AN INVESTIGATION OF DEET-INSENSITIVITY IN AEDES AEGYPTI

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Abstract

N,N-Diethyl-*m*-toluamide (DEET) is one of the most effective and commonly used mosquito repellents. However, during laboratory trials a small proportion of mosquitoes are still attracted by human odours despite the presence of DEET. In this study behavioural assays identified *Aedes aegypti* females that were insensitive to DEET. The selection of either sensitive or insensitive groups of females with males of unknown sensitivity over several generations resulted in two populations with different proportions of insensitive females. Crossing experiments showed the 'DEET-insensitivity' trait to be dominant. In addition to the finding of heritable DEET-insensitivity, unselected culture mosquitoes were shown to change their sensitivity to DEET after brief pre-exposure to the repellent. Female mosquitoes that were sensitive to DEET when first tested became insensitive when retested. Electroantennography showed that mosquitoes that were insensitive to DEET had a reduced response to DEET compared with mosquitoes that were sensitive to it. This was the case both for culture mosquitoes displaying insensitivity to DEET after brief pre-exposure to it, and for the sensitive and insensitive lines selected for several generations. Single sensillum recordings of the selected lines identified DEET-sensitive sensilla in the sensitive line that did not respond to DEET in the insensitive line. This study suggests that behavioural insensitivity to DEET in *Ae. aegypti* is a genetically determined dominant trait, which can also be temporarily induced by pre-exposure, and resides in changes in sensillum function. These results highlight the necessity for careful monitoring of DEET-insensitivity in the field, and caution when designing laboratory methods for repellency assays.

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Contents

Chapter 1. **General Introduction**

1.1 Mosquitoes as vectors of disease

Mosquitoes (*Diptera: Culicidae*) are haematophagous insects known worldwide as a biting nuisance, and also for their role in spreading diseases. Pathogens carried by mosquitoes cause diseases including malaria, West Nile fever or neuroinvasive disease, filariasis, yellow fever and dengue fever. The pathogens are transmitted between the mosquito and the host when the mosquito bites. Once a mosquito feeds from an infected host, the pathogens move from the mosquito's gut to the salivary glands, ready for transmission. As the mosquito probes with its proboscis, any pathogens present in the salivary glands are injected into the host. Specific mosquito species transmit specific diseases, because the pathogens ability to complete its lifecycle, by migrating through the gut wall to the salivary glands, is host dependant.

There are approximately 3200 known mosquito species, which vary widely in their habit and host range. Some mosquitoes are anthropophilic, feeding only on human beings, some are zoophilic, preferring animals, and some are opportunistic or generalist and feed on many different species (Service, 2000). Mosquitoes that bite human beings are of the greatest concern to public health, and these are mostly from the *Anopheles*, *Aedes* and *Culex* Genera (Fradin, 1998). Opportunistic and generalist mosquitoes, which feed on both human beings and other species, pose another problem to the control of diseases, as animals can act as reservoir hosts and maintain the disease even if it is being controlled in the human population. Opportunistic feeding on animals and human beings can also lead to further geographical spread of disease. A recent example of this can be seen with West Nile virus, which first emerged in North America in 1999 and spread quickly through the United States, Canada, Mexico and the Caribbean basin. The spread of the virus to Central and South America was due to the mosquito species which transmit the disease, *Culex pipiens* and *Culex quinquefasciatus*, feeding on both human and bird hosts. Mosquitoes carrying the pathogen passed it to avian hosts, which, after migrating long distances, transmitted the disease to mosquitoes in new

locations which then infected human beings (Granwehr *et al.*, 2004). However, most of the spread of vector-borne diseases is due to migration of the mosquitoes themselves as they are accidentally moved by human transportation (da Costa-Ribeiro *et al.*, 2006), or by infected humans travelling to an area where there is a vector mosquito population capable of transmitting that disease.

The most well known and thoroughly studied mosquitoes are of the *Anopheles* Genus, which are the vectors of malaria. However, mosquitoes in the *Aedes* Genus are also extremely important, being responsible for the transmission of a range of diseases including human lymphatic filariasis and the West Nile, yellow fever, dengue fever and Chikungunya viruses. There are over 700 species of *Aedes* mosquitoes, with *Aedes albopictus*, the Asian tiger mosquito, and *Aedes aegypti*, the yellow fever mosquito, the most commonly known. *Aedes aegypti* feeds on both human and animal hosts and is present in urban areas of Africa, South America, Australia and Asia, vectoring the yellow fever, dengue fever and Chikungunya viruses. Yellow fever is an acute viral haemorrhagic fever caused by a virus from the *Flaviviridae* family, with symptoms including fever, nausea, pain and, in some cases, liver damage which can lead to death. There are an estimated 200,000 cases of yellow fever, with 30,000 deaths, each year. Although there has been a vaccine available for over 60 years, it can rarely be applied to enough of the population to be effective, thus cases of yellow fever are increasing (WHO, Yellow fever factsheet). Dengue fever is a febrile disease caused by four virus serotypes of the Genus *Flavivirus* (*Flaviviridae*), with symptoms of muscle, joint and retro-orbital pain, fever and a rash. There are over 50 million cases of dengue fever per year, with two fifths of the world's population (2.5 billion people) now at risk (WHO, Dengue factsheet). Surviving one serotype of the virus gives only partial protection against the other serotypes, and having the virus multiple times increases the risk of developing the more severe dengue haemorrhagic fever (DHF). A smaller number of cases, approximately 500,000, of DHF occur, with up to a 20% mortality rate due to low blood pressure caused by blood loss if not treated correctly. There is no current specific treatment or vaccine for dengue fever or chikungunya (which presents with similar symptoms), therefore, the only way to prevent the disease is to control the mosquito vectors. The economic cost of these diseases can be high, with Brazil alone spending one billion dollars per year on the control of dengue fever.

As mosquitoes have such an important impact upon public health, there is a great interest in studying their biology, genetics and ecology to improve and develop methods of control. An area of particular interest is investigating how mosquitoes locate their hosts, and determining if the process can be interrupted. Some repellents, including the widely used DEET (*N,N*-Diethyl-*m*-toluamide), have been thought to work by interrupting the host-seeking process and preventing the mosquito from detecting attractive odours (Boeckh *et al.*, 1996; Ditzen *et al.*, 2008). However, different repellents work in different ways, and the modes of action of repellents are not fully understood (Davis, 1985), so it is important to study the mechanisms involved.

1.2 Current mosquito control

There are many intervention strategies used to try to control the spread of disease by mosquitoes. These mainly fall into the categories of chemical control, environmental control, genetic control and personal protection such as the use of repellents.

Chemical control of mosquitoes encompasses the use of insecticides and larvicides to kill the adults and larvae. For most mosquitoes, larvicides are the most commonly used form of control (Service, 2000), eliminating the insects before they mature and are able to transmit diseases or reproduce. The most commonly used larvicides are organophosphates, such as temephos, and carbamates, such as propoxpur. However, people are reluctant to contaminate drinking water with chemicals, and environmentally friendly alternatives such as growth regulators are comparatively expensive. Additionally, larvicides have not yet been fully developed and utilised for all mosquito species (Morrison *et al*., 2008). There are a wide range of insecticides available to target adult mosquitoes, including organochlorine compounds (such as dichlorodiphenyltrichloroethane, DDT), organophosphates, carbamates, pyrethroids, neonicotinoids and biological pesticides. Most of the non-biological pesticides are highly toxic to wildlife and persist in the environment, thus, the only

pesticides sanctioned for use by the World Health Organisation are pyrethroids, which have low mammalian toxicity and low persistence (WHO, 2005). The concentrations of pyrethroids used in household applications also show irritancy and repellent properties, even in pyrethroid-resistant strains, but little is known of the mechanisms responsible for these behaviours (Chareonviriyaphap *et al.*, 2004; Grieco *et al.*, 2007; Mongkalangoon *et al.*, 2009). The use of pyrethroid-treated bednets is widespread, as these ensure mosquitoes cannot reach the person inside. Even damaged nets give protection as they kill mosquitoes on contact. Although the use of insecticides, and insecticide-treated bednets (ITNs), has been very successful for mosquito control, widespread pyrethroid resistance has developed and there are many areas where these insecticides are no longer effective (Hemingway *et al*., 2004).

Environmental control of mosquitoes includes the removal or monitoring of containers or disused tyres which would serve as oviposition sites and the alteration of suitable mosquito habitats such as marshes or ponds by draining them, introducing predatory fish, or increasing water flow so that static pools cannot form (Service, 2000). Problems with environmental control include difficulties in maintenance, requiring health education and good communication with local people (Erlanger *et al*., 2008), and the fact that alteration of local habitat may lead to an undesirable rise in abundance of different mosquito species (Service, 2000).

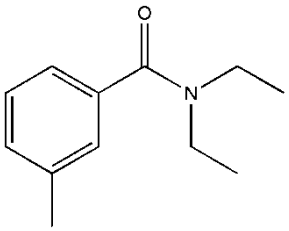
There are many studies on the genetic manipulation of mosquitoes, for example by altering their lifespan so that they are less likely to live long enough to transmit malaria (Corby-Harris *et al.*, 2010). The most common method of genetic control is the release of sterilized males into the wild, which will mate with females but not fertilise their eggs (Lofgren *et al*., 1974). This technique has only worked effectively alone in highly isolated areas such as islands, probably because of high genetic variability (Tabachnick and Powell, 1978) and the migration of insects (Bailey *et al*., 1980). Thus, the high expense and the difficulty in producing enough males to compete with wild mosquitoes (especially as they can have a lower fitness) makes it unpractical as a stand-alone control method, and it is often

recommended as part of an integrated control strategy alongside other control measures (Townson, 2009).

1.2.1 **Repellents**

Integrated control, combining several of the above methods, is considered the most effective way of controlling mosquito populations (Service, 2000). An important component of this is personal protection, such as the use of screens, bednets and repellents, to protect against mosquito bites. The use of ITNs and plant-derived repellents during the hours of greatest mosquito activity has been shown to decrease malaria transmission by up to 80% (Hill *et al.*, 2007), demonstrating that these methods can be effective against disease.

Although repellents are difficult to distribute to poor households in rural areas (McElroy *et al*., 2009), they are considered the first line of defence against mosquitoes (Curtis, 1992; Fradin, 2001), preventing biting and therefore stopping transmission of pathogens. There is a wide range of repellents available on the market, of varying levels of effectiveness and which last for different lengths of time. The five main active ingredients used in the production of most commercially available repellents are: DEET, IR3535 (ethyl butylacetylaminopropionate) and picaridin (1-piperidinecarboxylic acid, 2-(2-hydroxyethyl), 1-methylpropyl ester; KBR 3023), which are synthetic compounds, and citronella and *p*-menthane-3,8-diol (PMD), which are naturally derived from plant oils.



DEET
$C_{12}H_{17}NO$

Fig 1.1 Chemical structure and molecular formula of *N,N*-Diethyl-*m*-toluamide (DEET).

The most widely used and most effective repellent on the market is the synthetic compound *N,N*-Diethyl-*m*-toluamide (DEET) (Fig 1.1) (Fradin, 1998), now called *N,N*-diethyl-3-methylbenzamide (IUPAC). DEET was developed and patented by the US army in 1946 and became available as a broad-spectrum insect repellent on the worldwide market in the 1950s (Committee on Gulf War and Health, 2003). The US Environmental Protection Agency (1980) estimates that 200 million people worldwide use repellents containing DEET, with 30% of the population of the United States using DEET each year (Osimitz and Murphy, 1997). It has been shown that a commercial repellent containing 23.5% DEET provides 100% repellency against mosquitoes for at least 5 hours (Fradin and Day, 2002). The US military uses repellents containing up to 75% DEET (Committee on Gulf War and Health, 2003), and higher concentrations of DEET offer longer lasting protection (Fradin, 1998). As well as having repellent properties, DEET itself may have insecticidal effects. Studies have shown that mosquitoes coming into contact with DEET-impregnated materials (Licciardi *et al.*, 2006; N'Guessan *et al.*, 2008) and aerosol sprays (Xue *et al*., 2003) showed increased knock down and mortality. This indicates that DEET has a complex mode of action which is not fully understood. Despite the excellent safety history of DEET, there are concerns over possible toxic and carcinogenic effects when applying it to the skin, and of its properties as a plasticiser (Goodyer and Behrens, 1998). An alternative synthetic repellent to DEET, safer for use on human skin, is picaridin, which has a similar level of repellency as DEET against several mosquito species, although is less effective against others (Boeckh *et al.*, 1996; Badolo *et al.*, 2004; Frances *et al.*, 2004). Picaridin alone has been shown not to cause behavioural avoidance or irritancy, therefore this repellent may work by interfering with the detection of host odours (Boeckh *et al.*, 1996; Licciardi *et al.*, 2006). Another compound, IR3535 (ethyl 3-(N-buthylacetylaminopropionate)) is found in the Avon 'Skin So Soft' range of insect repellents and is an effective repellent against mosquitoes, although it has been found to be less persistent against *Anopheles* than DEET (Costantini *et al.*, 2004; Licciardi *et al.*, 2006).

Synthetic repellents may be undesirable to the public, both because they are commonly more expensive than natural alternatives and also due to the smell and greasy feeling when applied to the skin (Service, 2000). There are many natural, plant-derived compounds such as citronella oil, thyme

oil, and eucalyptus oil which show repellency against mosquitoes (Moore *et al.*, 2002; Frances *et al.*, 2005; Park *et al.*, 2005; Zhu *et al.*, 2006; Muller *et al.*, 2009). However, most plant-derived repellents generally give less protection, or protect for a shorter time, than the minimum 5 hours of protection provided by DEET (Fradin and Day, 2002; Frances *et al.*, 2005). For example, citronella oil is only effective against *Ae. aegypti* for 1-3 hours, and citronella candles only provide 14% repellency (Tawatsin *et al.*, 2001). The active ingredient of the most effective natural insect repellent is PMD, derived from lemon eucalyptus oil, which is the only naturally based insect repellent recommended by the Centre of Disease Control for protection against mosquitoes carrying West Nile virus. PMD was found to show a similar efficacy to DEET when DEET was tested at 15% (Carroll and Loye, 2006; Moore *et al.*, 2007a; Moore *et al.*, 2007b), but may not provide the same length of protection as higher concentrations of DEET (Barnard *et al.*, 2002).

The use of repellents has been shown to directly protect against the spread of pathogens by mosquitoes (Hill *et al.*, 2007), so any reduction in efficacy could affect the transmission of disease due to increased biting. With the increasing use of repellents, particularly DEET, there is the possibility that mosquitoes may develop resistance to repellents, in a similar way to insecticide resistance developing in response to the extensive use of insecticides. Some insects have been shown to no longer detect repellents after previous exposure to them (Stortkuhl *et al.*, 1999; Barbarossa *et al.*, 2007). In order to maintain the effectiveness of repellents, and develop new ones, it is necessary to understand as much as possible about mosquito biology and olfaction in order to determine their mode of action, especially as different repellents are likely to act in different ways (Davis, 1985; Licciardi *et al.*, 2006).

1.3 Mosquito biology

1.3.1 Life-cycle

Mosquitoes occupy multiple habitats during their life cycle, with aquatic larval and pupal stages after hatching from the egg, and a non-aquatic adult stage during which they will bite hosts and may transmit pathogens that cause disease (Fig 1.2).

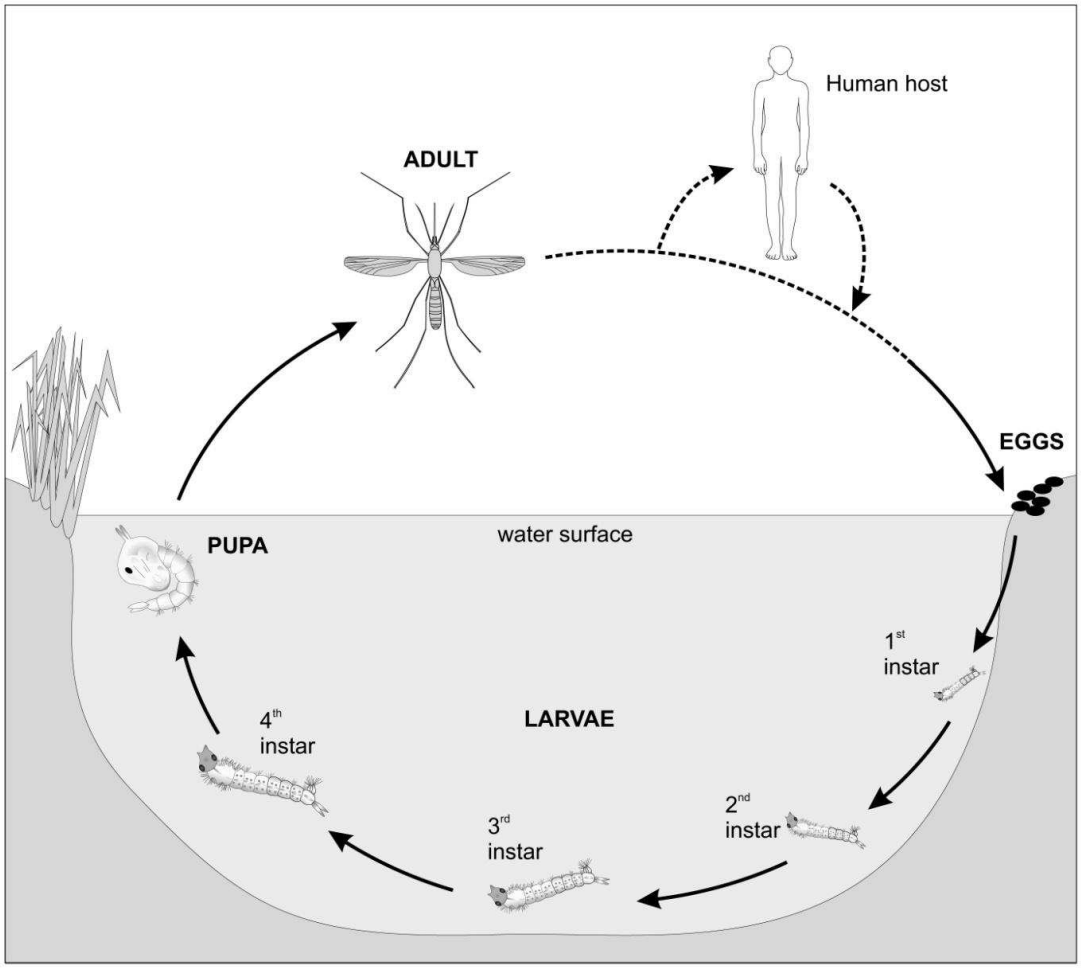


Fig 1.2 Life cycle of an anthropophilic mosquito species, e.g. *Aedes aegypti* (reproduced with permission from Lynda Castle, Rothamsted Research).

Aedes aegypti lays its eggs on damp substrate, just above the water line, around pools of water which are likely to flood (Service, 2000). This can be in disused containers and discarded tyres in urban settings, or around ponds and in tree holes in the countryside (Fradin, 1998). The eggs can remain dry

for months and still be viable when submerged, making it hard to guarantee mosquitoes are eliminated from an area and heightening the risk of them being transported between locations in containers or car tyres. When the water level rises to cover the eggs, the presence of bacteria in the deoxygenated water stimulates the eggs to hatch (Rozeboom, 1934) and larvae emerge. Various environmental conditions may cause hatching to be delayed (including photoperiods, temperature and the amount of food available) which staggers the emergence of larvae and keeps some eggs in reserve as a survival strategy (Gillett *et al.*, 1977).

There are four aquatic larval instar stages (Fig 1.2), which feed on plant and animal micro-organisms in the water and are frequently predated by larger aquatic organisms. Larvae breathe through the dorsal spiracles on the tenth, most posterior, body segment and they must return to the water surface to breathe. Depending on the amount of food available, larvae transform to pupae within 10 days (Service, 2000). At the end of the fourth stage, larvae become pupae, mostly staying at the surface of the water and no longer consuming food. After 1-3 days as a pupa, the fully formed adult mosquito emerges from the pupal casing.

During the first 24 h as an adult, mosquitoes will seek a sugar meal in order to sustain themselves. Males will feed only on nectar for their entire lifespan, whereas females require a blood-meal in order to develop viable eggs. Females only need to mate once to attain enough spermatozoa for their lifetime of reproduction. In the first two days after emergence, successful mating will take place and females will begin to search for a host, with different mosquito species varying in the time of day when they are most actively host seeking. *Aedes aegypti* is most active at dusk and dawn, but will also bite during the day. It is an endophilic species, naturally associating with humans, and also endophagic, willing to enter houses to bite its hosts. Once the mosquito has located a suitable host, it will attempt to feed by inserting its proboscis into the skin and withdrawing blood. If this process is interrupted, the female will return and make multiple attempts to feed until enough blood has been obtained to produce the eggs. Over the next 2-3 days, the mosquito becomes gravid with developing

eggs, and maintains a less active lifestyle. At the end of this time the mosquito will oviposit at a suitable site, possibly a site with a similar odour profile to where it emerged (McCall and Eaton, 2001; McCall *et al.*, 2001). A female mosquito can lay between 30-300 eggs (Service, 2000), with the size of the blood-meal affecting the number of eggs laid (Edman and Lynn, 1975).

1.3.2 **Host location**

Mosquitoes locate their hosts using heat, moisture, visual and olfactory cues (Eiras and Jepson, 1994; Service, 2000). For the latter, the volatile chemicals emitting from hosts are detected by the mosquito's antennae and maxillary palps (McIver, 1982). Such chemicals, which alter the behaviour of the insect when detected, are called semiochemicals (Takken, 1991; Takken and Knols, 1999), and the chemicals used by mosquitoes to find their host are kairomones, i.e. compounds which benefit the receiver (the mosquito) to the detriment of the organism releasing the compound (the human host). The ability of a mosquito to detect kairomones from hosts has a direct effect on its ability to transmit the pathogens that cause disease (Zwiebel and Takken, 2004; Bohbot *et al.*, 2007). Several semiochemicals that are released by vertebrates and attract mosquitoes have been identified and one of the most important, which is correlated with mosquito flight activity, is carbon dioxide (CO_2) (Takken and Kline, 1989; Eiras and Jepson, 1991). However, CO_2 from breath accounts for only 50% of the attraction to hosts in highly anthropophilic species (Costantini *et al.*, 1996), indicating that other chemical cues from the host must play an important role in olfactory host-seeking behaviour (Costantini *et al.*, 1998).

Aedes aegypti are attracted to volatile chemicals given off in odours from human skin (Maibach *et al.*, 1966; Mayer and James, 1969; Schreck *et al.*, 1990). The human body releases around 350 volatile compounds (Bernier *et al.*, 2000) and several of these have been found to be attractive to mosquitoes (Geier *et al.*, 1996; Bernier *et al.*, 2002; Logan *et al.*, 2008). Mosquitoes show preferences for certain individual human hosts, which may be due to variation in the ratios of these attractive chemicals (Qiu *et al.*, 2006; Williams *et al.*, 2006; Logan *et al.*, 2008). For some of the chemicals found in human

sweat, for example geranylacetone, 6-methyl-5-helten-2-one, octanal, nonanal and decanal, people with greater levels than normal are repellent to mosquitoes, and these human-derived compounds can be used as mosquito repellents (Logan *et al.*, 2008). One of the key ways to control insects is to manipulate their host-seeking abilities, either by interrupting their detection of attractants, or by causing direct repellency. However, much about the way mosquitoes detect, analyse and act upon chemical cues is still unknown, and this is an important subject to investigate further.

