wilson *et al*. 2014). Combining biting and oviposition repellents may be useful as part of the integrated approach to malaria control, by creating a more complete barrier between the vector and host, and is an area that may benefit from further investigations.

Learning may also be a factor affecting female mosquitos' choice of oviposition sites: studies have shown that associative learning has been demonstrated in *Culex quinquefasciatus* (Tomberlin *et al.,* 2006; Sanford & Tomberlin, 2011), *Aedes aegypti* (Menda *et al*., 2012) and *Anopheles gambiae* mosquitoes (Chilaka *et al.,* 2012). These studies all involve associative learning based on either food rewards (Tomberlin *et al.,* 2006; Sanford & Tomberlin, 2011; Chilaka *et al.,* 2012) or electric shocks (Chilaka *et al.,* 2012) demonstrating that both attraction and repellence can be learned behaviours. However, it is not clear if there is a potentially learned element to oviposition repellence or attraction.

Future studies could use observations (such as using the video recording methods described in Chapters 6 & 7) to determine if females that have been previously exposed to a high dose of 4-methylphenol in an oviposition assay are less inclined to oviposit or approach a target containing the same compound as part of a learned behaviour. It may also be useful to investigate if the avoidance

of 4-methylphenol by gravid females of *An. gambiae* is a trait selected for – i.e. is the oviposition repellence of 4-methylphenol observed greater in successive generations? This could potentially have real world applications in the design of future gravid traps, as each generation could become more susceptible to the repellent.

8.7 Conclusion

The research presented in this thesis has revealed much about the oviposition behaviour of the primary African malaria vector, *An. gambiae,* but has not been able to reveal an oviposition attractant for this species. In the decade since the first potential *An. gambiae* oviposition attractant was reported, there is still no clear explanation as to why this mosquito will oviposit preferentially into soil infusions over clean water. It is not even clear if the phenomenon is due to a single attractant, or if it is a more complex interaction of stimuli that leads up to *An. gambiae* selecting a particular oviposition site over others.

Prior to this study, the natural observations behaviour of *An. gambiae* and the potential for exploiting this behaviour in control techniques had not been well examined. This project has generated a significant about of new and detailed knowledge of the sequence of events that lead up to oviposition and the effects on oviposition of a range of potential oviposition stimuli. Until now the scientific 'weak link' was the lack of knowledge about these aspects of *An. gambiae* behaviour. The findings presented here can help in further understanding the choices that an ovipositing mosquito makes and assist in the development of a useful gravid trap as a tool for the control and monitoring of this species.

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APPENDIX: OVIPOSITION VIDEOS

A number of videos clips taken from oviposition video recordings have been uploaded to YouTube as supplementary material. The following web links can be followed for in-browser viewing.

	Video Description	Video URL
Video 1	*Anopheles gambiae* oviposition flight, viewed from the side	http://youtu.be/5My2_SGIdzs
Video 2	*Anopheles gambiae* oviposition flight, birds-eye view	http://youtu.be/CIgNgsn81mU
Video 3	*Anopheles gambiae* oviposition flight, ½ speed, viewed from the side	http://youtu.be/qjZ7eW-lSp8
Video 4	*Anopheles gambiae* oviposition while landed, viewed from the side	http://youtu.be/ERPPCqkrnzM
Video 5	*Anopheles gambiae* oviposition while landed, birds-eye view	http://youtu.be/okBE0FI4IWw
Video 6	*Anopheles gambiae* oviposition flight, alternative side view*	http://youtu.be/YZh81X9o5bs
Video 7	*Anopheles gambiae* landed oviposition, extended birds-eye view*	http://youtu.be/rVGz6Zs9LJg

* Videos 6 and 7 by courtesy of F. Hawkes