1.3.3 **Mosquito peripheral olfaction**

Thousands of tiny hair-like structures (sensilla) on the antennae and maxilliary palps of mosquitoes (Fig 1.3) contain 2-3 olfactory receptor neurones (ORNs) which are involved in the detection of semiochemicals (McIver, 1982). The chemosensitive trichoid and grooved-peg sensilla have been shown to detect behaviourally-active semiochemicals (Davis, 1976; Davis and Sokolove, 1976), which pass through the outer membrane of the sensilla and are transported by odorant binding proteins (OBPs) to the olfactory receptors (ORs) on the ORNs (McIver, 1982). When a semiochemical binds to the OR it causes the cell to depolarise and an action potential is sent along the olfactory nerve to the brain. This may result in a behavioural change, such as attraction or repellency.

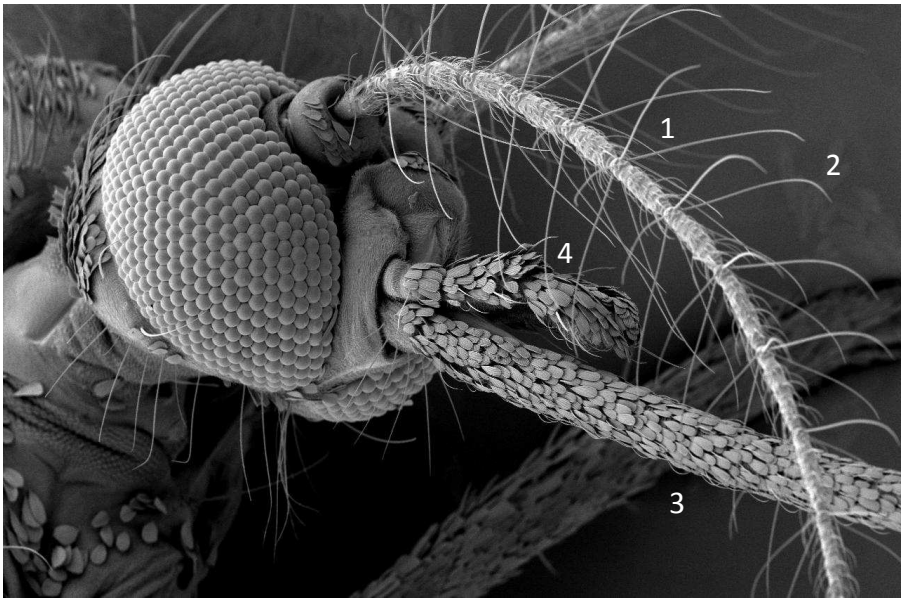


Fig 1.3 Scanning Electron Micrograph (courtesy of Jean Devonshire, Rothamsted Research) of *Aedes aegypti* female head, 1) antenna, 2) sensilla, 3) proboscis and 4) maxilliary palps.

The responses of *Ae. aegypti* antennal trichoid sensilla to a range of volatile semiochemicals have been categorised through single sensillum recordings (SSR), where a recording electrode is inserted into the sensillum so it is in contact with the ORNs and the electrical activity of the ORNs is monitored as the antenna is presented with odours (Ghaninia *et al.*, 2007; Siju *et al.*, 2010). Within each sensilla in *Ae. aegypti*, there are two ORNs (Fig 1.4), each of which has different ORs and thus responds to a different set of volatile chemicals. There are several different morphological types of sensilla in *Ae. aegypti*, and each morphological subtype has several functional subtypes, which differ in the ORNs they contain (Ghaninia *et al.*, 2007; Siju *et al.*, 2010). Sensilla of a specific morphological and functional subtype contain the same ORNs, and thus will respond to the same set of compounds. The primary olfactory centre in insects is the antennal lobe, which is divided into structural units called glomeruli. The ORNs project extensions, axons, which carry electrical impulses to the glomeruli. In *Ae. aegypti* the majority of axons from the antennal nerve, as well as axons from the maxilliary palp nerve, terminate in the antennal lobe, and likely relay olfactory information (Ignell *et al.*, 2005). ORNs expressing the same ORs will map to the same glomerulus within the antennal lobe, forming a spatial activity map of odour responses (Vosshall *et al.*, 2000; Couto *et al.*, 2005). Within the glomerular array, odour discrimination is based on interactions between ORN axons and dendrites of antennal lobe interneurons (Ignell *et al.*, 2005). Neurons from the antennal lobe project to higher brain centres such as the mushroom bodies, which are a pair of lobed neuropils (where synaptic connections are formed between axons and dendrites) involved in olfactory learning and memory (Zars *et al.*, 2000), though the role of mushroom bodies in behaviour is still little understood.

In mosquitoes, ORNs have been found which detect attractants and repellents, including those for DEET, picaridin, 1-octen-3-ol and lactic acid, (Davis and Rebert, 1972; Davis and Sokolove, 1976; Boeckh *et al.*, 1996; Ditzen *et al.*, 2008; Syed and Leal, 2008). Interestingly, in a study of *Ae. aegypti* which showed that both the A and B neuron in a specific sensillum type responded to DEET, the B neuron was also found to respond to picaridin (Boeckh *et al.*, 1996). These repellents may therefore share a similar mode of action upon the peripheral olfactory system to induce repellency. This could

be a suppression of the detection of attractive semiochemicals, or the direct detection of the repellents may induce avoidance behaviour (Davis, 1985).

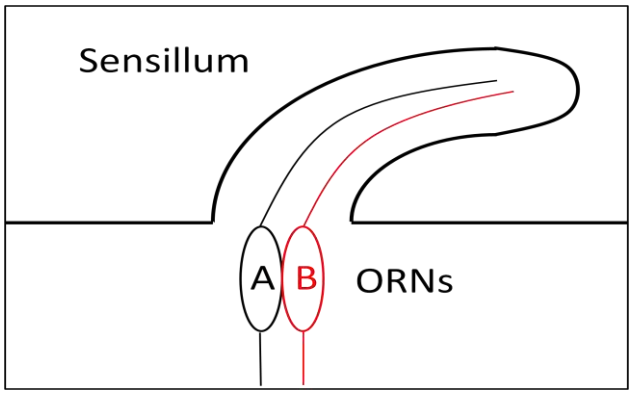


Fig 1.4 Representation of an *Aedes aegypti* trichoid sensillum, with two olfactory receptor neurons (ORNs) A and B.

1.4 DEET

1.4.1 The mode of action of DEET

Although DEET has been widely used to repel mosquitoes for over 60 years, until recently very little was known about its mode of action. There are several theories as to how DEET acts as a repellent. First, DEET was thought to act by affecting the receptors for lactic acid on the mosquito's antennae (Davis and Sokolove, 1976), thus inhibiting the mosquito's response to the normally attractive compound (Acree *et al*., 1968; Boeckh *et al*., 1996; Dogan *et al*., 1999). However, this was questioned by the finding that DEET acts as a repellent even when other attractants are present (Boeckh *et al.*, 1996; Hoffmann and Miller, 2003), indicating that DEET may have an effect upon multiple receptors. 1-Octen-3-ol is also an attractant for mosquitoes (Takken and Knols, 1999, Cook J.I, Pers comm), and an alternative explanation is that DEET could block the 1-octen-3-ol receptor, requiring a higher concentration of 1-octen-3-ol for detection in the presence of DEET (Ditzen *et al.*, 2008). Several studies have found that DEET directly stimulates ORNs in the antennae, suggesting it works as a repellent without necessarily affecting the receptors of other compounds (Davis and Rebert, 1972;

Boeckh *et al.*, 1996; Syed and Leal, 2008). A recent study, published after the completion of the experimental work for this thesis, with *Ae. aegypti* recombinant receptors, showed the AaOR2 receptor responded directly to DEET and the AaOR8 receptor was sensitive to 1-octen-3-ol and inhibited by DEET (Bohbot and Dickens, 2010). This suggests there may be more than one mode of action of DEET, especially as DEET has also been shown to suppress feeding in *Drosophila melanogaster* by stimulating the gustatory receptor neurons which respond to aversive compounds (Lee *et al.*, 2010), and DEET itself has insecticidal properties (Licciardi *et al.*, 2006).

In addition to being the most effective repellent on the market, DEET is undergoing trials as a combination-insecticide, with the aim of overcoming pyrethroid resistance (Bonnet *et al*., 2009). Pyrethroids are the only compounds currently recommended by the World Health Organisation Pesticide Scheme (WHOPES) for the treatment of insecticidal materials such as bednets (Zaim *et al.*, 2000). However, in many areas where mosquitoes are vectors for disease, resistance to pyrethroids has developed, causing problems with control by ITNs, indoor residual sprayings and space sprayings (N'Guessan *et al*., 2007; Marcombe *et al*., 2009). Finding an alternative insecticide as the resistance to pyrethroids spreads is urgent (Zaim and Guillet, 2002). One strategy being investigated is a combination of DEET with the carbamate propoxur, which shows a synergistic reaction and gives a significantly better performance than the pyrethroid deltamethrin (Pennetier *et al.*, 2005). The DEET-propoxur mixture showed a knockdown effect on mosquitoes which neither DEET nor propoxur induced separately. It has been suggested that cytochrome-p450 monooxygenases are responsible for the increased toxicity, with the mode of action of this still under investigation (Bonnet *et al.*, 2009). With the association of a non-pyrethroid insecticide and DEET exhibiting pyrethroid features, i.e. a fast killing effect and excito-repellency properties, with lower amounts of active compound, this strategy may be promising for the control of pyrethroid-resistant mosquitoes and the development of 'combination nets' in the field to combat insecticide resistance (Bonnet *et al*., 2009). DEET is, therefore, a versatile chemical with many possible applications in the field which is relied on for personal protection worldwide, but its increasing use could lead to the development of resistance.

1.4.2 **DEET Insensitivity**

Laboratory experiments with *Ae. aegypti* (Rutledge *et al.*, 1978; Rutledge *et al.*, 1994) and with *D. melanogaster* (Becker, 1970; Reeder *et al*., 2001), have demonstrated the presence of DEET-insensitive individuals, i.e. insects which are not repelled by DEET and, in the case of mosquitoes, that will still bite protected individuals. Rutledge *et al.* (1994) tested *Ae. aegypti* for insensitivity to DEET and found that after several generations with no selection, inbred strains were actually less insensitive to DEET than their parent strains, though this was likely to have been due to inbreeding depression. This study was merely an observation of repellent tolerance in the inbred offspring in comparison to the original parent strains, thus it is still unknown if insensitivity to DEET would increase in mosquitoes if it was selected for. Studies on *D. melanogaster* have shown DEET-insensitivity in the offspring of mutagenized males (Reeder *et al.*, 2001), and heritable DEET-insensitivity when the most insensitive *D. melanogaster* in a population were selected over several generations according to a choice-test (Becker, 1970). Becker (1970) found by the 11th generation the population was uniformly insensitive to DEET. Although the above studies have identified DEET-insensitive insects, and the inheritance of the trait was examined in *D. melanogaster*, there have been no attempts to identify the physiological or genetic basis of the insensitivity to DEET in mosquitoes to date.

The mechanism of 'insensitivity' to repellents is unknown, but it may be of interest to consider it in relation to the mechanisms that underlie resistance to insecticides. Insecticide resistance is caused by either mutations conferring changes to the insecticide target protein (Hemingway *et al.*, 2004), or increased detoxification (Hemingway, 2000). The mutations are generally in only a few genes with large effects (Raymond *et al*., 2001) and are thought to be rare or possibly unique events, which spread by selection and migration (Pasteur and Raymond, 1996). Whilst a mutation for insecticide resistance may have a high fitness benefit in areas where the insecticide is present, it can also have a fitness cost in the absence of selection, limiting the spread of the genotype (Gazave *et al*., 2001; Berticat *et al*., 2002). Insecticide resistance involves a high selection pressure, as only those insects which are resistant will survive, so the resistance can spread quickly through a population in the wild

due to the enormous reproductive advantage it confers, i.e. the ability to survive. Insensitivity to repellents also confers a reproductive advantage by increasing the probability of finding a blood-meal. However, since being repelled by DEET is not fatal, insensitivity is unlikely to spread through a population to the same degree as insecticide resistance unless the selection pressure is increased, for example by the extensive use of DEET-impregnated bed nets (Pennetier *et al.*, 2007; Bonnet *et al.*, 2009) resulting in only DEET-insensitive females being able to obtain a blood-meal where they can get through damaged nets.

1.5 Aims of this study

The aim of this project is to investigate the cause of insensitivity to DEET in the mosquito *Ae. aegypti*. The null hypothesis is that all mosquitoes are equally repelled by DEET, with no difference in responses between individuals.

In order to achieve this aim and test the null hypothesis, the objectives are:

- **To determine the heritability of DEET-insensitivity in *Ae. aegypti* mosquitoes.** Mosquitoes with different responses to the repellent DEET will be selected in laboratory bioassays. Bidirectional selection will continue for several generations to monitor the frequency of the trait in the population and crossing experiments will determine the mode of inheritance **(Chapter 2)**.

- **To determine if the behavioural insensitivity is caused by a change in the olfactory system.** Electroantennogram recordings will be carried out on DEET-sensitive and DEET-insensitive mosquitoes **(Chapter 3)**.

- **To discover if the alteration in response to DEET involves a specific olfactory receptor neuron.** DEET-sensitive and DEET-insensitive mosquitoes will be examined with single sensillum recordings **(Chapter 4)**.

- **To investigate if there are non-genetic factors which can also cause DEET-insensitivity.** Mosquitoes will be tested behaviourally and electrophysiologically after multiple exposures to DEET **(Chapter 5)**.

- **To draw conclusions on the causes and possible ramifications of DEET-insensitivity in mosquitoes (Chapter 6)**.

Chapter 2.**Selection of DEET-Insensitivity and Crossing Experiments**

2.1 Introduction

N,N-Diethyl-*m*-toluamide (DEET) is one of the most effective and widely used mosquito repellents available (Fradin, 1998). However, insensitivity to DEET has been shown in *Aedes aegypti* mosquitoes (Rutledge *et al.*, 1978; Rutledge *et al.*, 1994) and in *Drosophila melanogaster* (Becker, 1970; Reeder *et al.*, 2001), both of which are normally repelled by the compound (Boeckh *et al.*, 1996; Ditzen *et al.*, 2008). For *Ae. aegypti*, DEET was tested at varying concentrations on membranes covering warm blood, and the number of mosquitoes feeding was observed (Rutledge *et al.*, 1978; Rutledge *et al.*, 1994). Two parent strains, and six new strains resulting from single-pair brother-sister mating for 10 generations, were tested for DEET-insensitivity, and the inbred strains were shown to be less insensitive to DEET than the parent strains (Rutledge *et al.*, 1994). This was concluded to be at least partially due to inbreeding depression and a corresponding loss of fitness. Heritability of DEET tolerance (heritability in the broad sense, H^2) of 0.05 with incomplete dominance of the characteristic was found. Some *Ae. aegypti* mosquitoes were shown to be insensitive to DEET in this study, but there was no selection for tolerance to DEET, merely an observation of the inbred offspring in comparison to the original parent strains (Rutledge *et al.*, 1994). For *D. melanogaster,* individuals insensitive to DEET were selected using a maze of connected Y-tube choice tests, with DEET-coated material in one arm of each Y-tube and the other containing a control (Becker, 1970). After 10 choice tests the insects were sorted according to how many times they had chosen the DEET arm, with the flies that had made the highest number of choices of the DEET arm being the most insensitive. The insensitive individuals were then bred in two separate selection experiments, and the offspring tested in the same choice test. This selection was continued for 12 generations, and the flies in generations 11 and 12 of the two replicates were uniform in their DEET-insensitivity, being just as likely to choose a DEET-arm as a control arm. Flies from the two replicates were then crossed with each other in a complementation test, and from the proportion of insensitive offspring it was concluded that the genetic basis for insensitivity was the same in both replicates. Crosses between the selected lines and

control lines showed that the trait was at least partially dominant. In another experiment with *D. melanogaster*, Reeder *et al.* (2001) performed choice tests between DEET-impregnated paper and a control using the offspring of mutagenized males to detect those which were insensitive to DEET. Crossing experiments with the selected offspring and other strains showed that the insensitivity was recessive and located on the X chromosome. One of the strains used, C(1)DX, was found to have DEET-insensitivity present at a naturally high level, and this was theorised to be autosomal and only partially dominant.

Although DEET insensitivity has been selected for in *D. melanogaster*, it was noted but never fully investigated in studies with mosquitoes. Thus, the cause and mechanisms of insensitivity to DEET in insects are still largely unknown. The aim of this chapter was to investigate the heritability of insensitivity to DEET in *Ae. aegypti* through selection of insensitive mosquitoes and the crossing of DEET-sensitive and insensitive lines. The life history of the selected lines would also be recorded to monitor changes in fitness. The hypothesis was that there would be a difference in sensitivity to DEET between mosquitoes and that this would be passed on to their offspring.

2.2 Methods

2.2.1 Predictions of genetic inheritance of DEET-insensitivity

In order to determine how many generations of selection would be required to show clear heritability of the DEET-insensitive trait, and also to determine the likely mode of inheritance, calculations were performed to predict the frequency of the DEET-insensitive trait in the population at each generation. Predictions were calculated assuming the trait was polygenic, with multiple genes contributing to the insensitive phenotype, or monogenic, with only one gene responsible (Calculations by John Brookfield, The University of Nottingham). For the polygenic trait model four different levels of heritability, 20%, 40%, 60% and 80%, were calculated (Appendix 1.1.1-4). These levels of heritability showed how much of the insensitivity was due to genetics and the environment, with mosquitoes of the highest level of heritability being most genetically similar to each other, and most different from the rest of the population. For a monogenic trait, predictions were made for if DEET-insensitivity was dominant, needing only one allele for insensitivity to confer the phenotype, or recessive, which would require two insensitivity alleles to confer the phenotype (Appendix 1.1.5-6). The level of insensitivity in the culture population was assumed to be 9% based on preliminary experiments (data not shown).

2.2.2 Insects

The mosquitoes used in this study were *Ae. aegypti* [REFM strain obtained from the Liverpool School of Tropical Medicine (Macdonald and Sheppard, 1965), in culture at Rothamsted Research since 2001 and replenished with new mosquitoes from Liverpool School of Tropical Medicine in 2007] reared in 30x30x30 cm Bugdorm 1 cages (Megaview®) in rooms maintained at 27.5°C ± 1°C, 60-80% RH and a 12:12 light:dark cycle. Adults were fed 10% sucrose solution. Females in culture were fed with sheep's blood using a Hemotek® system. Behavioural experiments used 5-12 day old nulliparous females which were shown to respond to human odours (Appendix 2).

2.2.3 **Repellency bioassay for selection of DEET-insensitive mosquitoes**

A repellency bioassay based on a previously established method (James G. Logan, pers. comm.) was used to identify, and separate, females insensitive to DEET. The method was adapted from a WHOPES repellency test where an arm was inserted into the test cage (WHO, 1996) and based on previous repellency work where the attractant was outside the cage (Chou *et al.*, 1997), as this was shown to be an effective method for separating DEET-insensitive mosquitoes (Appendix 3). Mosquitoes were placed in a 30x30x30cm cage with clear plastic sides (adapted from Megaview® Bugdorm 1), with a removable 6x12cm section of stainless steel mesh (wire diameter: 0.2 mm, aperture: 0.8 mm) on the top, and a netting sleeve covering an opening (15 cm diameter) on one of the sides (Fig 2.1). The experimental room was maintained at 50-70% humidity and 27°C ± 1°C. An extractor duct was placed 3" from the netting sleeve on the cage with an air flow of 0.18 m sec^{-1}, drawing air from above the mesh into the cage (including volatiles from the arm and DEET, when present) down through the cage and out of the opening with the netting sleeve. This prevented a build up of human and repellent volatiles in the cage and surrounding area.

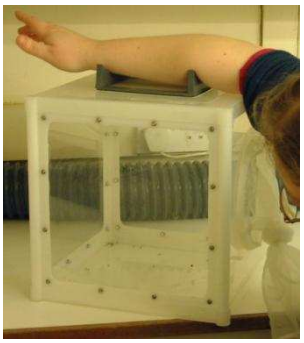


Fig 2.1 Repellency assay to detect female *Aedes aegypti* insensitive to DEET. The forearm was covered with 0.5ml 20% DEET and any mosquitoes attempting to feed during a 2 minute period were collected.

Ten female *Ae. aegypti* were placed in each test cage and left to acclimatise for 2 h. An arm was then placed over the metal mesh, held on a cradle at a height of 1.5 cm above the mesh. Nina Stanczyk was the volunteer for all behavioural experiments. DEET in ethanol (20%, 0.5 ml) was applied evenly over the forearm (the control was 0.5 ml ethanol), and the ethanol was allowed to evaporate for 30 s. The arm was then immediately placed over the mesh and the behaviour of the mosquitoes was observed

for two mins. Mosquitoes that landed on the mesh and attempted to probe the arm were considered to be insensitive to DEET and removed by using a mouth-aspirator into a separate cage, causing as little disturbance as possible to the other mosquitoes. At the end of the two mins, the mosquitoes that had not landed and probed on the mesh were considered to be sensitive to the repellent. Two duplicate experiments, A and B, were done, testing an initial 600 individuals for A, and 480 for B. The selected sensitive and insensitive mosquitoes were then used to establish the s and i lines, with each generation being tested in the selection bioassay and only the offspring of selected individuals forming the next generation. Unselected culture mosquitoes were also tested for comparison.

Bi-directional selection continued in this way for nine generations (the F_4 generation of experiment B was not selected because of low numbers). The s and i lines were reared without selection for the F_{7-8} generations of experiment A, and the F_{6-7} generations of experiment B, and then the following generation was tested with the repellency bioassay.

2.2.4 60% penetrance explanations

After the results of the selection experiment (Section 2.3.2) suggested incomplete penetrance of the trait, a new set of calculations was performed to assess the predicted frequencies of insensitivity, in order to see which best explained the data. These calculations assumed 60% penetrance of the DEET-insensitive trait, rounded up from the observed level of insensitivity in the i line (53-59%) (Section 2.3.2). With 60% penetrance of the trait, mosquitoes which had the insensitive genotype would only appear phenotypically insensitive 60% of the time. These calculations were done for dominant, intermediate dominance, and recessive models (Appendix 1.1.7,1.1.9 and 1.1.10). In the model for intermediate dominance, there would be 60% penetrance of the trait in a homozygote with two insensitivity alleles, and 30% penetrance in a heterozygote with one insensitivity allele and one sensitivity allele. Taking into account the response of the s line to selection, a model was also produced assuming the trait was dominant, with 60% penetrance in the i line and 8% penetrance in the s line (Appendix 1.1.8). In this case 8% of s mosquitoes would be phenotypically insensitive while having the sensitive genotype. The figure of 8% was chosen as the level of insensitivity in the s line does not go below this by the F_9 generation (Section 2.3.2).

2.2.5 **Fitness of selected lines**

For each generation of the selection experiments the number of surviving pupae and adults was recorded. The survival from egg to pupae was then calculated from the number of pupae collected divided by the number of eggs on the egg paper(s) used. The survival from pupae to adult was calculated from the number of live adults, 48-72 h after the pupae bowl was placed in the cage, divided by the number of pupae collected. The overall survival from egg to adult was calculated from the number of live adults divided by the number of eggs on the egg paper.

After selection, DEET-insensitive females from the i line and DEET-sensitive females from the s line were bloodfed and the proportion feeding noted. The number of eggs was counted after oviposition. An estimate of the number of eggs laid per female was obtained by dividing the number of eggs oviposited by the number of bloodfed females. Any additional factors such as changes in environment were noted down. For the two generations in which there was no selection, only the number of eggs laid was recorded.

2.2.6 **Crossing**

Females and males from the F_9 generation were separated into individual containers as the adults emerged, and reciprocal crosses performed with individuals from the s and i lines. The offspring from each cross were then tested in the repellency bioassay to determine the proportion of offspring insensitive to DEET. Fifty females of each line in both replicates were crossed, but only 10–16 females in each had enough surviving female offspring to be tested.

2.2.7 **Statistics**

In the selection trials the proportion of insensitive mosquitoes in different replicates selected in the F_1 i and s lines were analyzed with a Student's t test (Genstat®, 12th edition) to determine differences between the lines. The number of insensitive F_1 mosquitoes in the selected i line was also compared

to the number of insensitive mosquitoes in the laboratory culture. A one-way ANOVA was used to ascertain if there was any difference between the proportion of the i line insensitive to DEET before and after the two-generation gap in selection. Differences were judged to be significant when the difference between means was greater than the least significant difference (LSD). The proportion of insensitive mosquitoes in the F9 selected lines and the offspring of crossing experiments were analyzed with a Student's t-test as above.

For the fitness data, a two way unpaired Student's t-test was performed to compare the s and i lines for each characteristic across all generations recorded. The difference in the survival or number of eggs laid between the lines was considered significant when $p<0.05$. To analyse trends in the data from F_0-F_9, a regression analysis (with groups) was performed. As the lines of best fit were not significantly different for the s and i lines, they were presented and further analysed as the common line of best fit. The gradient of the line was determined to be significantly different from 0 if the t value was such that $p<0.05$ for variation explained by the slope. Data from some generations were excluded from the regression analysis where there was a known source of outside stress (such as severe temperature fluctuations caused by a power cut); this included analyses of egg to pupae or egg to adult in generations F_3 s, F_4 i and F_6 s of experiment A and F_3 s and F_9 s of experiment B. Also, generations F_4 s and i of experiment B were excluded from analyses of pupae to adult and egg to adult.

2.3 Results

2.3.1 Predictions of genetic inheritance of DEET-insensitivity

The expected levels of insensitivity in the population at each generation, assuming that the trait was either polygenic or monogenic (recessive or dominant), were predicted (Fig 2.2) (Appendix 1.1.1-6). From these calculations it would be expected that the trait would have spread fully through the population by the 10th generation, in all modes of inheritance, so it was decided to continue selection to the 10th generation. At this point the level of insensitivity in each generation would show the mode of inheritance of the trait. If the trait was polygenic, it would have a variety of inheritance patterns depending on the level of heritability. At 80% heritability, the trait would be present in 35% of the population in the F_1. If the trait was monogenic, and recessive, the frequency of the trait in the F_1 population would be ~30%. If the trait was dominant, the frequency of the trait in the F_1 population would be ~55%.

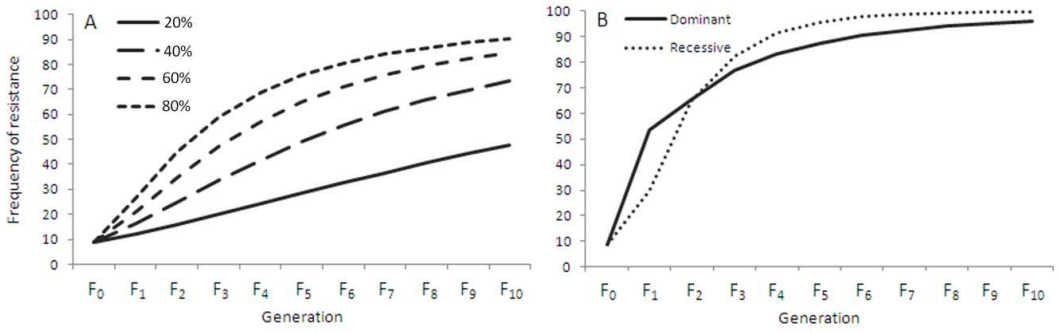


Fig 2.2 Predictions of DEET-insensitivity trait frequency in the population if there is A) polygenic inheritance with different levels of heritability, and B) monogenic dominant or recessive inheritance.

2.3.2 **Selection of DEET-insensitive mosquitoes**

In the initial selection from the laboratory culture, approximately 13% of the mosquitoes in the culture were found to have the DEET-insensitive phenotype in both experiment A and B. In the F_1 generation of the i line, insensitivity to DEET rose to 50% of females in experiment A and 33% in experiment B (Fig 2.3). In both experiments there were significantly more females ($p<0.001$) probing in the F_1 i line than in either the s line or in the unselected culture. In successive generations in the i line the insensitive phenotype plateaued at 53% and 59% in experiments A and B respectively. After the two generations without selection, there were no significant changes in the proportion of the population insensitive to DEET.

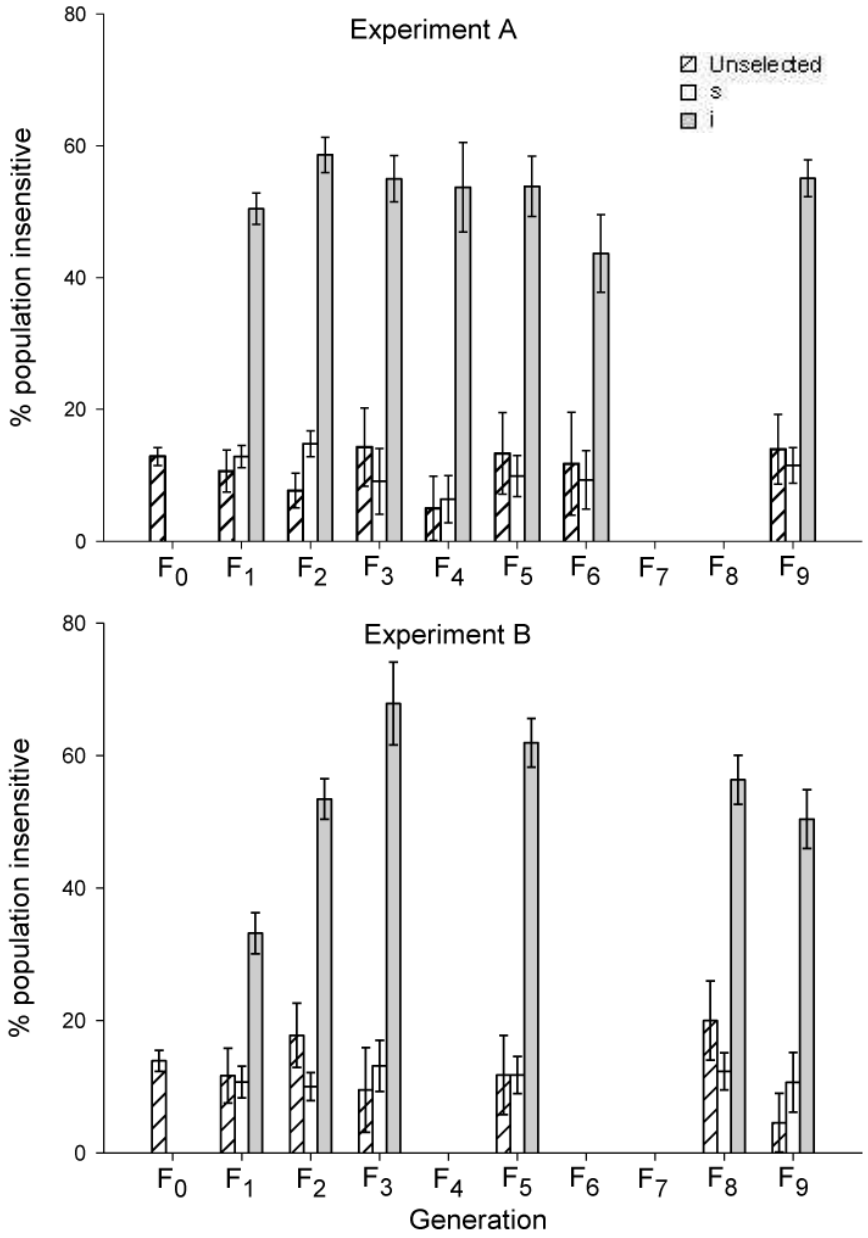


Fig. 2.3. Percentage of female *Aedes aegypti* insensitive to DEET in unselected and selected populations in two experiments, A and B (Stanczyk *et al.*, 2010). Unselected = female mosquitoes from standard culture. s = line bred from female mosquitoes sensitive to DEET at each generation. i = line bred from female mosquitoes insensitive to DEET at each generation. F_4 in experiment B was not tested because of low numbers. F_7 and F_8 of experiment A and F_6 and F_7 of experiment B were reared without selection. N (F_1) = 600 experiment A, 480 experiment B. N (F_2–F_9) = 100–400 per generation. Means are given ± SEM.

2.3.3 60% penetrance explanations

These calculations assumed 60% penetrance of the DEET-insensitive trait (Appendix 1.1.6-10). In predictions for the dominant mode of inheritance, the frequency of the trait in the population was at 35% in the F_1 generation of the i line, and plateaued at about 55% by the F_5 generation (Fig 2.4A). The trait gradually decreased in frequency in the s line, and had effectively disappeared from the population by the F_5 generation.

In the dominant model where 8% of the s line are phenotypically insensitive to DEET, and only 60% of mosquitoes with the insensitive genotype appearing insensitive, the s line maintained the same level of insensitivity found in the unselected culture (Fig 2.4B). The increase of insensitivity in the i line was slower, only reaching 20% in the F_1 and 50% by the F_6, as most phenotypically insensitive mosquitoes selected were of the s genotype.

In the case of intermediate dominance, with the heterozygote at 30% penetrance being an exact intermediate of the two homozygotes at 60% and 0%, there was a more gradual rise of the i line than in the dominant or recessive models, with insensitivity reaching ∼50% at the F_7 generation in the i line, and a gradual fall to zero in the s line (Fig 2.4C).

In the predictions of a recessive trait, DEET insensitivity in the i line showed a rapid rise from 30% in the F_1 to ∼55% in the $F_{3/4}$ at which point it plateaued (Fig 2.4D). Insensitivity in the s line showed a gradual decline over 10 generations, but never entirely disappeared through selection.

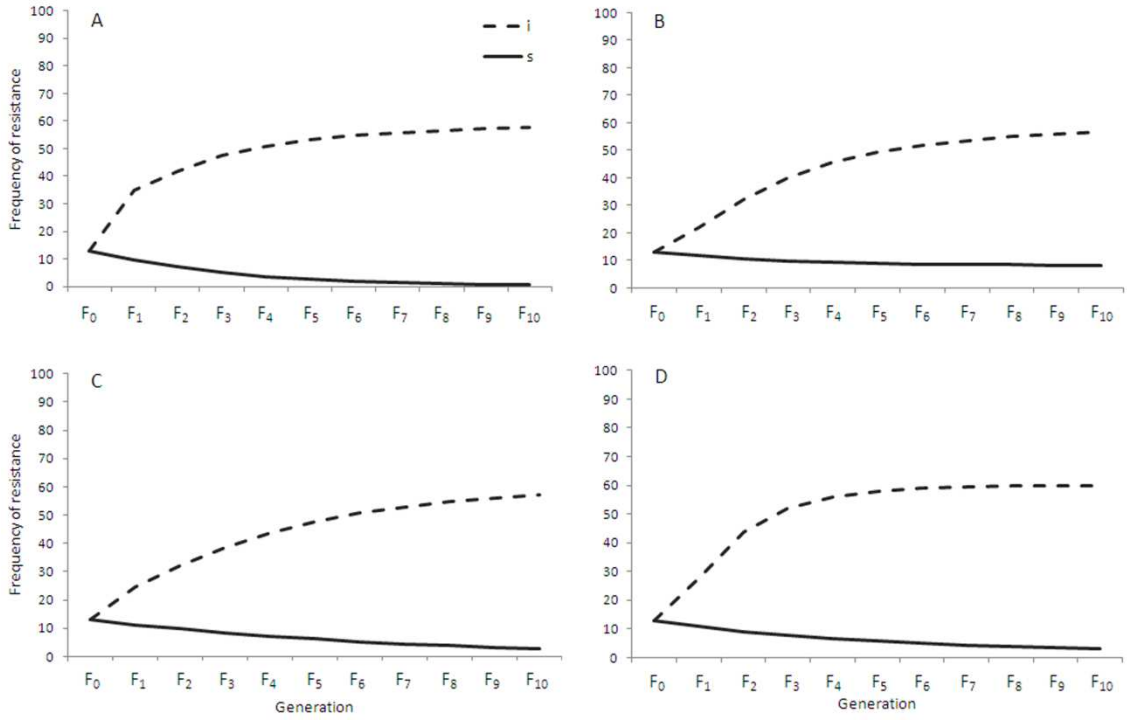


Fig 2.4 Predictions of trait frequency in the population with monogenic inheritance assuming A) Dominant 60% penetrance, B) Dominant 60%/8% penetrance, C) Intermediate dominance 60%/30%, and D) Recessive 60% penetrance.

2.3.4 **Fitness of selected lines**

There were no significant differences in the survival from eggs to pupae, pupae to adult, or overall survival from egg to adult between the s and i lines in experiment A or B (Fig 2.5). On average 20% of eggs survived to become pupae (Fig 2.5 A+B), and ~85% pupae became adults (Fig 2.5 C+D). The overall survival from egg to adult was 15-20% (Fig 2.5 E+F). There were no significant differences in the mean number of eggs laid per female between the s and i lines in experiment A or B, with each female laying an average of 80 eggs after bloodfeeding (Fig 2.5 G+H). There were no significant changes in fitness over the course of the generations for any of the characteristics measured.

In generations F_4 i and F_6 s of experiment A, and F_9 s of experiment B there was a poor rate of emergence from the eggs. In generation F_3 s of experiments A and B there was unexplained large-scale larval death. In the F_3 i, and in the F_4 s and i generations of experiment B, there was a high rate of adult mortality within the first 48 hours, before selection could occur.

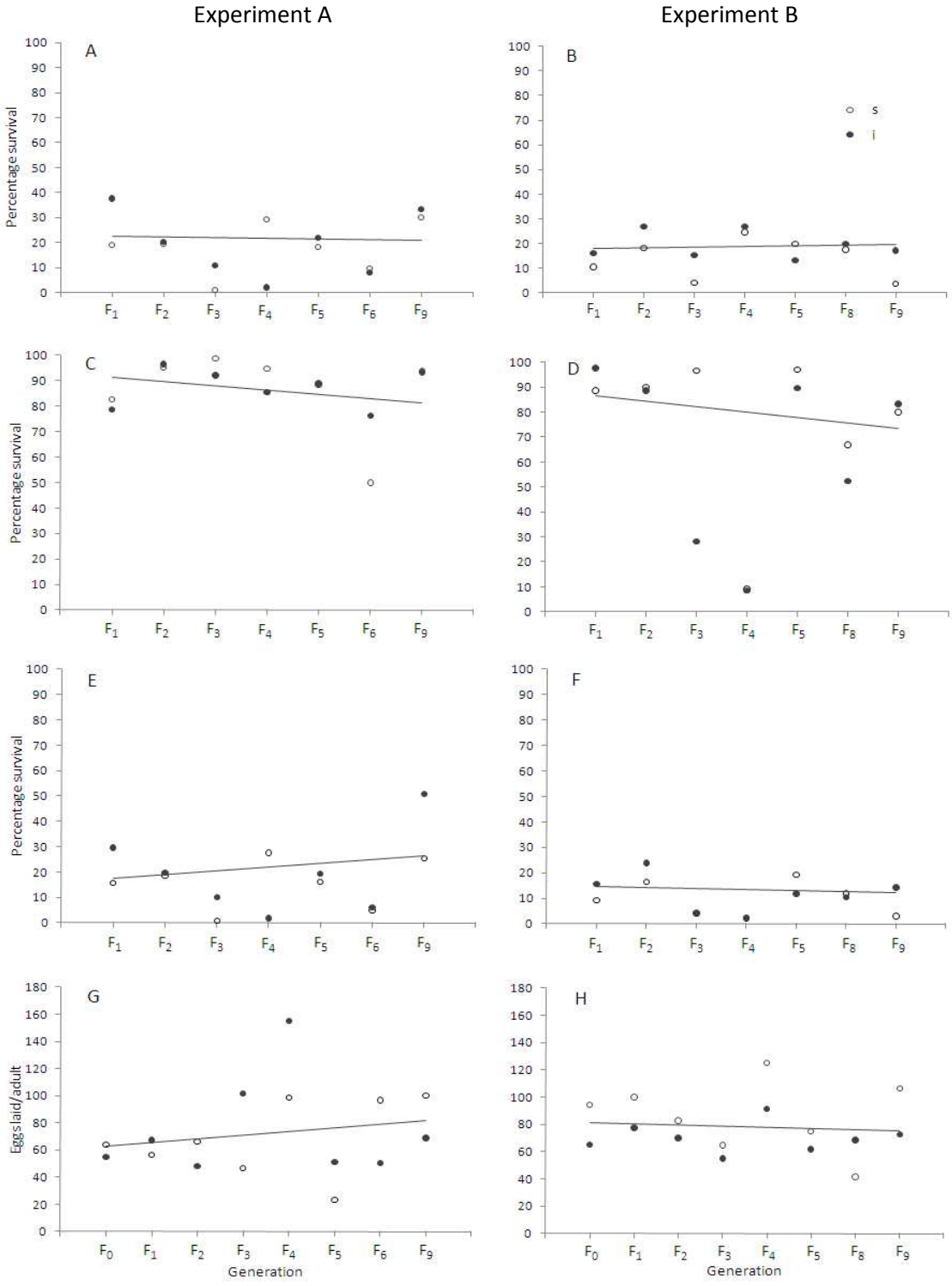


Fig 2.5 Percentage survival from egg to pupae in each generation in A) experiment A and B) experiment B. Percentage survival from pupae to adult in C) experiment A and D) experiment B. Percentage survival from egg to adult in E) experiment A and F) experiment B. Number of eggs laid per adult female in G) experiment A and H) experiment B. The common line of best fit, to show changes in fitness over the generations, is presented as there was no difference in the line of best fit between the s and i lines.

2.3.5 **Crossing**

The F_1 of reciprocal crosses between the F_9 s and i lines showed a mean percentage insensitivity in the populations between 45% and 55% (Table 2.1). This insensitivity was not significantly different from the level of insensitivity in the F_9 i line (p=0.42).

Table 2.1. Mean percent insensitivity in offspring from reciprocal crosses between *Aedes aegypti* from selected lines in experiment A and B (Stanczyk *et al.*, 2010).

Experiment	**Parents**	**Number of offspring tested**	**Mean percent insensitive**
A	♀s♂i	118	55
	♀i♂s	118	49
B	♀s♂i	109	45
	♀i♂s	209	50

2.4 Discussion

The use of behavioural assays to bidirectionally select for olfactory responses has been used in previous studies with insects to determine mode of inheritance through changes in the frequency of the trait (Becker, 1970; Hoffmann, 1983; Margolies et al., 1997; Wang et al., 2003). In our study the arm-on-cage method was adapted from previous repellency studies and a WHOPES (1996) method (World Health Organisation, 1996; Chou *et al.*, 1997; Dogan and Rossignol, 1999). This method was ideal for measuring the repellent effects of DEET without allowing direct contact or probing of the volunteer's arm, and allowed for detecting differences in individual mosquito's responses to DEET (Appendix 3). The selection experiments reported in our study show that breeding DEET-insensitive females with males of unknown sensitivity increases the proportion of the insensitive phenotype in the population (Fig 2.3). This demonstrates that, in *Ae. aegypti*, the DEET-insensitivity is a heritable trait, something also seen previously *in D. melanogaster* (Becker, 1970; Reeder *et al.*, 2001). The rapidity of the increase in insensitivity from 13% to >50% in *Ae. aegypti* females between the F_0 and F_2 generations matched the predictions for the trait being monogenic (Fig 2.2).

There was a difference in the rapidity of the increase of DEET insensitivity in the populations between the A and B experiments. In experiment B only 33% of the population was insensitive in the F_1, compared to 50% of experiment A. According to the predictions for the various modes of inheritance (Fig 2.2), this suggests there could be a difference in the gene(s) being selected. The increase to 50% in experiment A suggests a dominant characteristic, whilst the increase to 33% in experiment B suggests a recessive allele. Whilst it is possible that there are two separate genes which can be responsible for DEET-insensitivity, the low mutation rate makes this unlikely, and it is more likely that random chance affected the F_1 generation of experiment B. This is supported by the fact that fluctuations around the mean ~55% level of insensitivity are seen throughout later generations.

In order to further examine the mode of inheritance in experiments A and B, the s and i lines were reciprocally crossed (Table 2.1). This demonstrated that the insensitive trait was dominant, giving a

level of insensitivity in the F_1 indistinguishable from that in the i line and significantly higher than that in the s line. The finding that insensitivity was dominant differs from previous work with *D. melanogaster*, where the trait was found to be either recessive and on the X chromosome (Reeder *et al.*, 2001) or autosomal and partially dominant (Becker, 1970). This may mean that that the mutations giving rise to DEET insensitivity are found in different genes both within *D. melanogaster*, due to the mutagenesis technique (Reeder *et al.*, 2001), and between *D. melanogaster* and *Ae. aegypti*. DEET-insensitivity has also been previously examined in *Ae. aegypti*, with the conclusion that there was low heritability of DEET tolerance, and the suggestion that the trait was incompletely dominant (Rutledge *et al.*, 1994). The results showed that after 10 generations of brother-sister mating, the inbred lines formed were less insensitive to DEET than the original parent lines. As no selection for insensitivity took place, it is unsurprising that the inbred lines were not more insensitive than the parents and the fact that the inbred lines were in fact less insensitive to DEET can be attributed partially to their lack of fitness due to inbreeding. Incomplete dominance would be a possible explanation for our selection results, but the crosses between the i and s line show insensitivity to be a dominant trait in our study.

In our selection experiments, the frequency of insensitivity to DEET in the population plateaued at ~55% by the third generation in both experiments A and B. This could be because only 55% of the population has the insensitive genotype, or alternatively because all of the insects have the insensitive genotype but, for some reason, only 55% show the insensitive phenotype. In a previous study with DEET-insensitivity in *D. melanogaster*, by generation 12 the insensitive insects chose the DEET-treated arm of the experimental maze 40% of the time, showing no difference in their end distribution in the maze from flies tested with control treatments (Becker, 1970). The authors, therefore, assumed that they had achieved complete DEET insensitivity, with no difference between DEET and the control. This is clearly different to our study in which the proportion of females showing DEET insensitivity plateaued at ~55% (Fig 2.3). In the *D. melanogaster* study by Reeder *et al*. (2001), offspring of a DEET-insensitive mutagenized male were insensitive to DEET in 80% of choice assays and one of the strains used was also found to be insensitive to DEET in 40% of choice assays without any selection taking place (Reeder *et al.*, 2001). It is unknown if insensitivity to DEET would have

increased further with selection in either of these strains. In *Cotesia glomerata,* flight orientation towards a specific odour was selected over four generations and the initial response of an insect rose from less than 20% in the F_0 to 40% in the F_4. The upwards trend suggests that the percentage of wasps successfully orienting towards the odour would have continued to rise if selected further. With *Phytoseiulus persimilis*, the movement of mites towards prey-infested leaves rose from 70% to ~90% after one generation of selection (Margolies *et al.*, 1997). However, there was no further change in response when the mites were selected for another two generations, indicating the trait had plateaued as with DEET-insensitivity in our study. Selection for increased attraction to acetaldehyde or ethanol *in D. melanogaster* increased the proportion of the population responding to these chemicals (Hoffmann, 1983). In some of the selected lines, the response plateaued by the 11th generation, while in other lines it was still increasing by the 20th generation. In our study, there are at least two possible explanations for the plateau in the proportion of i line mosquitoes with the DEET-insensitive phenotype. First, it is possible that the trait is single-locus dominant but homozygous lethal, preventing it from spreading further in the population. However, in the absence of selection a homozygous lethal allele would be expected to fall rapidly in frequency, and this was not seen when the populations were left unselected for two generations. In addition, it would be expected that a recessive lethal allele would have caused a reduction in survival from egg to adult in the i line compared to the s line which was not seen (Fig 2.5). The second possibility is that all of the mosquitoes in the population have the same genotype that can confer insensitivity to DEET, but that there is incomplete penetrance and so the genotype does not always confer an insensitive phenotype. In the case of incomplete penetrance, some DEET-insensitive females would not express the trait, possibly due to non-heritable epigenetic differences or environmental factors. Insensitivity was maintained in the s line at a similar level to that of the base culture, despite downwards selection. A possible explanation for this is that females homozygous for DEET-sensitivity are not always sensitive, with a small proportion of 8% displaying an insensitive phenotype. If there is incomplete penetrance of the insensitive or sensitive traits, a different set of predictions for the expected frequency of the trait in each generation can be compared to the selection results (Fig 2.4). Incomplete penetrance of the insensitive and sensitive traits would explain the plateau in insensitivity, and the lack of downwards selection, but also lead to a slower increase of insensitivity in the i line to 20% in the F_1

and 50% by the F_6 generation, due to sensitive females being selected as insensitive. This is similar to the slower initial increase of insensitivity seen in experiment B, but does not explain the rapid increase in experiment A, or that both experiments plateaued at ~55% by the F_2 generation. It is possible that the trait has incomplete penetrance, and that random chance was responsible for more mosquitoes being insensitive in the early generations than would be predicted in this model. The pattern seen in our study of increased response in upwards selection, but no significant difference from the base population in response to downwards selection after several generations, was also seen in the predatory mite *P. persimilis* (Margolies *et al.*, 1997). In the study on *P. persimilis* they suggested the phenomenon could be caused by unbalanced, additive genetic variation, but whether this might explain our results is unknown.

In our experiments there was no significant change in the fitness of the s or i lines throughout the generations of selection. The two lines did not differ in their survival rates throughout the life cycle, and females did not differ in the number of eggs laid. In both experiments, the average number of eggs laid per female was 75-80. This is comparable to other work on *Ae. aegypti* where the average number of eggs laid per female fed on sheep's blood was 81 (Greenberg, 1951). There was overall poor survival from egg to adult, which was largely due to a low percentage of the eggs hatching. The proportion of *Ae. aegypti* eggs which hatch successfully in uncontaminated water varies from study to study, being as high as 75-90% in some, (Gerberg *et al.*, 1994), and as low as 2% in others (Rozeboom, 1934). The hatching of *Ae. aegypti* eggs has previously been described as erratic, with hatching times and the number of larvae emerging varying with no discernable cause (Gillett *et al.*, 1977). We do not have an exact figure for the rate of successful hatching in our study, but as the average survival from egg to pupae was ~20% (excluding generations with known environmental problems), it is unlikely the number of eggs successfully hatching was higher than 30%. Since this rate is constant between the two lines and experiments, it appears to be characteristic of the strain used here and not caused by the selection. The number of eggs hatching may have been affected by the method used, with water uncontaminated by inorganic matter or bacteria, which have been shown to be important for hatching of the eggs (Rozeboom, 1934). There were several generations with a particularly poor rate

of emergence from the eggs, possibly due to room temperature variance caused by uncontrollable variables such as power cuts. Generations with unexplained larval death may have been affected by contamination of the water or variance in room temperature.

2.5 Conclusion

DEET-insensitivity is a heritable trait in *Ae. aegypti* and therefore the proportion of insensitive females increases in the population when selected. Here two selected lines were established with differing sensitivity to DEET, the s and i lines, that can be used for further examination of DEET-insensitivity. The rapidity of the spread of the trait throughout the population suggests a monogenic trait and crossing experiments show it to be dominant. DEET-insensitivity does not appear to confer any fitness disadvantages on the mosquitoes.

Chapter 3. Using Electroantennography to Characterise DEET-Insensitivity

3.1 Introduction

It was originally thought that DEET acted as a repellent by blocking the olfactory receptors which respond to lactic acid in the antennae of *Aedes aegypti* mosquitoes (Davis and Sokolove, 1976). However, other studies have shown that DEET can function as a repellent for several species of mosquito, including *Ae. aegypti*, even when attractive compounds other than lactic acid are present (Boeckh *et al.*, 1996; Hoffmann and Miller, 2003). 1-Octen-3-ol is a component of human sweat (Cork and Park, 1996) which in combination with CO_2 acts as an attractant for *Anopheles gambiae* (Takken and Kline, 1989; Takken and Knols, 1999). A recent electrophysiological investigation in *An. gambiae* has suggested that the olfactory receptor neuron (ORN) for 1-octen-3-ol is blocked by DEET, such that a higher concentration of 1-octen-3-ol is then required for detection by mosquitoes (Ditzen *et al.*, 2008). Syed and Leal (2008) also investigated this using *Culex quinquefasciatus* and suggested that the reduction in response to 1-octen-3-ol in this species is not due to a diminished response of the ORN but to interactions between the two compounds when DEET and 1-octen-3-ol were tested in the same odour cartridge during the electrophysiological recordings. Additionally, single sensillum recordings have identified ORNs that respond directly to DEET in *Cx. quinquefasciatus* (Syed and Leal, 2008) and *Ae. aegypti* (Davis and Rebert, 1972; Boeckh *et al.*, 1996), indicating that these mosquito species are able to actively detect DEET. A recent study expressing recombinant *Ae. Aegypti* olfactory receptors (ORs) AaOR2 and AaOR8 in *Xenopus* oocytes found AaOR2 responded to DEET in a concentration dependent manner, but not to 1-octen-3-ol, and AaOR8 responded to 1-octen-3-ol but not DEET (Bohbot and Dickens, 2010). DEET was also shown to inhibit the detection of 1-octen-3-ol by AaOr8. Thus, based on these studies we concluded that the behavioural difference in sensitivity to DEET between the selected s and i lines of *Ae. aegypti* (Section 2.2.3) could result from either a change in the blocking activity of DEET, or a difference in the detection of DEET.

An effective way to determine the mode of action of DEET on the olfactory system is to use electroantennography (EAG) to measure the response of the ORs on the antennae of insects (Schneider, 1957). Olfactory receptors located in the ORNs on antennae detect semiochemicals (McIver, 1982), which, as they bind to the OR, cause the cell to depolarise and an action potential to be transmitted along the olfactory nerve to the brain. As an odour stimulates receptors on the antennae, the EAG records the sum of the electrical potentials generated by all receptors on the antennae (Nagai, 1985). This allows for a measurement, displayed as a depolarisation, of the insect's response to individual odours. This method has been used successfully to identify attractants and repellents for mosquitoes (Cork and Park, 1996; Bernier *et al.*, 2000; Meijerink *et al.*, 2000; Logan *et al.*, 2008). Thus, since previous EAG work has demonstrated that semiochemicals are detected by ORs on the antennae (McIver, 1982; Takken and Knols, 1999), and that a DEET-sensitive neuron is present on the antennae of *Ae. aegypti* (Davis and Rebert, 1972; Boeckh *et al.*, 1996) and *Cx. quinquefasciatus* (Syed and Leal, 2008), EAG was selected as an appropriate initial method of analysis for the s and i lines in our study.

The aim of this chapter was to investigate the DEET insensitivity found in behavioural experiments (Section 2.3.2) through electroantennography, in order to see if the difference between the s and i lines lay in the peripheral olfactory system. The hypothesis was that there would be a difference in EAG responses between the two lines.

3.2 Methods

3.2.1 Insects

The mosquitoes used in this study were female *Ae. aegypti* reared as described in section 2.2.2. For EAG recordings, 7-15 day-old females were chosen from the s and i lines which had been selected previously for DEET-sensitivity or insensitivity in behavioural assays 1-5 days prior (Section 2.2.3).

3.2.2 Preparation

Female *Ae. aegypti* mosquitoes were chilled on ice for approximately 30 s before removal of the head, the tips of both antennae and the proboscis. The electrodes were formed by two glass pipettes filled with ringer solution (129 mM sodium chloride, 8.58 mM potassium chloride, 1.98 mM calcium chloride, 18.17 mM magnesium chloride, 10.24 mM sodium bicarbonate, 3.72 mM sodium orthophosphate, pH 7.4) with Ag/AgCl (silver/silver chloride) electrodes inserted. The indifferent electrode was inserted into the back of the mosquito head and the ends of both antennae were inserted into the recording electrode. A continuous airflow (1 L min^{-1}) was passed over the head and antennae from a glass tube positioned 0.5 cm away, with the air stream charcoal filtered and humidified. Signals from the antennae were recorded and analysed (amplified x 10,000) using a software package (EAG v2.6, Syntech®, The Netherlands).

3.2.3 Stimulus Delivery

The test compound (in redistilled hexane, 10 µl) was applied to a strip of filter paper (Whatman® 55 x 3 mm) and 30 s was allowed for the solvent to evaporate. The filter paper was then placed in a glass pipette cartridge (volac® 230 mm). Using a stimulus controller, a 2 second air-puff was passed through the glass cartridge into the continuous air-stream, through a hole in the glass tube 7 cm away from the mosquito preparation, and the response to the stimulus was recorded. A compensatory air flow system ensured that, when the stimulus airflow (840 ml min^{-1}) was triggered, the continuous airflow

dropped by the corresponding amount to maintain a constant airflow. The method for stimulus delivery was adapted to pass the stimulus airflow through a split airflow delivery system (Fig 3.1) (Syed and Leal, 2008) into the continuous air-stream, allowing two compounds to be tested together. Where there was only one test compound, or when testing the control, the second cartridge contained a control (hexane). The control and standard (methyl salicylate) stimuli were applied at the beginning of each test to determine whether the mosquito was responding. The control and standard stimuli were then applied, in that order, after every two test treatments.

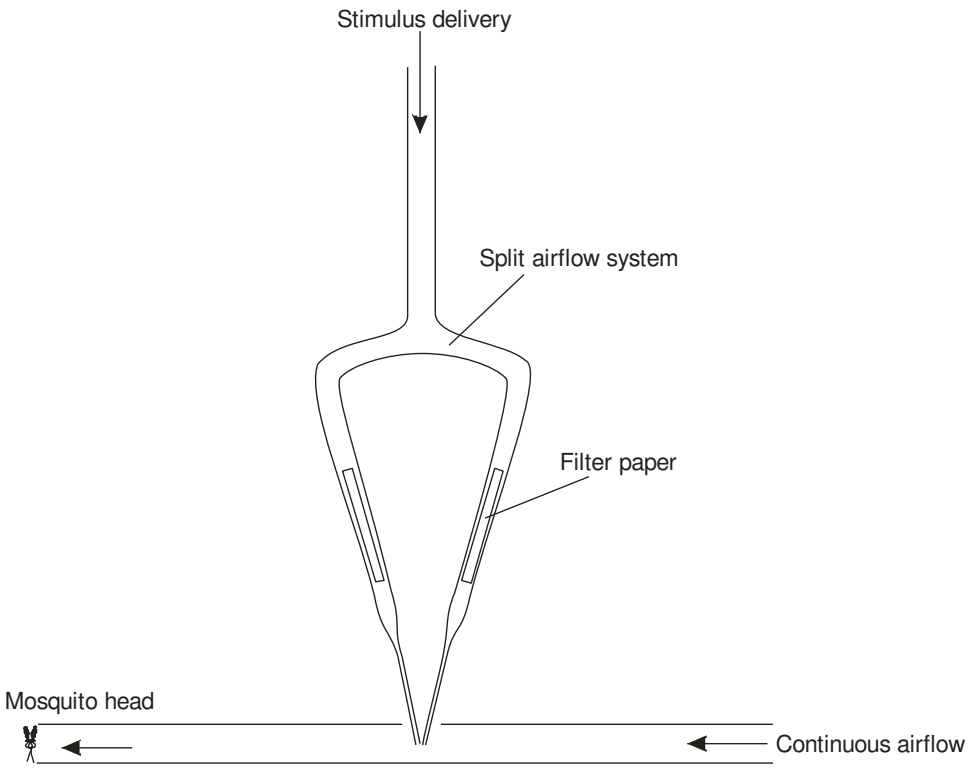


Fig 3.1 Split airflow delivery system consisting of PTFE tubing, with ends capped by lids, in which the 2 s stimulus airflow was run simultaneously through two separate cartridges, enabling two compounds to be tested together without being in the same cartridge.

3.2.4 **Treatments**

To test the hypothesis that DEET interferes with a mosquito's responses to the known attractant 1-octen-3-ol (Takken and Knols, 1999; Ditzen *et al.*, 2008; Bohbot and Dickens, 2010, Cook J.I, Pers comm) the two compounds were tested together in the same cartridge. They were also tested simultaneously in separate cartridges to see if there was an interaction between the two compounds before they reach the receptor when they were in the same cartridge. This interaction would give the appearance of DEET interfering with the 1-octen-3-ol receptor (Syed and Leal, 2008).

Behaviourally selected DEET-sensitive mosquitoes from the s line, and insensitive mosquitoes from the i line were tested with EAG in the F_1-F_5 generations of experiment A, and from the F_0-F_3, and F_8 of experiment B (Section 2.3.2). Mosquitoes from the F_9 of experiments A and B were also tested, as this was the first generation when the mosquitoes from experiments A and B could be recorded from simultaneously.

Dose response experiments

Dose response experiments were carried out on unselected culture mosquitoes for DEET ($1x10^{-6}$ g - 10^{-3} g), methyl salicylate ($1x10^{-7}$ g - 10^{-3} g) and 1-octen-3-ol ($1x10^{-7}$ g - 10^{-3} g) to establish the optimal concentration for testing the selected mosquitoes, where $1x10^{-n}$ g was the amount of compound present on the filter paper. A single stimulus cartridge was used (instead of the split airflow system). According to these results (Section 3.3.1), $1x10^{-4}$ g of methyl salicylate and 1-octen-3-ol, and $1x10^{-3}$ g of DEET were selected as appropriate in recording from the selected lines, and, therefore, used for treatment set 2.

Treatment set 1

For the F_1 and F_2 generations of Experiment A, and F_0 and F_1 generations of Experiment B, each mosquito was tested with six treatments. 1) Control (hexane), 2) Standard (methyl salicylate $1x10^{-4}$ g), 3) DEET ($1x10^{-5}$ g), 4) 1-octen-3-ol ($1x10^{-4}$ g), 5) DEET ($1x10^{-5}$ g) + 1-octen-3-ol ($1x10^{-4}$ g) in the same cartridge, 6) DEET ($1x10^{-5}$ g) + 1-octen-3-ol ($1x10^{-4}$ g) in different cartridges.

Treatment set 2

For all further generations, including the F_9, each mosquito was tested with six treatments; these were the same as treatment set 1 but with a different, optimal, concentration of DEET. 1) Control (hexane), 2) Standard (methyl salicylate $1x10^{-4}$ g), 3) DEET ($1x10^{-3}$ g), 4) 1-octen-3-ol ($1x10^{-4}$ g), 5) DEET ($1x10^{-3}$ g) + 1-octen-3-ol ($1x10^{-4}$ g) in the same cartridge, 6) DEET ($1x10^{-3}$ g) + 1-octen-3-ol ($1x10^{-4}$ g) in different cartridges.

3.2.5 **Air entrainment of pipette cartridge**

Air entrainment is a method of collecting volatiles from a subject (headspace collection) with a dynamic, continuous airflow. To establish the amount of each chemical that would be passing over the antennae during the 2 s stimulus pulse, a pipette cartridge with filter paper treated with DEET ($1x10^{-3}$ g) or 1-octen-3-ol ($1x10^{-4}$ g) was air entrained for 2 s. The pipette cartridge was prepared as described in Section 3.2.3. Air was pulled for 2 s (840 ml min^{-1}) through the pipette into a glass tube containing 50 mg Tenax to trap the volatiles. The apparatus was connected using PTFE tubing and brass Swagelock fittings. The Tenax tubes had been previously heated at 210°C for 2 hours while attached to a constant supply of nitrogen to 'condition' them. Cotton gloves were used to handle all equipment. After the 2 s entrainment the tube was sealed immediately with swagelock compression fittings to prevent any contamination and the samples were run on a gas chromatograph (GC). Four entrainments were done for each chemical. The GC analyses were done using an Agilent Technologies 6890N GC containing an HP1 column (50 m x 0.32 mm, 0.5 µm film thickness) and an Optic 2 Atas programmable injector providing optic thermal desorption. The oven temperature was maintained at

30°C for 1 min and then raised by 5°C min^{-1} to 150°C, then by 10°C min^{-1} to 250°C, where it was held for 20 mins. An external standard quantification method was used to calculate the amount of each chemical collected in the test samples.

3.2.6 **Statistical Analyses**

EAG responses were corrected by dividing the response in millivolts by the average of the control values before and after the stimulation of each treatment, so that the control had a value of 1 and the response to treatments was expressed as a proportion of 1. The mean responses of the s and i lines to each treatment, between the treatment and control, and between lines for treatments, were compared using a two-way ANOVA in Genstat® (12th edition), using replicates as blocks. The data were log (base10) transformed to correct for skewed data with a large range showing heterogeneity of variances. Differences were deemed significant when the difference between means was greater than the least significant difference (LSD).

3.3 Results

3.3.1 EAG dose-response experiments

Unselected *Ae. aegypti* females showed dose-dependent responses to methyl salicylate, 1-octen-3-ol and DEET (Fig 3.2). For methyl salicylate, all doses elicited a response that was significantly different from the control ($1x10^{-7}$ g p=0.04, $1x10^{-6}$ g p=0.02, $1x10^{-5}$–$1x10^{-3}$ g p<0.001) (Fig 3.2A). The responses to $1x10^{-6}$ g and $1x10^{-5}$ g were not significantly different from those to $1x10^{-7}$. Methyl salicylate at $1x10^{-4}$ g gave significantly greater responses than at all lower concentrations (p<0.001), and there were no differences between responses to $1x10^{-3}$ g and $1x10^{-4}$ g.

For 1-octen-3-ol (Fig 3.2B), EAG responses to the second lowest concentration ($1x10^{-6}$ g) were not significantly different from the control. The response to all other concentrations was significantly higher than the response to the control ($1x10^{-7}$ g p=0.04, $1x10^{-5}$–$1x10^{-3}$ g p<0.001). The response to $1x10^{-4}$ g was significantly greater than $1x10^{-7}$–$1x10^{-5}$ g (p=0.007, p<0.001, p<0.001 respectively). The response to $1x10^{-3}$ g was significantly greater than $1x10^{-7}$ g (p=0.002) and $1x10^{-6}$ g (p<0.001) but not significantly different from $1x10^{-5}$ g or $1x10^{-4}$ g.

The EAG responses of the mosquitoes to DEET at $1x10^{-6}$g were not significantly different from the control (Fig 3.2C). For all other concentrations the mosquitoes showed a significantly higher response compared with the control (p<0.001). The response to $1x10^{-5}$g was significantly higher than to $1x10^{-6}$g (p=0.016). There were no significant differences between the responses to $1x10^{-5}$g and $1x10^{-4}$g or between $1x10^{-4}$g and $1x10^{-3}$g. Responses to $1x10^{-3}$ g were significantly greater than the responses to $1x10^{-5}$g (p<0.001).

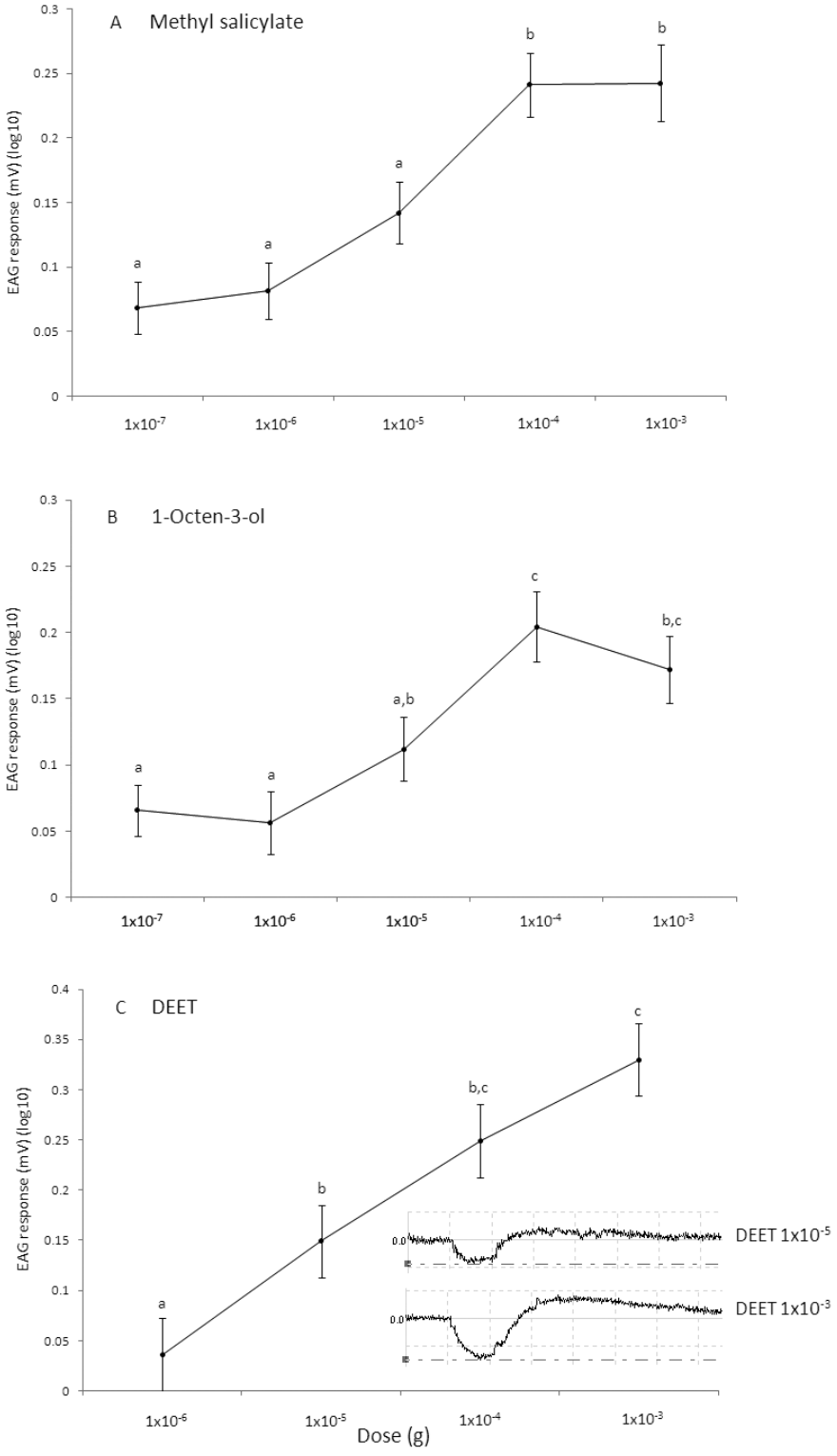


Fig 3.2 EAG dose responses (mV/average control value, data $\log^{10}$ transformed) of female *Aedes aegypti* to different doses of: A) Methyl salicylate (N=15), B) 1-Octen-3-ol (N=15) and C)

DEET (N=10) with typical EAG recordings of DEET $1x10^{-5}$ g and DEET $1x10^{-3}$ g. Means are ± SEM. Means with different letters are significantly different from each other ($p<0.05$).

3.3.2 EAG recordings from selected lines (generations F_0-F_8)

3.3.2.1 *EAG with treatment set 1*

In the F_1 generation of experiment A there were no significant differences in responses between the s and i lines for any of the treatments (Fig 3.3A). The response to DEET in the s and i lines was not significantly different from the response to the control. There was a significant difference between 1-octen-3-ol plus DEET tested in the same and different cartridges for the i line ($p=0.016$), but not for the s line. In the F_2 generation the i line response to DEET was not significant compared to the control, and responded significantly less than the s line ($p=0.026$) (Fig 3.3B). There were no significant differences between lines for any of the other treatments, and no differences within lines between the same and separate cartridge methods.

In the F_0 generation of experiment B the i line responded significantly less to DEET than the s line ($p<0.011$), and was not significantly different from the control (Fig 3.3C). There were no significant differences in response to the other treatments between lines, and no differences within lines between the same and separate cartridge methods. In the F1 generation there was a significant difference ($p=0.0018$) in response between the s and i lines to 1-octen-3-ol and DEET in the same cartridge, with the i line having a lower response (Fig 3.3D). There were no significant differences in responses between lines for the remaining treatments. In the i line, the mosquitoes had a significantly lower response to 1-octen-3-ol and DEET tested together in the same cartridge than when the two compounds were tested in separate cartridges ($p=0.025$). There were no differences between methods for the s line.

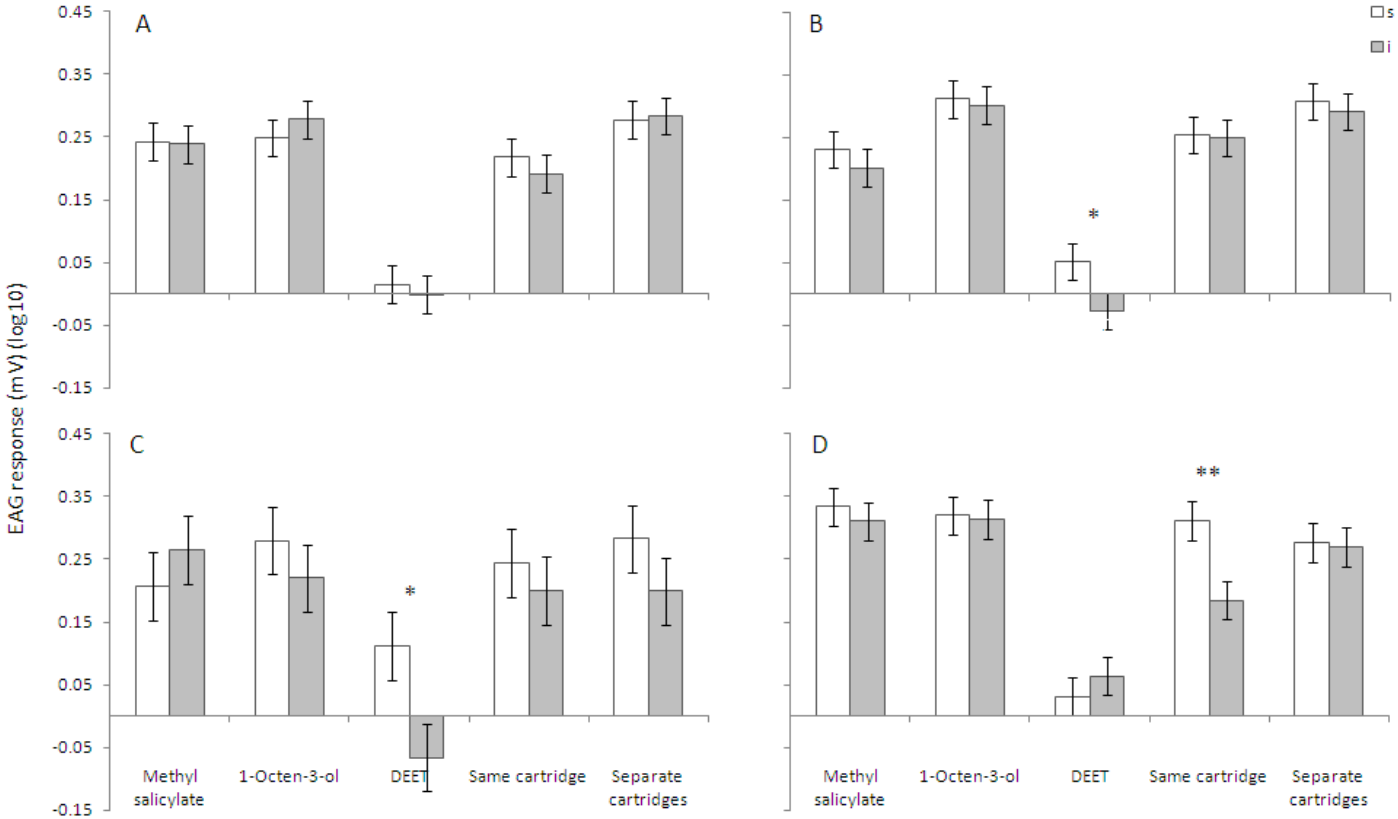


Fig 3.3 EAG responses (mV/average control value, data $\log^{10}$ transformed) of *Aedes aegypti* females to methyl salicylate, 1-octen-3-ol and DEET from the A) F_1 (N=9) and B) F_2 (N=10) generations of the s and i lines of experiment A, and the C) F_0 (N=8) and D) F_1 (N=14) generations of experiment B. Same cartridge and separate cartridges refer to DEET and 1-octen-3-ol tested simultaneously by different delivery methods (Section 3.2.2). Means are ± SEM. * indicates a significant difference between lines ($p<0.05$), ** ($p<0.01$).

3.3.2.2 *EAG with treatment set 2*

There was a significant difference between the s and i lines in response to DEET in the F_3 generation of experiment A ($p=0.002$), but no differences between the delivery methods (Fig 3.4A). In the F_4 generation there were no differences in response between lines, but a significantly lower response was seen to 1-octen-3-ol and DEET when tested together in the same cartridge as opposed to when delivered by separate cartridges for both the s and i lines ($p=0.023$, $p=0.043$ respectively) (Fig 3.4B). For the F_5 generation the i line had a significantly lower response to DEET than the s line ($p<0.001$) (Fig 3.4C). There was also a difference between lines, with the i line having a lower response, when 1-octen-3-ol and DEET were tested in separate cartridges ($p=0.003$). Within the s line, the response to 1-octen-3-ol and DEET tested in separate cartridges was significantly higher than the response to the two compounds tested in the same cartridge ($p=0.037$).

In the F_2 generation of experiment B (Fig 3.5A) there was a significantly lower response in the s line than in the i line when 1-octen-3-ol and DEET were tested together in the same cartridge ($p=0.009$). In the s line, there was a significantly greater response when 1-octen-3-ol and DEET were tested in separate cartridges compared to when tested together in the same cartridge ($p<0.001$). In the F_3 generation (Fig 3.5B) there was a significant difference in response between lines when tested with 1-octen-3-ol and DEET in the same ($p=0.004$) cartridge, with the i line having a lower response than the s line. The i line response to 1-octen-3-ol and DEET in separate cartridges was significantly greater than the i line response to the compounds tested in the same cartridge ($p=0.033$). There were no differences in responses between lines in the F_8 generation (Fig 3.5C), and no difference according to whether treatments were administered in the same or separate cartridges.

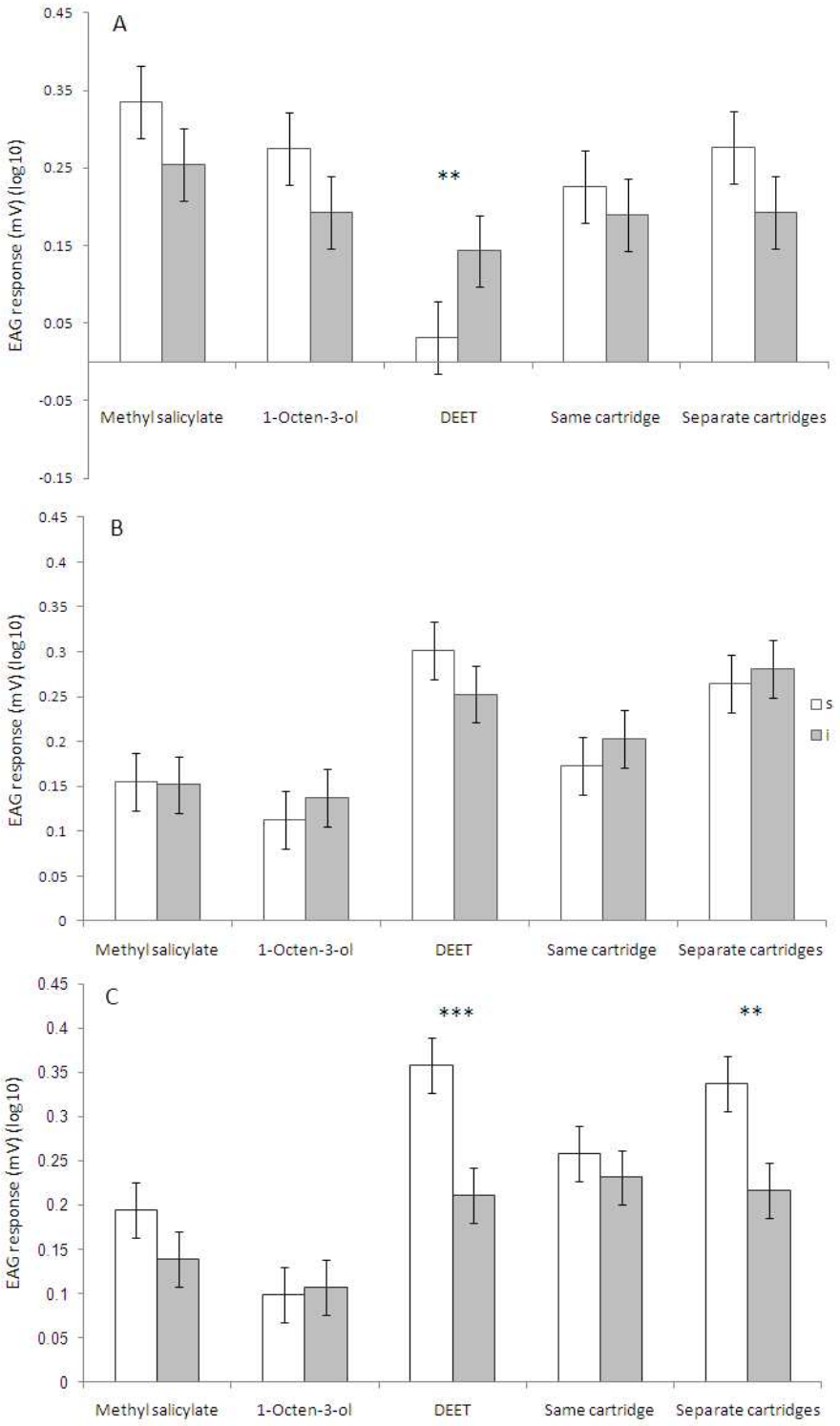


Fig 3.4 EAG responses (mV/average control value, data $\log^{10}$ transformed) of *Aedes aegypti* females to methyl salicylate, 1-octen-3-ol and DEET from the A) F_3 (N=9), B) F_4 (N=10) and C) F_5 (N=10) generations of the s and i lines of experiment A. Same cartridge and separate cartridges refer to DEET and 1-octen-3-ol tested simultaneously by different delivery methods (Section 3.2.3). Means are ± SEM. ** indicates a significant difference between lines ($p<0.01$), *** ($p<0.001$).

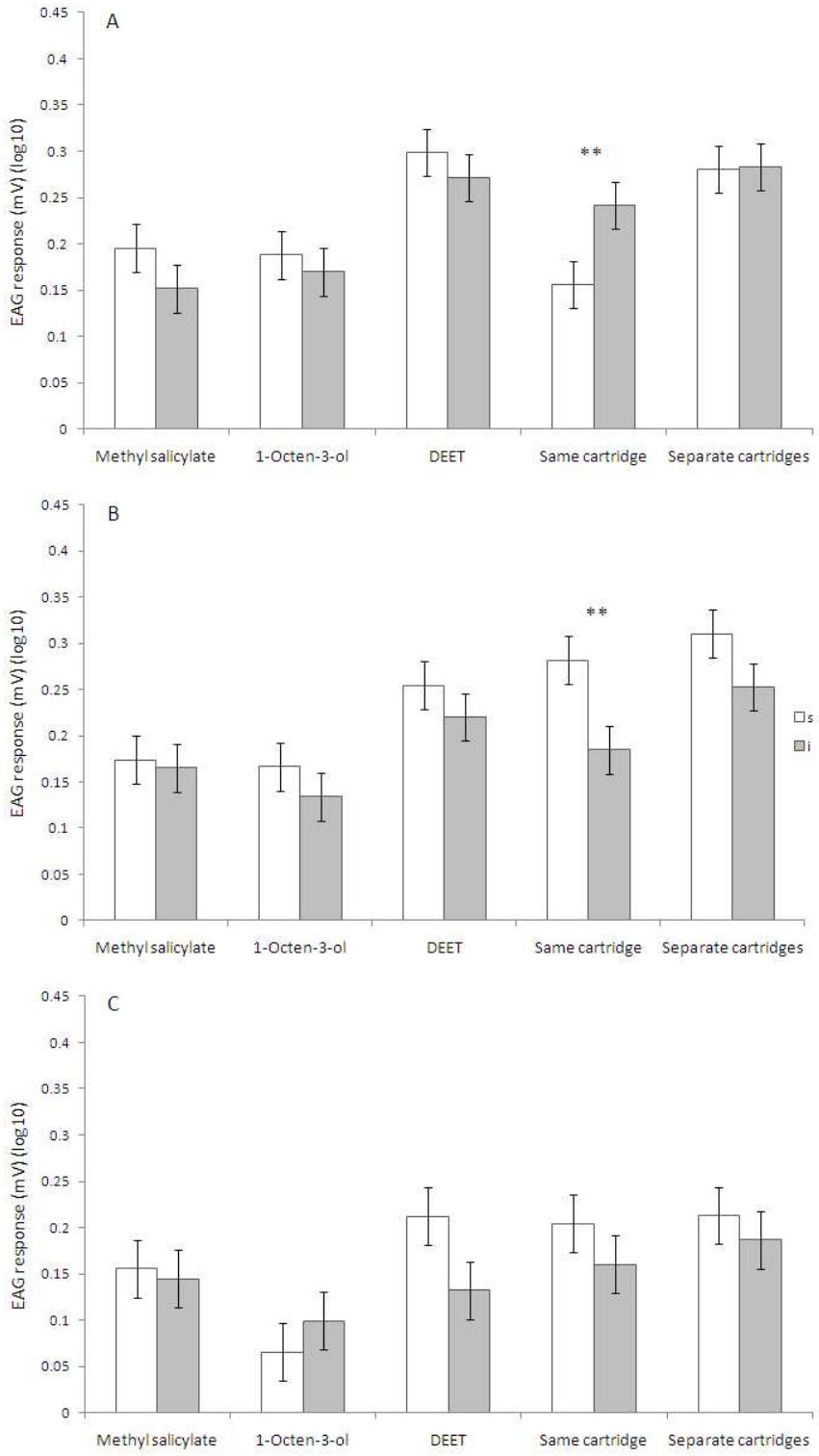


Fig 3.5 EAG responses (mV/average control value, data $\log^{10}$ transformed) of *Aedes aegypti* females to methyl salicylate, 1-octen-3-ol and DEET from the A) F_2 (N=15) and B), F_3 (N=10) and C) F_8 (N=9) generations of the s and i lines of experiment B. Same cartridge and separate cartridges refer to DEET and 1-octen-3-ol tested simultaneously by different delivery methods (Section 4.2.2). Means are ± SEM. ** indicates a significant difference between lines ($p<0.01$).

3.3.3 EAG with selected lines (F_9)

Responses to the standard compound, methyl salicylate, and to 1-octen-3-ol were not significantly different in the s and i lines of either experiment of F_9. However, for DEET, in both experiments the i line had a significantly lower ($p<0.001$) response than the s line (Fig 3.6). In both experiments the s lines showed lower responses to DEET with the 1-octen-3-ol in the same cartridge than when the compounds were in different cartridges (experiment A, $p=0.002$, experiment B, $p=0.001$). There were no such differences in the i line. In experiment B there were also differences between the responses of the s and i lines to DEET with 1-octen-3-ol when tested in the same ($p<0.001$) or different ($p<0.001$) cartridges, with the i line being lower (Fig 3.6B).

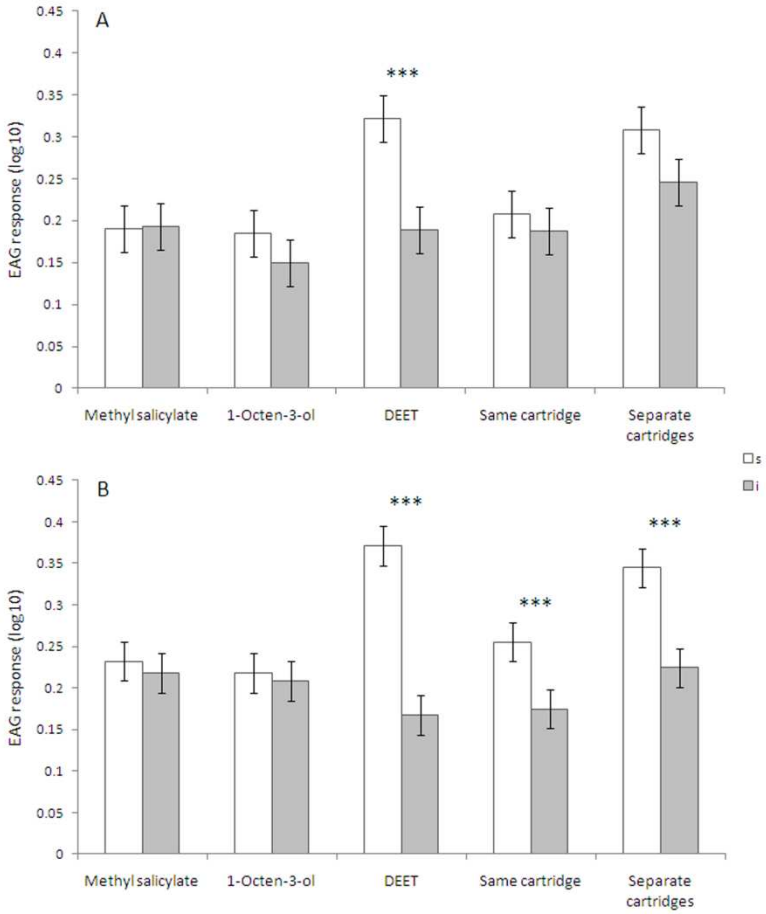


Fig 3.6 EAG responses (mV/average control value, data $\log^{10}$ transformed) of *Aedes aegypti* females to methyl salicylate, 1-octen-3-ol and DEET from the F_9 generation of the s and i lines of experiments A and B (N=20). Same cartridge and separate cartridges refer to DEET and 1-octen-3-ol tested simultaneously by different delivery methods (Section 3.2.3). Means are ± SEM. *** indicates a significant difference between lines ($p<0.001$).

3.3.4 **Pipette air entrainments**

In the time of the 2 s stimulus pulse (Section 3.2.3), the average amount of DEET being released from the filter paper and collected from the pipette was 0.612 ng. The average amount of 1-octen-3-ol was 619.88 ng.

3.4 Discussion

Electroantennography has been used to determine if insects respond to volatile compounds (Schneider, 1957), to compare the strength of responses to different volatiles (Logan *et al.*, 2008), and, when coupled with gas chromatography, to identify behaviourally important chemicals in complex mixtures (Pickett and Woodcock, 1996; Logan *et al.*, 2008). For haematophagous insects, EAG has been used to record from a range of species of mosquitoes (Cork and Park, 1996; Bernier *et al.*, 2000; Meijerink *et al.*, 2000), midges (Blackwell *et al.*, 1997; Bhasin *et al.*, 2000; Mands *et al.*, 2004), tsetse flies (Bursell *et al.*, 1988; Gikonyo *et al.*, 2002) and also the sandfly *Lutzomyia longipalpis* (Sant'Ana *et al.*, 2002) to examine compounds involved in attraction or repellency.

In the present study, a dose response experiment with unselected culture mosquitoes was done for each of the chemicals being tested to establish the appropriate concentrations to use in further electrophysiological recordings with the selected lines. Methyl salicylate has been shown to elicit an EAG response in several insect species (Henning and Teuber, 1992; Light *et al.*, 1992; Blackwell *et al.*, 1997), including the mosquito *Cx. pipiens*, where it was found to be an attractant (Jhumur *et al.*, 2008). In the present dose response experiment, $1x10^{-4}$ g of methyl salicylate was found to be the optimal concentration, giving significantly greater responses than all lower concentrations, but there was no further increase with $1x10^{-3}$ g (Fig 3.2). This is unlike a previous study that found *Cx. Pipiens* responses continued increasing when presented with methyl salicylate from $1x10^{-5}$ g to $1x10^{-1}$ g (Jhumur et al., 2008). The present study also shows that methyl salicylate is detected at lower concentrations in *Ae. aegypti* than reported for the midge *Culicoides impuntatus*, where the lowest dose detected by the insect was $1x10^{-3}$ g (Blackwell *et al.*, 1997). This greater sensitivity to methyl salicylate in *Ae. aegypti* could indicate the compound is ecologically relevant at different levels in different species, possibly for identifying host plants (Chamberlain *et al.*, 2000) for nectar feeding.

1-Octen-3-ol is a compound found in human sweat (Cork and Park, 1996) and has been shown to elicit electrophysiological responses in *C. impuntatus* (Blackwell *et al.*, 1996), *An. gambiae* (Meijerink and

van Loon, 1999; Ditzen *et al.*, 2008) and in recombinant receptors of *Ae. aegypti* (Bohbot and Dickens, 2009). In our study, racemic 1-octen-3-ol showed an optimal response in *Ae. aegypti* at $1x10^{-4}$ g. This is consistent with previous work on the enantiomers (optical isomers) of 1-octen-3-ol with *Ae. aegypti* OR8 (Bohbot and Dickens, 2009).

The data presented here show that for *Ae. aegypti*, responses to DEET at $1x10^{-5}$ g were significantly different from the control. However, DEET at $1x10^{-3}$ g was chosen for use in further EAG recordings because it gave significantly greater responses than $1x10^{-5}$ g, and therefore differences in response between lines would be easier to detect. An examination of the volatiles collected from the filter paper in the pipette cartridge in the two second stimulus pulse demonstrated that a thousand-fold less DEET is passed over the mosquito's antennae in the duration of the pulse compared to 1-octen-3-ol. This disparity in the volatility of the two substances probably explains why a higher concentration of DEET is needed.

The repellent action of DEET has previously been attributed to DEET interfering with the receptors of 1-octen-3-ol (Ditzen *et al.*, 2008). However, DEET has been found to elicit a direct response in *Ae. aegypti* (Davis and Rebert, 1972; Boeckh *et al.*, 1996) and *Cx. quinquefasciatus* (Syed and Leal, 2008) when tested with single sensillum recordings. Whilst behavioural insensitivity to DEET has been observed in *Drosophila melanogaster* (Becker, 1970; Reeder *et al.*, 2001) and *Ae. aegypti* (Rutledge *et al.*, 1994), there have been no previous reports on the electrophysiological basis of this trait. In the present study two alternative hypotheses were tested; 1) that DEET interferes with the receptor which also detects 1-octen-3-ol (Ditzen *et al.*, 2008), and 2) that DEET itself elicits an electrophysiological response and acts without affecting the response to 1-octen-3-ol (Syed and Leal, 2008). In our study, *Ae. aegypti* females in the s and i lines of experiments A and B, selected to be sensitive or insensitive to DEET in behavioural bioassays, were tested simultaneously with EAG in the F_9 generation once it was established that the trait was stable in the population, and responded significantly to DEET (Figs 3.3 - 3.6), supporting the second hypothesis. The significantly lower

response in the F_9 generation of the i line to DEET in both experiments A and B (Fig 3.6) shows that the behaviourally insensitive mosquitoes were no longer able to detect DEET to the same extent as that found in the s line. This could result from a mutation affecting the function of an OR or an odorant binding protein, and this is discussed further in Chapter 4. The difference between the s and i lines in response to DEET was also seen in several of the F_1-F_8 generations, but this was not consistent. This is possibly because of the low numbers of mosquitoes tested and the putative incomplete penetrance of the trait which means only ~55% of mosquitoes with the insensitive genotype would appear insensitive (Section 2.3.2). If insensitive mosquitoes are only visibly behaviourally insensitive 55% of the time, there is the possibility that i line mosquitoes which are insensitive will not be displaying the phenotype when tested with EAG, and that s line mosquitoes which are behaviourally selected as sensitive are in fact insensitive, but not displaying the insensitive phenotype when selected. If females tested with EAG have a different sensitivity to DEET than when they were selected behaviourally, it would mask the difference in EAG responses between the lines.

To test the hypothesis that DEET acts by blocking or interfering with the 1-octen-3-ol ORN as found in *An. gambiae* (Ditzen *et al.*, 2008) and in the recombinant *Ae. aegypti* receptor AaOR8 (Bohbot and Dickens, 2010), in our study DEET was tested simultaneously with 1-octen-3-ol on *Ae. aegypti*. The two chemicals were presented either together in the same cartridge, or in two separate cartridges to rule out any interaction between the chemicals affecting the results (Syed and Leal, 2008). The differences in response between the s and i lines in the F_1 and F_3 generations of experiment B and the F_5 generation of experiment A were due to a lower response in the i line (Figs 3.3 - 3.5). This was also seen in the F_9 generation of experiment B, where tests in both the same and separate cartridges resulted in a significantly lower response in the i line (Fig 3.6). In all generations where there was a difference between the s and i lines, the i line response was not significantly different from that seen with DEET alone, indicating that the i line was responding consistently to DEET. These differences in response between lines to 1-octen-3-ol and DEET tested simultaneously were due to a greater response of the s line to the compounds, which was an unexpected result. These differences were not seen in all generations as the responses of the s line were not constant, likely due to the unavoidable

inclusion in the s line of insensitive mosquitoes not displaying the insensitive phenotype at the time of selection.

Syed and Leal suggested that the differences shown in responses of *An. gambiae* to 1-octen-3-ol delivered with DEET (Ditzen *et al.*, 2008) were due to the method of delivery of the compounds (Syed and Leal, 2008). An analysis of the volatiles in the air flow from a cartridge containing both DEET and 1-octen-3-ol showed a reduced amount of 1-octen-3-ol, which could cause the appearance of DEET inhibiting responses to 1-octen-3-ol. A recent study using the recombinant *Ae. aegypti* receptor AaOR2 showed that this receptor is directly activated by DEET, and that DEET inhibited the response of AaOR8 of 1-octen-3-ol (Bohbot and Dickens, 2010). In the present study with *Ae. aegypti*, over half of the tested F_1-F_8 generations showed a significant difference (within at least one of the lines) between the response to DEET and 1-octen-3-ol delivered in the same cartridge and DEET and 1-octen-3-ol delivered in separate cartridges. This was also seen in the F_9 generation of experiments A and B, where there was a lower response in the s line to DEET and 1-octen-3-ol in the same cartridge compared with the compounds in separate cartridges. The stimulus delivery method did appear to alter the response in *Ae. aegypti*, supporting Syed and Leal's (2008) conclusions in *Cx. quinquefasciatus* that the interaction of the two chemicals, when present in the same cartridge, directly affects the response of the insect. It seems likely that, when delivered in the same cartridge, DEET prevents the dispersion of 1-octen-3-ol, affecting the amount of test material reaching the insect's antennae. However, when they are presented in separate cartridges the mosquito responds normally to 1-octen-3-ol without any inhibition (Pickett *et al.*, 2008). The reason that only the s line of the F_9 showed this difference could be that the i line has a lower response to DEET alone. Thus, the likelihood of detecting differences between delivery methods here using EAG is small compared to the use of single sensillum recordings, where differences would be easier to detect in recordings taken from the 1-octen-3-ol olfactory receptor neuron (Syed and Leal, 2008). In our study no inhibition of 1-octen-3-ol in the presence of DEET, as shown in the *Ae. aegypti* recombinant receptor AaOR8 (Bohbot and Dickens, 2010), is seen in the separate cartridge method, possibly because Bohbot and Dickens used a lower concentration of 1-octen-3-ol ($1x10^{-7}$ g) at which the inhibition may

have been visible. When working with ORs expressed in *Xenopus* oocytes, DEET and 1-octen-3-ol were found to have no chemical interactions in their dissolved state, so this would not have caused the observed inhibition of 1-octen-3-ol (Bohbot and Dickens, 2010). It seems that there is both an inhibition of responses to 1-octen-3-ol in AaOR8 by DEET at low concentrations (Bohbot and Dickens, 2010), and also an effect on single sensillum and EAG responses caused by the delivery method (Syed and Leal, 2008). It is unclear which is responsible for the reduction in response in *An. gambiae* (Ditzen *et al.*, 2008).

3.5 Conclusion

Although there was some variation in the early generations of selected *Ae. aegypti*, the EAG recordings from the F_9 generation showed that behaviourally insensitive female mosquitoes from the i line had a significantly reduced EAG response to DEET in experiments A and B. This indicates that the observed behavioural insensitivity results from a change in the mosquitoes' ability to detect the compound at the peripheral olfactory level.

The hypothesis that DEET could act as a repellent by affecting the 1-octen-3-ol receptors suggested by Ditzen *et al*. (2008) in *An. gambiae* is shown in this study with *Ae. aegypti* to possibly be an artefact of the method used to deliver the odorants, supporting the conclusions of Syed and Leal (2008) in *Cx. quinquefasciatus*. It also supports the view that mosquitoes have receptors which respond directly to DEET (Davis and Rebert, 1972; Boeckh *et al.*, 1996; Syed and Leal, 2008; Bohbot and Dickens, 2010). If DEET both directly activates a receptor, and also causes inhibition of the 1-octen-3-ol receptor (Bohbot and Dickens, 2010), then it is unknown which of these causes the repellent effect, although since DEET still functions as a repellent even when 1-octen-3-ol is not present (Rutledge *et al.*, 1976; Klun *et al.*, 2005) it seems likely to be the former.

Chapter 4. Single Sensillum Recordings from DEET-Sensitive and DEET-Insensitive Individuals

4.1 Introduction

Single sensillum recordings (SSR) can be used to measure responses from individual olfactory receptor neurons (ORNs). Volatile odours are detected by the ORNs in the sensilla located on an insect's antennae and maxilliary palps. Odour molecules move through cuticular pores into the sensillum, and are transported by odorant binding proteins (OBPs) to odorant receptors (ORs) located on the membranes of the ORNs. OBPs recognise a broad range of odour molecules, but bind some with higher affinity due to the individual structure of the ligand pocket (Pelosi *et al.*, 2006), which may determine odour responses (Biessmann *et al.*, 2010). In *Drosophila*, and possibly many other insect species, antennal ORNs generally express only one functional OR (Hallem *et al.*, 2004; Fishilevich and Vosshall, 2005; Goldman *et al.*, 2005). As odours stimulate the ORs, a signal is transmitted downstream to produce a behavioural response.

Single sensillum recordings utilise sharp tungsten microelectrodes to penetrate the sensillum and establish contact with the ORNs. Electrical activity from the neurons is seen as spikes in the recording, correlating with the action potentials generated in the ORNs (Boeckh, 1962, as reviewed by Mustaparta, 1984). Single sensillum recordings in a range of species including mosquitoes, moths, flies and the honey bee, *Apis melifera*, have shown that different ORNs respond in an excitatory or inhibitory fashion to different compounds with different response levels (Clyne *et al.*, 1997; Meijerink and van Loon, 1999; van den Broek and den Otter, 1999; Meijerink *et al.*, 2001; Laurent *et al.*, 2002; Stensmyr *et al.*, 2003; Baker *et al.*, 2004).

There are five morphological types of sensilla. *Sensilla chaetica*, *sensilla ampullacea* and *sensilla coeloconica* have been shown to contain mechanoreceptor, thermoreceptor or hygroreceptor cells,

and *sensilla trichodea* and grooved peg sensilla, which constitute 90% of antennal sensilla, have been shown to respond to behaviourally active compounds (Davis, 1976; Davis and Sokolove, 1976). There are 2-3 ORNs grouped within a trichoid or grooved peg sensillum. The ORNs project extensions, axons, to carry electrical impulses to glomeruli, which are the first site for synaptic processing, in the antennal lobe. The ORNs in a sensillum do not necessarily respond to similar compounds, but similar receptors tend to map to glomeruli which are physically close to each other in the antennal lobe (Vosshall *et al.*, 2000; Couto *et al.*, 2005).

For each morphological type of sensillum, there are several functional subtypes, responding to different sets of compounds, which cannot be distinguished visually. The morphological and functional subtypes of sensilla in mosquitoes have been characterised with single sensillum recordings in *Anopheles gambiae* (Qiu *et al.*, 2006), *Aedes aegypti* (Ghaninia *et al.*, 2007) and *Culex quinquefasciatus* (Hill *et al.*, 2009). In *Ae. aegypti* a second characterisation revealed an alternative, different number of functional subtypes with different response panels, within some morphological types of trichoid sensilla (Siju *et al.*, 2010). The technique has also been used to compare the responses of two groups, or strains, of insects to a set of compounds in mosquitoes and moths (van den Broek and den Otter, 1999; Baker *et al.*, 2004; Karpati *et al.*, 2008; Siju *et al.*, 2010).

Single sensillum recordings have identified an ORN that responds directly to DEET in *Cx. quinquefasciatus* (Syed and Leal, 2008) and *Ae. aegypti* (Davis and Rebert, 1972; Boeckh *et al.*, 1996), and shown that AaOR2 responds to DEET in *Ae. aegypti* (Bohbot and Dickens, 2010), indicating that these mosquito species are actively detecting DEET. In *Ae. aegypti* the A neuron in the short blunt sensilla was found to respond to DEET (Boeckh *et al.*, 1996), however, this study did not distinguish between the morphological types of short blunt type I and II sensilla, and the recordings from these sensilla were analysed together rather than divided into functional groups. Thus, the responses of different neurons would have been combined, making it difficult to detect if, and to what degree, each neuron was responding. In earlier work the short blunt sensilla were categorised, by length, into three morphological types suggested to correspond with different functional types, only two of which

responded to DEET (Davis and Rebert, 1972). In *Cx. quinquefasciatus* a DEET-sensitive A ORN was located in a short sharp trichoid sensillum and also responded to plant-derived chemicals thujone, 1,8-cineole and linalool (Syed and Leal, 2008), however, no attempt was made to differentiate between the functional subtypes which have since been identified in this species (Hill *et al.*, 2009).

Mosquitoes selected from the s and i lines, identified as behaviourally sensitive or insensitive to DEET in the present study (Section 2.2.3), had shown different antennal responses to DEET by electroantennography (Section 3.3.3). The aim of this chapter was to compare the responses of sensilla that responded to DEET in the s and i lines. In light of the inconclusive identifications of DEET-sensitive sensilla in the literature, all *Ae. aegypti* sensilla were screened to identify the specific morphological and functional subtypes that responded to DEET. The hypothesis was that the s and i lines would respond differently to DEET in a DEET-sensitive sensillum.

4.2 Methods

4.2.1 Insects

SSRs were done on the antennae of 7-15 day old female *Ae. aegypti* (reared as described in 2.2.2) from the s and i lines, which had been selected for DEET-sensitivity or insensitivity respectively, in behavioural assays (Section 2.2.3).

4.2.2 Preparation

A female mosquito was chilled for 1 min at -5°C, then placed on double-sided tape on a microscope slide (76x26 mm). A strip of double-sided tape was used to secure the abdomen and thorax to the slide. A cover slip (18x18 mm) with double sided tape was then placed in a raised position on the slide and manipulated so that the antennae rested on top (Fig 4.1), allowing insertion of the electrode without movement, and reducing vibration. The long hydroscopic sensilla were lightly raked off to reduce interference with the electrode. An Olympus light microscope (BX51W1) was used to view sensilla on the antennae at 750x magnification.

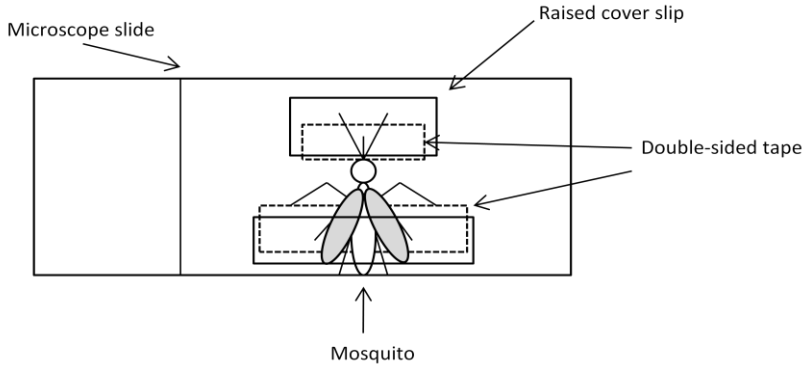


Fig 4.1 Diagram showing a mosquito preparation for single sensillum recordings, with antennae placed upon a raised cover slip.

4.2.3 **Single sensillum recordings**

Single sensillum recordings were performed according to standard protocols described by Stensmyr *et al.* (2003). Two tungsten electrodes were electrolytically sharpened by immersing the tip repeatedly into a 10% KNO_2 solution (2-10V) until the tip was approximately 1µm in diameter. One of the electrodes was inserted into the eye of the mosquito, close to the recording electrode (to reduce background noise), and the other into the shaft of the sensillum, until contact with the ORNs was achieved. Good contact with the ORNs was characterised by clear and distinct spikes from both the A and B neuron, with minimal background interference.

All recordings were carried out on the F_6 generation of experiment A (experiment B was not tested due to time restrictions).

4.2.4 **Stimulus**

The stimulus cartridge was prepared by pipetting each compound (in redistilled hexane or paraffin oil, 10 µl) onto filter paper (Whatman®, 5x20 mm), allowing 30 s for the solvent to evaporate and then placing the filter paper into a Pasteur pipette (230 mm, volac®). The main airflow (0.5 m sec^{-1}) (charcoal filtered and humidified) was passed over the head and antennae from a glass tube positioned at a distance of 0.5 cm. A stimulus controller (Syntech, Germany), was used to deliver a 0.5 s puff from the pasteur pipette into a hole in the glass tube of the main continuous airflow 10 cm away from the preparation.

The compounds tested are described in Table 4.1. All compounds were dissolved in redistilled hexane, except indole which was dissolved in paraffin oil. Controls were hexane (10 µl) and paraffin oil (10 µl).

Table 4.1. Compounds used in electrophysiological recordings.

Stimulus compound	**Purity (%)**	**Concentration (g/10 µl)**	**CAS***	**Supplier**
α Thujone	99	$1x10^{-3}$	546-80-5	Fluka
αβ Thujone (technical)	70 α thujone basis 10 β thujone basis	$1x10^{-3}$	76231-76-0	Aldrich
(±)-Linalool	95 (70.2% R, 29.8% S)	$1x10^{-3}$	78-70-6	Fluka
(-)-Linalool	98.5	$1x10^{-3}$	126-91-0	Fluka
1,8-Cineole	99	$1x10^{-3}$	470-82-6	Fluka
Racemic 1-octen-3-ol	98	$1x10^{-3}$	3391-86-4	Alfa Aesar
Acetic acid	99	$1x10^{-3}$	64-19-7	Fluka
N,N-diethyl-*m*-toluamide (DEET)	97	$1x10^{-7}$-10^{-3}	134-62-3	Aldrich
Indole	99	$1x10^{-4}$	120-72-9	Aldrich

*CAS numbers are unique numerical identifiers for compounds

4.2.5 Screening antennae

The ORNs in all morphological types of trichoid sensilla (Fig 4.2) were screened for a response to DEET. Once a neuron responding to DEET was located in a short blunt type II sensillum (sbtII), further recordings were focused on this morphological type.

Recordings were made from 23 s sbtII sensilla and 11 i sbtII sensilla with a panel of 9 compounds (Table 4.1) to determine the functional class of each sensillum and its response to DEET. A DEET dose–response from $1x10^{-7}$ g to $1x10^{-3}$ g was done to ascertain sensitivity.

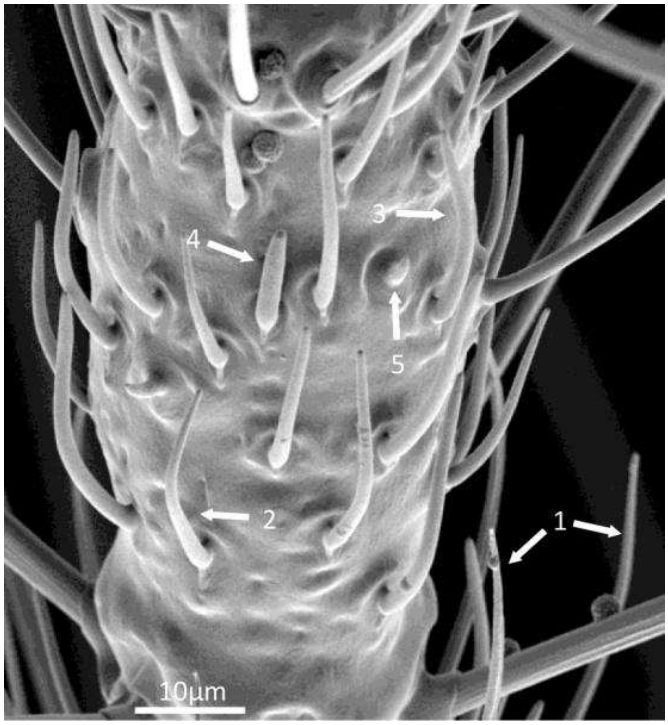


Fig 4.2 Scanning Electron Microscopy picture (courtesy of Jean Devonshire, Rothamsted Research) (Stanczyk *et al.*, 2010) of *Aedes aegypti* sensilla morphological types: (1) long sharp sensilla; (2) short sharp sensilla; (3) short blunt type I sensilla; (4) short blunt type II sensilla; (5) grooved peg sensilla.

4.2.6 **Analysis**

Two spontaneously active ORNs were present in each *Ae. aegypti* trichoid sensillum, designated A and B (Ghaninia *et al.*, 2007), where A was the ORN with spikes of higher amplitude and B was the ORN with spikes of lower amplitude. Spikes were counted by hand in order to differentiate between A and B by shape and amplitude (Fig 4.3).

ORN response to an odour was measured as the difference between the number of spikes 0.5 s before and 0.5 s after the stimulus was applied (by using software: AutospikeTM; Syntech) and presented as spikes/s. ORNs were characterized as non-responding if the response failed to exceed 15 spikes/s. Responses were classified as inhibitory when the response was diminished by 10 spikes/s or more. Recordings were not included in further analyses if there was a response of > 15 spikes/s to the control.

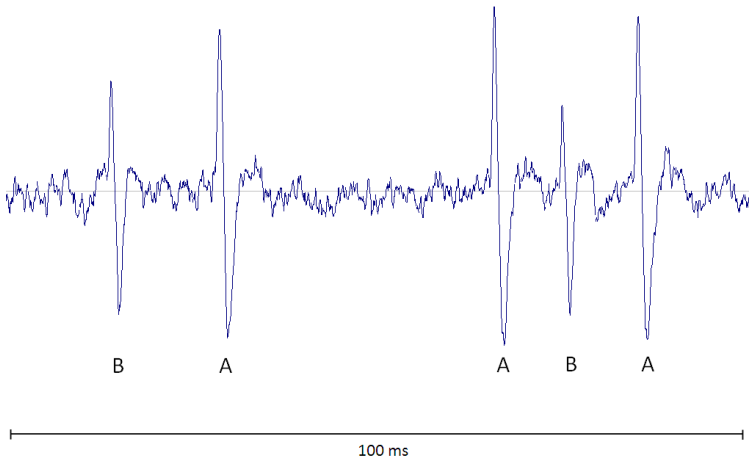


Fig 4.3 Microelectrode recordings from sbtII 4 sensilla in response to hexane control, showing the A and B neurons firing.

4.2.7 **Determining behavioural response to Linalool**

Mosquitoes from the F_{10} generations of the s and i lines were tested in a repellency bioassay (see Section 2.2.3 for methods) to determine the repellent effect of linalool. Linalool was tested at a concentration which had been found previously to be repellent to mosquitoes when applied to the skin (Park *et al.*, 2005). As multiple chemicals were being tested they could not be applied directly to the arm, to avoid cross contamination. Each chemical in redistilled hexane (0.5 ml) was spotted evenly over a section of tights (Boots brand 97% nylon, 3% LYCRA®, small/medium, nude, Denier 10, 12cm long when unstretched) (Appendix 4) and allowed to dry for 2 min. A layer of untreated material was placed over the arm and then the treated layer placed on top. After testing each cage of mosquitoes, both layers of tights were removed before the next treatment. In each block of 8 cages tested, cages of s or i females were tested with the following treatments : 1) tights on control arm, 2) (-)-linalool (2%) applied to tights on arm, 3) (±)-linalool (2%) applied to tights on arm, 4) DEET (20%) applied to tights on arm. Mosquitoes attempting to probe were considered insensitive to the repellent being tested, and mosquitoes not attempting to probe were considered sensitive.

4.2.8 **Statistics**

Cluster analysis

Based on the response spectra of all ORNs in both the s and i lines, a hierarchical cluster analysis was used to classify the sensilla into functional types. Forty six s ORNs and twenty two i ORNs housed in the sbtII morphological subtype were grouped using Genstat® (12th edition) and the group average method according to their responses to a set of 8 compounds (Table 4.1). Responses to DEET were excluded from the analysis, as it was hypothesized these would differ between lines.

Difference between s and i lines

In each of the 5 functional groups of sbtII sensilla the s and i line were examined for differences in their responses to compounds including DEET. The first functional group was excluded from this analysis as none of the sensilla from the i line fell in this group. The other four functional groups were analysed with a two-way ANOVA (Genstat® 12th edition) for line and treatment, using replicates as blocks, transforming the data with log+25 in order to adjust for negative values. Differences were judged significant when the difference between means was greater than the least significant difference (LSD).

Linalool behavioural experiment

The mean responses to treatments between the s and i lines, and between treatments within lines were compared using a two-way ANOVA in Genstat® (12th edition), using replicates as blocks. Differences were judged significant when the difference between means was greater than the LSD.

4.3 Results

4.3.1 Functional subtypes of SbtII

Recordings from short blunt type II sensilla were sorted by cluster analysis (Fig 4.4) according to their responses to a panel of compounds (Table 4.1) excluding DEET. The response profiles of sensilla clustered together were compared to ensure closely grouped sensilla had similar spike counts in the A and B ORNs to indole, α thujone and acetic acid, which are key compounds in distinguishing sbtII functional types (Ghaninia *et al.*, 2007; Siju *et al.*, 2010). Where sensilla did not have similar responses to these compounds compared with the sensilla they were most closely grouped with, they were compared with the next closest cluster until a match was found. This resulted in the identification of five functional groups of sensilla, which were assigned as subtypes sbtII 1-5 (Fig 4.4) based on comparisons with work done previously on *Ae. aegypti* sensilla (Ghaninia *et al.*, 2007; Siju *et al.*, 2010).

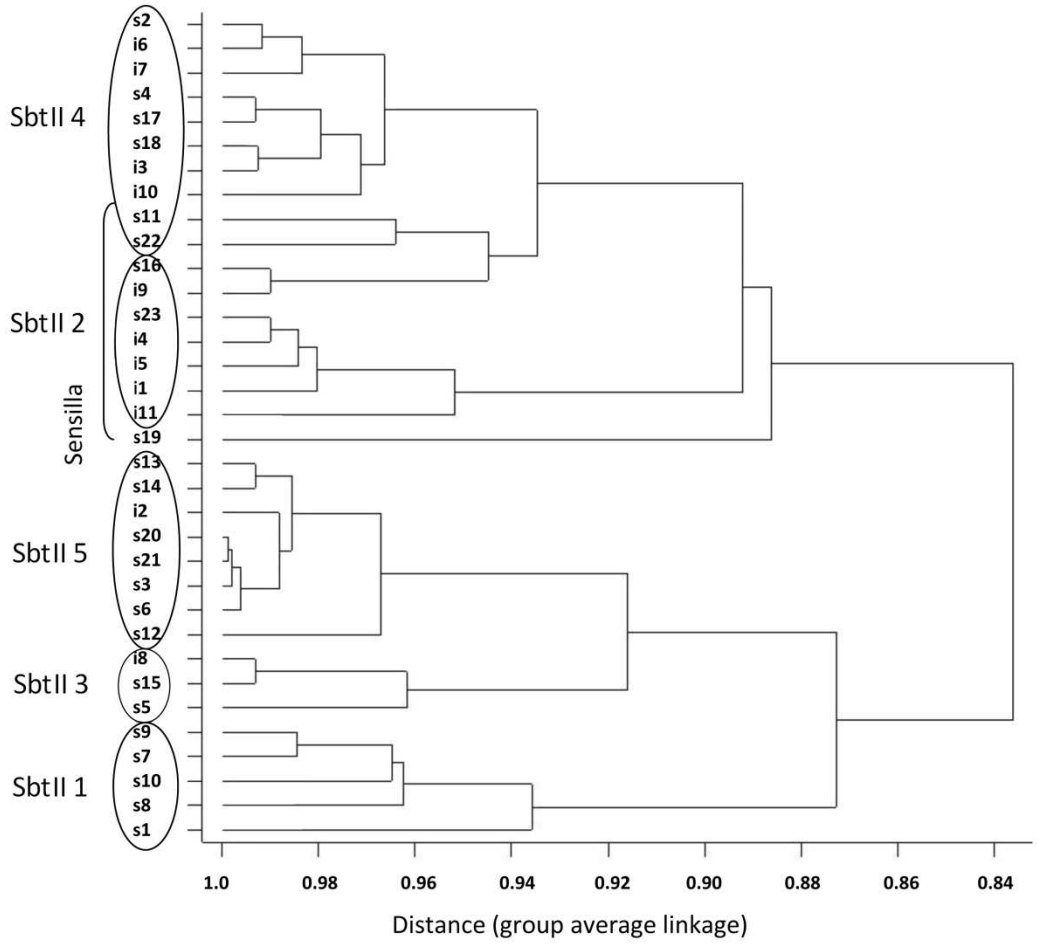


Fig 4.4 Dendrogram displaying 33 *Aedes aegypti* female sensilla grouped according to their responses to 8 compounds. Sensilla were sorted into 5 groups and labelled as functional types sbtII 1-5. Sensilla are labelled according to whether they are s or i line, and identifying number.

4.3.2 **SbtII responses in *Ae. aegypti***

The five functional subtypes of sbtII had distinct response spectra to the compounds tested (Table 4.2, Fig 4.5) for the s and i lines combined.

Table 4.2. Compounds found to elicit responses in the *Aedes aegypti* females from the s and i lines in short blunt type II sensilla functional subtypes 1-5, olfactory receptor neurons A and B. + = increase of 15-50 spikes/s, ++ 50-100 spikes/s, +++ >100 spikes/s

	SbtII 1		**SbtII 2**		**SbtII 3**		**SbtII 4**		**SbtII 5**	
ORN	**Compound**	**Response**	**Compound**	**Response**	**Compound**	**Response**	**Compound**	**Response**	**Compound**	Response
A	α Thujone	+	α Thujone	+	α Thujone	+	α Thujone	+	α Thujone	+
	αβ Thujone	+	αβ Thujone	+	αβ Thujone	++	αβ Thujone	+	αβ Thujone	+
	1-Octen-3-ol	+++	1,8-Cineole	+	1,8-Cineole	++	1,8-Cineole	+		
	(-)-Linalool	+	Indole	++	(±)-Linalool	+	(±)-Linalool	+		
	(±)-Linalool	++					Indole	+		
							Acetic acid	+		
B	1-Octen-3-ol	+	Indole	++	1,8-Cineole	+	Indole	+++		

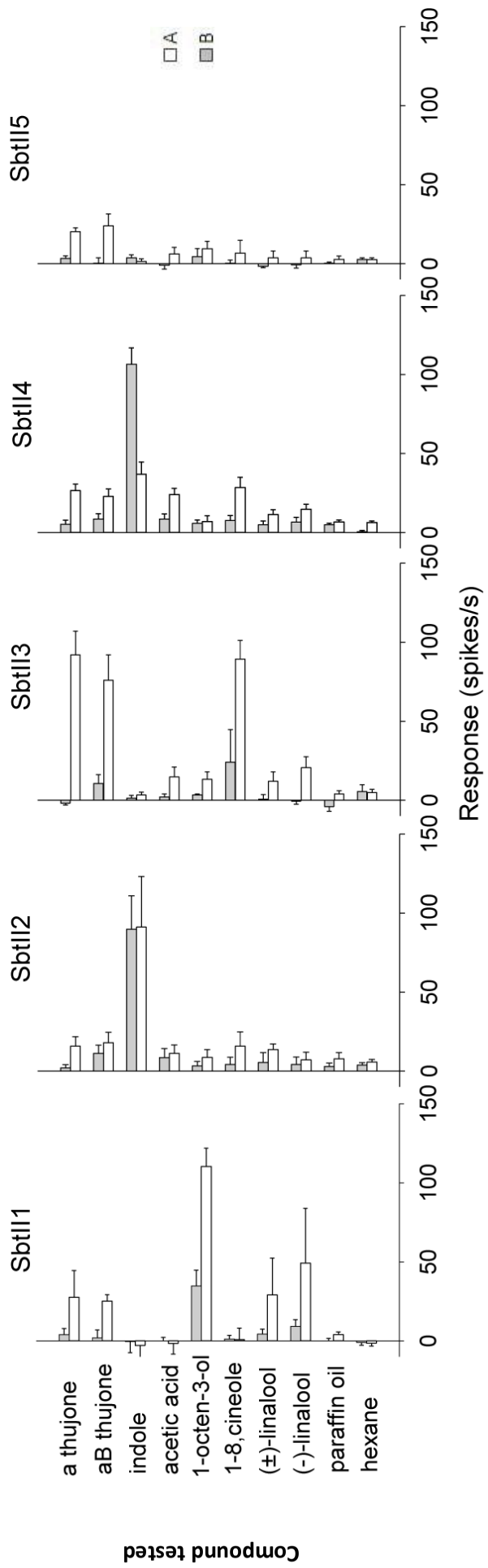


Fig 4.5 Response spectra of olfactory receptor neurons housed in short blunt type II *sensilla trichoda* of the combined *Aedes aegypti* s and i lines (Stanczyk *et al.*, 2010). The neuronal responses of the two neurons, A and B, housed in five functional classes are shown as an average over N replicates. SbtII 1, N = 5 s, 0 i. SbtII 2, N = 2 s, 5 i. SbtII 3, N = 2 s, 1 i. SbtII 4, N = 7 s, 4 i. SbtII 5, N = 7 s, 1 i. Means ± SE.

Within each functional class the s and i lines were examined for their response to the compounds tested, including DEET, and the responses of the two lines were compared. This was not possible for sbtII1, as no i line sensilla were found in this group. However, the s line in sbtII1 did not respond to DEET. For functional subtypes 2, 3, and 5, the s and i lines showed no response to DEET, and there were no differences in response between the lines to any of the compounds tested (results not shown).

In the functional type sbtII 4, the A neuron in the s line responded to DEET in a dose-dependent fashion (Fig 4.6), showing an excitatory response to DEET at $1x10^{-4}$ g ($p=0.04$) and $1x10^{-3}$ g ($p=0.048$). There was a difference in spikes/s between the response to DEET at these two concentrations in the s and i lines, with the i line having a significantly lower response ($p=0.007$ and $p=0.02$, respectively) which was not significantly different from the control ($p=0.32$ and $p=0.24$, respectively (Figs 4.6, 4.7). There was also a significant difference ($p=0.02$) in the response of this neuron between the two lines to (±)-linalool, which elicited a significant response in the s line ($p=0.022$), but not in the i line response, which showed no significant difference from the control ($p=0.28$) (Fig 4.8).

There was no difference between the s and i lines in the response of sbtII 4 to any other compounds tested. SbtII 3 and 4 responded to (±)-linalool but not (-)-linalool, while sbtII 1 responded to both (Fig 4.4). There were no differences in responses in any functional subtype of sensilla between the stereoisomers α thujone and αβ thujone.

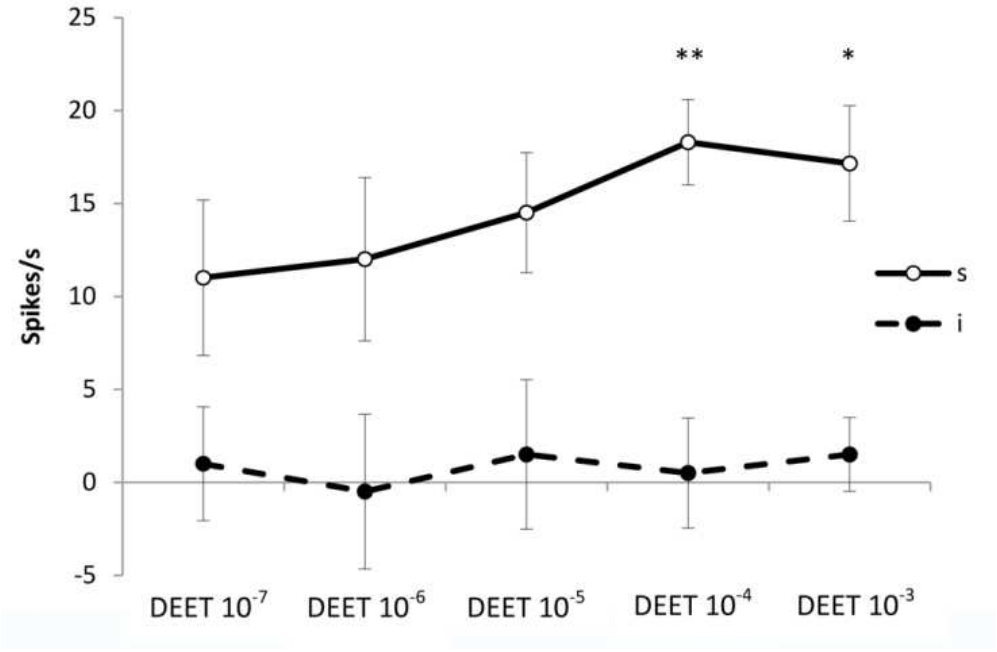


Fig 4.6 Responses of sbtII type 4 in behaviourally selected *Aedes aegypti* females from the s and i lines (Stanczyk *et al.*, 2010). Dose–response curve of sbtII 4 to DEET in the s and i lines (N=7 s, 4 i). Here only the responses of the A neuron are shown, as the B neuron showed no difference in response to the control. The s line showed an excitatory response to DEET in comparison to the control at 1×10^{-4} g and 1×10^{-3}g ($p < 0.05$). Means are ± SEM. * indicates a significant difference in response by the s and i lines ($p < 0.05$), ** ($p < 0.01$).

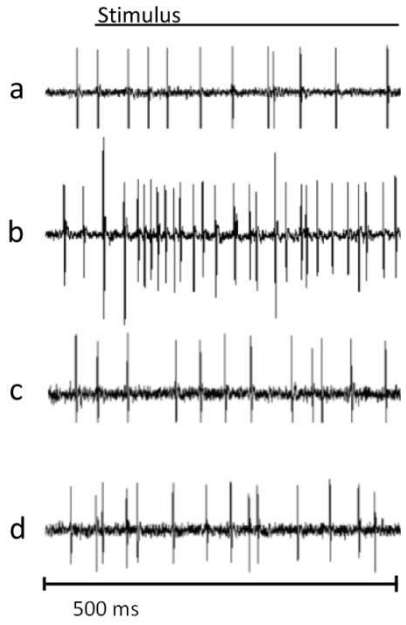


Fig 4.7 Responses of sbtII type 4 in behaviourally selected *Aedes aegypti* females from the s and i lines (Stanczyk *et al.*, 2010). Microelectrode recordings from sbtII 4 sensilla. (a) s line control. (b) s line tested with 1×10^{-3} g DEET. (c) i line control. (d) i line tested with 1×10^{-3} g DEET.

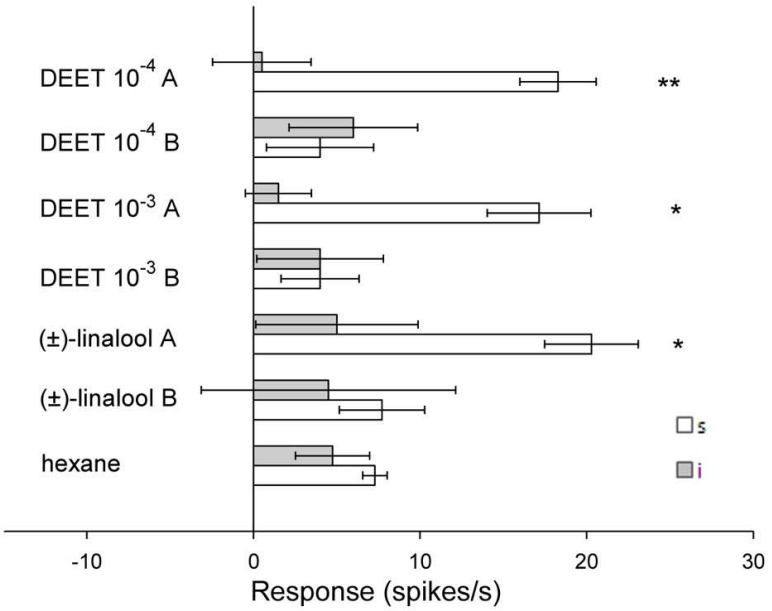


Fig 4.8 Responses of sbtII type 4 in behaviourally selected *Aedes aegypti* females from the s and i lines (Stanczyk *et al.*, 2010). Neuronal responses to DEET and (±)-linalool (g/10 µl) by the A and B neurons of sbtII 4 sensilla in mosquitoes from the s and i lines. N = 7 s, 4 i. Means are ± SEM. * indicates a significant difference in response by the s and i lines ($p < 0.05$), ** ($p < 0.01$).

4.3.3 **Linalool behavioural experiment**

There was no difference in the proportion of mosquitoes probing in response to the control arm or (-)-linalool between the s and i lines. Both lines had significantly less mosquitoes probing when tested with (-)-linalool, (±)-linalool and DEET compared to the control arm ($p<0.001$) (Fig 4.9). Mosquitoes in the i line were significantly less sensitive to (±)-linalool and DEET than mosquitoes in the s line. Females in the i line showed no difference in the level of insensitivity to (±)-linalool and DEET. In the s line, 20% DEET was a more effective repellent than 2% (-)-linalool or (±)-linalool, with significantly fewer females probing ($p<0.01$).

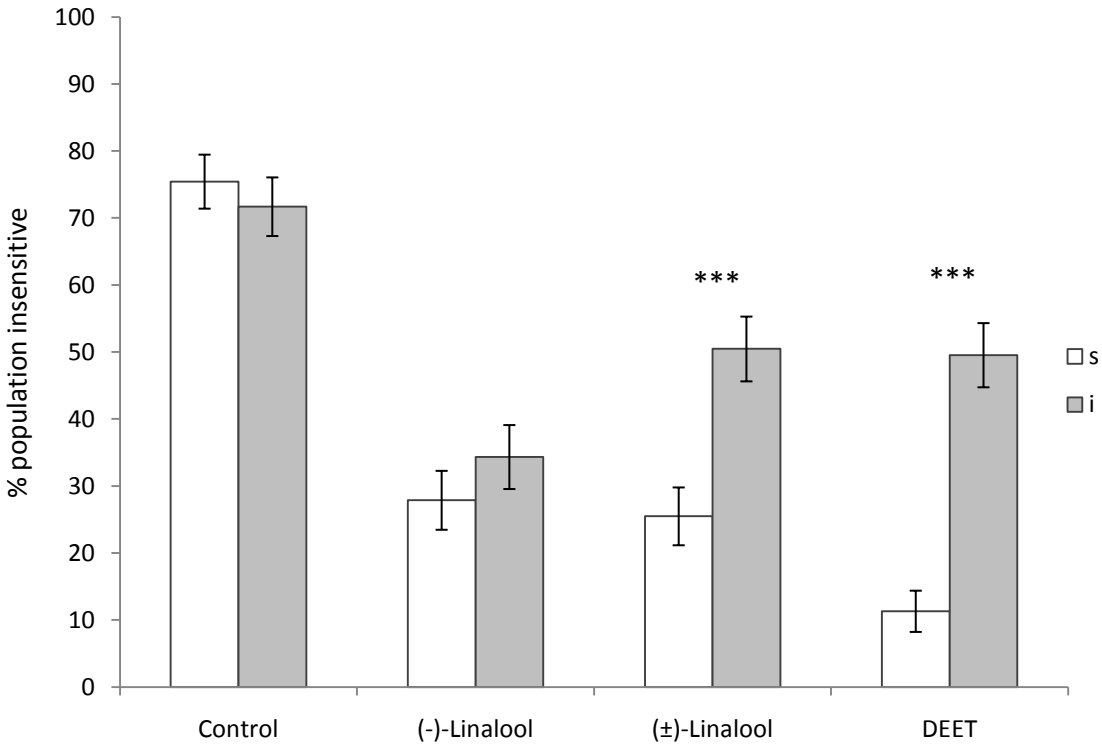


Fig 4.9 Percentage of female *Aedes aegypti* mosquitoes from the s and i lines probing in response to a control arm, an arm with (-)-linalool (2%) applied, an arm with (±)-linalool (2%) applied, and an arm with DEET (20%) applied. Means are ± SEM. *** indicates a significant difference between the s and i lines ($p < 0.001$)

4.4 Discussion

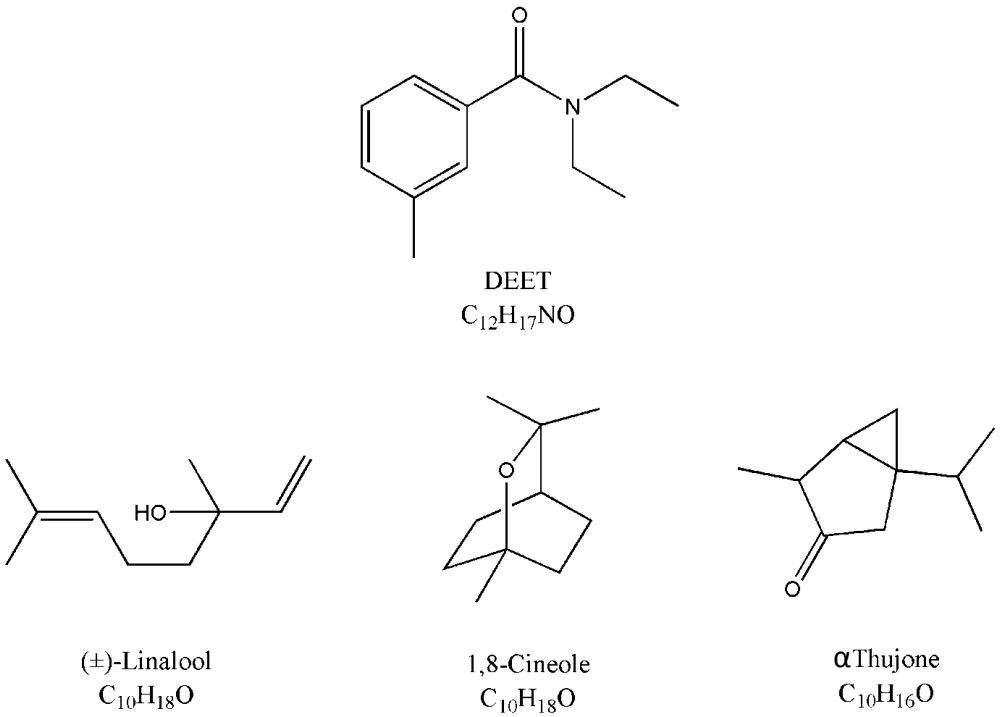
Single sensillum recordings have been used in previous studies to detect and ascertain the sensitivity of ORN responses to a variety of compounds (Meijerink and van Loon, 1999; Stensmyr *et al.*, 2003), and to compare responses between strains of insects (Baker *et al.*, 2004; Karpati *et al.*, 2008). The responses of the peripheral olfactory system have been characterised through SSR in *Ae. aegypti* (Ghaninia *et al.*, 2007; Siju *et al.*, 2010), *An. gambiae* (Qiu *et al.*, 2006), and *Cx. quinquefasciatus* (Hill *et al.*, 2009), allowing for comparisons of specific sensilla and ORNs to be made between species. In our study all trichoid sensilla were screened to search for an olfactory receptor neuron (ORN) sensitive to DEET (Fig 4.2). ORNs sensitive to DEET have been described previously in *Cx. quinquefasciatus* (Syed and Leal, 2008) and *Ae. aegypti* (Davis and Rebert, 1972; Boeckh *et al.*, 1996), with *Ae. aegypti* AaOR2 responding directly to DEET (Bohbot and Dickens, 2010). In *Cx. quinquefasciatus* the ORN sensitive to DEET was the A neuron located in a short sharp sensillum (Syed and Leal, 2008), but the three functional types of this sensillum (Hill *et al.*, 2009) were not distinguished. In *Ae. aegypti,* Boeckh *et al.* (1996) located a DEET-sensitive ORN in a short blunt sensillum, but did not differentiate between the morphological types sbtI and sbtII, nor the different functional types (3 and 5 respectively) (Siju *et al.*, 2010). A previous study separated short blunt sensilla into three morphological categories according to length, S, M and L, and suggested this indicated functional types, of which only S and L were sensitive to DEET (Davis and Rebert, 1972). In our study with *Ae. aegypti*, a DEET-sensitive A neuron was located in the sbtII sensilla. As neither of the previous studies on *Ae. aegypti* fully differentiated between the two morphological types of short blunt sensilla, it is unclear whether we have identified the same sensillum. The short blunt S sensilla identified by Davis and Rebert (1972) are likely to be the same morphological type as sensilla here identified as sbtII, but the responses of the A and B neurons were not detected separately so it is unclear which were responding. In the Boeckh *et al*. (1996) study both the A and B neurons responded to DEET, whereas in our study only the A neuron responded, thus, we cannot rule out the possibility that we have identified a different DEET-sensitive sensillum. The sbtII sensillum recorded from in our study responded to DEET with a lower spikes/s increase than that found by Syed and Leal in *Cx. quinquefasciatus* (Syed and Leal, 2008) but at a similar level to those found in *Ae. aegypti* (Davis

and Rebert, 1972; Boeckh *et al.*, 1996). There are likely to be species differences, but it is also possible that the DEET-sensitive sbtII sensillum identified in *Ae. aegypti* is not the analogue of the DEET-sensitive sensillum in *Cx. quinquefasciatus.*

In this study *Ae. aegypti* sbtII sensilla were screened with a panel of compounds (Table 4.1) chosen for their known effect on mosquito behaviour or ability to elicit an electrophysiological response. Linalool and 1,8-cineole are repellent to some mosquito species (Park *et al.*, 2005; Traboulsi *et al.*, 2005; Muller *et al.*, 2009), and thujone has been shown to be attractive to *Culex pipiens* (Bowen, 1992). All of these plant-derived compounds affect the DEET-sensitive ORN in *Cx. quinquefasciatus* (Syed and Leal, 2008). 1-Octen-3-ol, which is an attractant for several species of mosquitoes in combination with CO_2 (Takken and Kline, 1989; Takken and Knols, 1999, Cook J.I, Pers comm), was suggested to be important in the mechanism of DEET repellency in *An. gambiae* (Ditzen *et al.*, 2008), and in *Ae. aegypti* (Bohbot and Dickens, 2010). Indole and acetic acid, found in human sweat (Cork and Park, 1996; Meijerink *et al.*, 2000), were tested because *Ae. aegypti* trichoid sensilla have been shown to respond to these compounds previously (Davis, 1976; Ghaninia *et al.*, 2007), allowing for the differentiation between functional subtypes.

The *Ae. aegypti* peripheral olfactory system has been characterised previously, with four (Ghaninia *et al.*, 2007) or five (Siju *et al.*, 2010) sbtII functional subtypes identified. In the present study, the sbtII sensilla of the *Ae. aegypti* s and i lines were sorted into five functional types according to their responses to compounds excluding DEET, and these functional groups were categorized as subtypes sbtII 1–5 (Fig 4.4). This is consistent with the more recent characterisation of sbtII subtypes (Siju *et al.*, 2010), but the response profiles were more similar to the functional types identified in previous work (Ghaninia *et al*., 2007) (Fig 4.5), so the latter was used as the primary source for designating the subtypes sbtII 1-5.

The DEET-sensitive A neuron identified by Syed and Leal (2008) in *Cx. quinquefasciatus* also responded in a dose-dependent manner to the plant derived compounds thujone, 1,8-cineole and linalool, and the B neuron in the same sensillum responded to 1-octen-3-ol. In the *Ae. aegypti* sbtII 4 sensillum, found to respond to DEET in our study, the DEET-sensitive A neuron also responded to thujone, 1,8-cineole and (±)-linalool but not to (-)-linalool. The B neuron of this sensillum type did not respond to 1-octen-3-ol. As the DEET-sensitive A neuron responded to these plant-derived compounds in both of these mosquito species, it could be that DEET is binding with the receptor for these compounds (Fig 4.10). DEET was found to inhibit feeding in *D. melanogaster* by stimulating the gustatory receptor neurons for other, aversive compounds (Lee *et al.*, 2010). By binding with the ORN that normally responds to plant-derived repellents (Park *et al.*, 2005; Traboulsi *et al.*, 2005; Muller *et al.*, 2009), DEET could be inducing a similar effect.



DEET
$C_{12}H_{17}NO$

(±)-Linalool
$C_{10}H_{18}O$

1,8-Cineole
$C_{10}H_{18}O$

αThujone
$C_{10}H_{16}O$

Fig 4.10 Chemical structures and molecular formulae of DEET, (±)-linalool, 1,8-cineole and α thujone.

The sbtII 4 sensilla of the female *Ae. aegypti* in the s and i lines responded in the same way to all of the odours tested except for DEET and (±)-linalool (Figs 4.4-4.6). For both compounds the A neuron in the i line did not respond significantly to DEET or (±)-linalool, but did in the s line. It is, therefore, likely

that the observed reduction in response in the sbtII 4 sensilla is responsible for the difference in behavioural response to DEET seen in the insensitive female mosquitoes. With regard to (±)-linalool, the A neuron in the i line responded significantly less than that of the s line. If, as suggested by Syed and Leal (2008), DEET is being recognized by a neuron that naturally responds to plant compounds (Fig 4.10), it is possible that the alteration in the i line that leads to lowered recognition of DEET is also affecting the response to the plant-derived compound (±)-linalool. Linalool enantiomers are present in the essential oils of different plants and have distinct scents that insects can differentiate between (Bichao *et al.*, 2005) or that can be detected at different thresholds (Ulland *et al.*, 2006). The ability to detect the enantiomers separately may serve a purpose in the ecology of the insect. In our study, the trait that we selected was likely to be relevant only to (+)-linalool because the difference between the selected lines occurred only in response to (±)-linalool and not (−)-linalool (with (−)-linalool eliciting no response in either line). A behavioural study of the s and i line response to (±)-linalool and (−)-linalool showed significantly more individuals in the i line than the s line were insensitive to (±)-linalool, and no difference in response between the lines to (−)-linalool (Fig 4.9). It is, therefore, possible that the behavioural and electrophysiological insensitivity to DEET and (±)-linalool results from a mutation affecting the way in which both of these compounds are detected.

This alteration in the responsiveness of ORNs after selection has also been observed in other insects, with a behavioural change in response to the sex pheromone in the male Cabbage looper moth, *Trichoplusia ni*, resulting from a reduction in response in the ORNs, likely adapting to cope with the higher levels of the pheromone present in captivity (Domingue *et al.*, 2009). This has also been observed in other rare phenotypes of this moth (Domingue *et al.*, 2007a; 2007b). The authors of these moth studies theorise that changes in the conformation of ORs, or in the number of ORs expressed, could cause the alterations in responsiveness to a given compound. Similarly, therefore, an alteration in the ORs of the i line in our study could be responsible for the difference in responsiveness to DEET. Alternatively, there could be a mutation in the gene encoding an OBP that normally delivers DEET to the receptor, leading to the OBP transporting less DEET to the receptor, and thus a lowered response to the compound. This has been demonstrated in *An. gambiae*, where silencing OBP1 led to mosquitoes no longer responding electrophysiologically to indole (Biessmann *et al.*, 2010). The

hypothesis that the i line mosquitoes have a change in their OBPs rather than the ORs is supported by the fact that the sbtII 4 A ORN, likely only expressing one functional type of OR (Hallem *et al.*, 2004; Fishilevich and Vosshall, 2005; Goldman *et al.*, 2005), in the i line mosquitoes responds normally to all compounds other than DEET and (±)-linalool. It is, therefore, more likely that an OBP which binds with higher affinity (Pelosi *et al.*, 2006) to DEET and (±)-linalool, but not the other compounds, has been altered.

4.5 Conclusion

The SSRs have shown that the behavioural and electroantennography differences between the s and i lines were caused by a difference in the detection of DEET by the sbtII type 4 trichoid sensilla in *Ae. aegypti*. There was a similar difference between the lines in the ability to detect and respond behaviourally to the plant-derived compound (±)-linalool, making it likely that the same mechanism was affected in the i line for both compounds. This may be due to a change in an OR or an OBP.

Chapter 5. **Altered Behavioural Responses after Pre-Exposure to DEET**

5.1 Introduction

Research has shown that some insects, such as *Ae. aegypti* and *D. melanogaster* (Becker, 1970; Rutledge *et al.*, 1994; Reeder *et al.*, 2001) (Chapter 2), are able to ignore the repellent effect of DEET when moving towards an attractant. During preliminary repellency trials for the selection experiment described in Chapter 2 (data not shown), the same mosquitoes were tested multiple times with DEET to ensure all insensitive mosquitoes were identified. In some cases there was a change to DEET-insensitivity in previously sensitive mosquitoes being re-tested with DEET, and investigating this phenomenon was of interest. In previous studies, and in the current work (Becker, 1970; Rutledge *et al.*, 1994; Reeder *et al.*, 2001) (Chapter 2), a genetic cause of DEET-insensitivity was discovered. However, it is possible that the change in behavioural sensitivity to DEET found when re-testing mosquitoes which have been previously exposed, could be due to other, non-genetic, factors. Many studies have focused on non-genetic changes in behaviour in response to compounds, through forms of conditioning or learned behaviour. For example, Pavlovian conditioning has been shown in the mosquito *Culex quinquefasciatus* (Tomberlin *et al.*, 2006), where the mosquitoes were able to associate a novel odour with a sugar or blood meal, and learn to respond to that odour even when the food stimuli were not present. Other host-seeking insects which have shown Pavlovian conditioning are the triatomine, *Rhodnius prolixus* (Abramson *et al.*, 2005; Aldana *et al.*, 2008), and the parasitic wasp, *Microplitis croceipes* (Lewis and Takasu, 1990).

Mosquitoes have been shown to display a preference for returning to a host they have previously successfully fed on for a bloodmeal (Kelly and Thompson, 2000). In the wild, this behaviour has been shown to be learned rather than inherent, with naïve offspring not displaying the host preference (Mwandawiro *et al.*, 2000). Mosquitoes have also been shown to oviposit at sites with the same chemical cues as the site where they hatched (McCall and Eaton, 2001; McCall *et al.*, 2001). They are

not deterred by the repellent citronella at oviposition sites if citronella was present in the site where they emerged (Kaur *et al.*, 2003). Similarly, pre-exposure of *D. melanogaster* to a repellent can change the response when the insect is re-tested, causing the flies to no longer be repelled by the odour (compared to naïve flies) (Stortkuhl *et al.*, 1999; Devaud *et al.*, 2001). Stortkuhl *et al.* (1999) showed that this adaptation does not occur in flies with a mutant transient receptor potential (Trp) calcium channel. Such channels are permeable to cations which pass through and depolarise cell membranes in response to sensory stimuli, leading to synapses sending signals to the brain. Stortkuhl *et al.* suggested that a lack of Trp during the developmental phase led to the flies being unable to adapt to the repellent. Devaud *et al.* (2001) exposed *D. melanogaster* to an extended stimulus and found there was a difference in the volume of glomeruli, which are the first site for synaptic processing of odours, in the antennal lobes. They suggested that the behavioural change was caused by a reduction in the number of selected synapses present, and thus a reduction in the ability to process odours, after exposure to a concentrated stimulus.

The peripheral olfactory system of insects with altered behaviour after pre-exposure to a chemical has been examined using electroantennography (EAG). In one study on *D. melanogaster* (Devaud *et al.*, 2001) and in *Microplitis croceipes* (Park *et al.*, 2001), which had changed their behavioural response after exposure to, or conditioning with, an odour, there was no difference in the EAG response to the odour compared with naïve insects. However, in a different study on *D. melanogaster* (Stortkuhl *et al.*, 1999), and in *Apis mellifera* (Bhagavan and Smith, 1997) and *Protophormia terraenovae* (Barbarossa *et al.*, 2007), insects showed a decreased EAG response to an odour after pre-exposure. It is not known if pre-exposure to DEET affects the behavioural or electrophysiological responses of mosquitoes, but it was important to investigate this, both for the scope of the project and because the repellency bioassay recommended by WHOPES repeatedly tests insects (WHOPES, 2009).

The aim of this chapter was to determine if multiple exposures to DEET would cause behavioural and EAG changes in female *Ae. aegypti* mosquitoes. Repellency bioassays were used to ascertain behavioural DEET-sensitivity, and EAG experiments were conducted to determine the responses of the peripheral olfactory system. The hypothesis was that pre-exposing mosquitoes to DEET would alter their behaviour or EAG responses to subsequent exposure.

5.2 Methods

5.2.1 Insects

The mosquitoes used were female *Ae. aegypti* reared as described in Section 2.2.2 and which had been shown to be responding to human odours (Appendix 2).

5.2.2 Repeat exposure to DEET experiments

5.2.2.1 *Experiment 1: Pre-exposure using DEET; behavioural selection for EAG*

Female *Ae. aegypti* were tested using the arm-on-cage repellency assay (Section 2.2.3) (Appendix 5) with either 0.5 ml ethanol (control) or 0.5 ml DEET (20%) on an arm. The initial test was carried out at 0h, and then 3h later the same mosquitoes were retested with either a control arm or with DEET on the arm (Cages 2, 3 + 5, Table 5.1). At 3hs, cages of mosquitoes which had not been previously exposed were tested with a control or DEET arm (Cages 1 + 4, Table 5.1). Ten blocks of experiments were carried out, with 10 mosquitoes per cage. Mosquitoes which were insensitive to DEET at 0h in Cage 5 were removed from the cage, so that only previously sensitive mosquitoes were retested with DEET to see if their response had changed. Thus, the number of mosquitoes probing when retested with DEET, after pre-exposure to it, can be expressed as a proportion of the remaining mosquitoes, to get the percentage which have changed their sensitivity (ie. changed from DEET-sensitive to insensitive). At the end of each of the 3h trials, mosquitoes were removed (by mouth aspirator) from the cage to be used in EAG experiments (Table 5.1). Mosquitoes collected were either sensitive or insensitive to DEET, or collected at random. All mosquitoes were tested with EAG within 3h following the behavioural experiment.

Table 5.1. Treatments for experiment 1. Female *Aedes aegypti* were tested at 0h and/or 3h with a control arm (0.5 ml ethanol) (control) or DEET on an arm (0.5 ml, 20%) (DEET). N = 10. Individuals collected for EAG were sensitive (sens) or insensitive (ins) to DEET, or collected at random.

Cage	Treatment at 0h	Treatment at 3h	Tested with EAG
1		control	Random
2	control	control	
3	control	DEET	ins
4		DEET	ins
5	DEET	DEET	sens and ins

5.2.2.2 *Experiment 2: Pre-exposure to DEET alone*

To eliminate any effect of pre-exposure to the heat and volatiles from the human arm as being responsible for the change in mosquito behaviour seen in these experiments, an additional experiment was conducted using the arm-on-cage method (Section 2.2.3). Treatments were the same as in experiment 1, with the removal of experiment 1's Cage 2, and the addition of Cage 5, a cage tested with DEET without a human arm or heat source in the initial exposure (Table 5.2). A section of tights (4 cm unstretched, Boots brand 97% nylon, 3% LYCRA®, small/medium, nude, Denier 10) spotted evenly with 0.5 ml DEET (20% in redistilled hexane) (allowed 2 min to evaporate) was placed over the mesh on the cage at 0h and left for a 2 min exposure of the mosquitoes before being removed. For the duration of this treatment, no volunteer was present in the room, so that no human volatiles or heat source were presented to the cage. The tights were handled with nitrile gloves to prevent contamination with human volatiles. At 3h the cage was tested with DEET on an arm. Ten blocks of experiments were carried out, with 10 mosquitoes in each cage. Mosquitoes which were insensitive to DEET at 0h in Cage 4 were removed from the cage so that only previously sensitive mosquitoes were retested with DEET to see if their response had changed.

Table 5.2. Treatments for experiment 2. Female *Aedes aegypti* were tested at 0h and/or 3h with a control arm (0.5 ml ethanol) (control), DEET on an arm (0.5 ml, 20%) (DEET) or DEET on a section of tights with no arm present (only DEET). N = 10.

Cage	Treatment at 0h	Treatment at 3h
1	control	
2	control	DEET
3		DEET
4	DEET	DEET
5	only DEET	DEET

5.2.2.3 *Experiment 3: Pre-exposure to DEET and an artificial heat source*

To eliminate the presence of human volatiles during either pre-exposure or re-testing with DEET as a cause of altered DEET-sensitivity, an experiment was carried out using heat from a Hemotek® artificial heating system. A section of tights (see 5.2.2.2) covered the heating block reservoir (3.5 cm diameter, stainless steel), and this was used as the attractant in place of the human arm present in experiments 1 and 2. The tights were either a control, treated with redistilled hexane (0.5 ml), or were treated with DEET (0.5 ml, 20% in redistilled hexane), allowed 2 min to evaporate before they were stretched over the Hemotek and held in place with an 'o' ring. The Hemotek reservoir was maintained at 27°C, and positioned 0.5 cm above the mesh of the experimental cage (it was necessary to place the Hemotek® closer than the 1.5 cm distance of the human arm in order for mosquitoes to probe in response to the control). Ten blocks of experiments were carried out, with 10 mosquitoes per cage (Table 5.3). Mosquitoes which were insensitive to DEET at 0h in Cage 5 were removed from the cage so that only previously sensitive mosquitoes were retested with DEET to see if their response had changed.

Table 5.3. Treatments for experiment 3. Female *Aedes aegypti* were tested at 0h and/or 3h with a control section of tights on a heat source (0.5 ml redistilled hexane) (Heat), or DEET on a section of tights on a heat source (0.5 ml, 20% in redistilled hexane) (DEET). N = 10.

Cage	Tested at 0h	Tested/retested at 3h
1	Heat	
2	Heat	Heat
3	Heat	DEET
4		DEET
5	DEET	DEET

5.2.3 EAG recordings from mosquitoes

5.2.3.1 *EAG Preparation and stimulus delivery*

Mosquitoes collected as described in Table 5.1 were tested with EAG as described in Section 3.2.2, except that a single stimulus delivery cartridge was used rather than the split airflow system (Section 3.2.3). Treatments were 1) redistilled hexane (control), 2) methyl salicylate (positive control), and 3) DEET ($1x10^{-3}$ g) (Section 3.3.1). The two control stimuli were applied at the beginning of each test to determine if the mosquito was responding, and again after DEET had been tested.

5.2.4 Statistics

For the behavioural experiments, the number of mosquitoes successfully probing during each treatment was analysed using regression analysis in a generalised linear model (GLM) in Genstat® (12th edition), modelling binomial proportions with a logit transformation using replicates as blocks. This was used to obtain predicted means and standard errors of the means (SEMs). Differences were deemed to be significant when the difference between means was greater than the least significant difference (LSD).

EAG responses were corrected by dividing the response in millivolts by the average of the control values before and after the stimulation of each test treatment. Thus, the control value was 1 and the response was expressed as a proportion of 1. The mean responses between treatments were compared by using a one-way ANOVA in Genstat® (12th edition), using replicates as blocks. The data were log (base10) transformed. Differences were deemed to be significant when the difference between means was greater than the LSD.

5.3 Results

5.3.1 Experiment 1: Pre-exposure to DEET on a human arm

There was no significant difference between the proportion of mosquitoes probing in response to the control arm (control) compared with the control retested with the control treatment after 3h (control/control) (Fig 5.1). There was also no significant difference between the number of mosquitoes probing in response to DEET without pre-exposure (DEET) and to DEET tested 3h after pre-exposure to a control arm (control/DEET). However, mosquitoes tested with DEET 3h after pre-exposure to DEET (DEET/DEET) probed significantly more in response to DEET than cages tested with DEET 3h after pre-exposure to a control (control/DEET) or DEET without pre-exposure (DEET) ($p<0.001$), but there were still less mosquitoes probing than in response to the control ($p<0.001$).

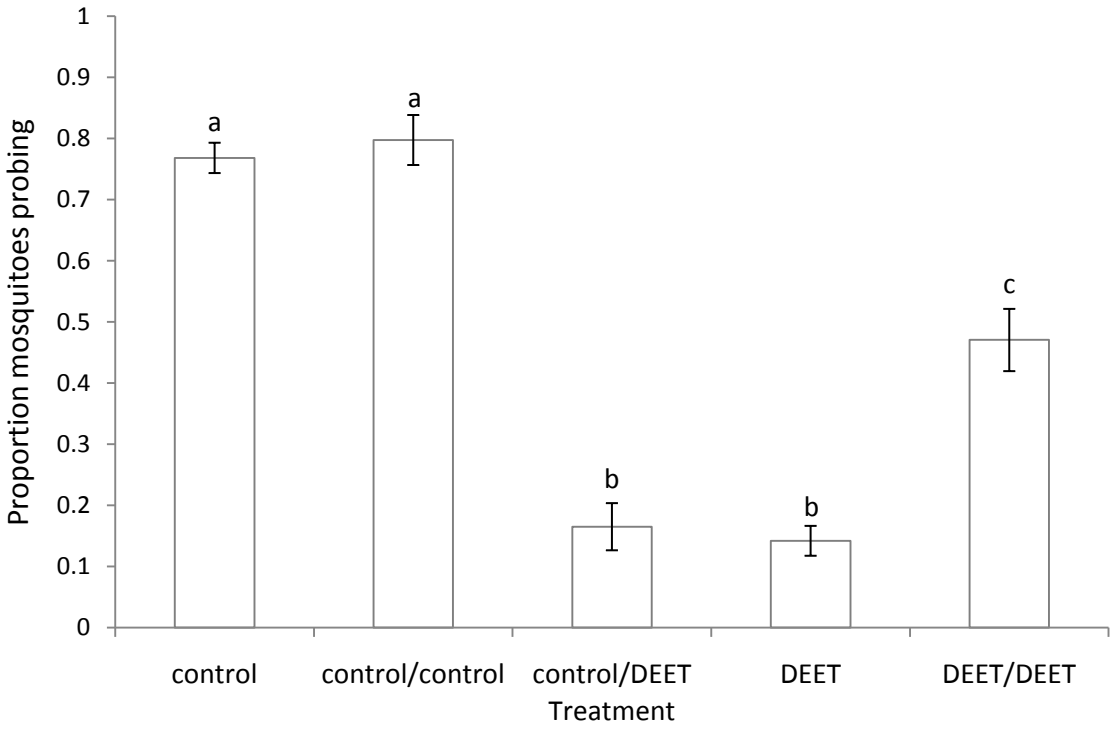


Fig 5.1 Proportion of female *Aedes aegypti* mosquitoes probing in response to a control arm (0.5 ml ethanol) (control), a control arm after being tested with a control arm 3h previously (control/control), an arm treated with DEET (0.5 ml in ethanol) after being tested with a control arm 3h previously (control/DEET), a DEET treated arm with no pre-exposure (DEET), and an arm treated with DEET after being tested with DEET 3h previously (DEET/DEET). Means are ± SEM. Means with different letters are significantly different from each other ($p<0.05$).

5.3.2 **Experiment 2: Pre-exposure to DEET alone**

The proportion of mosquitoes probing in response to being retested with DEET, having been previously exposed to a control arm (control/DEET), a DEET arm (DEET/DEET), or a DEET impregnated section of tights (only DEET/DEET), was significantly greater than mosquitoes tested with DEET for the first time (DEET) ($p<0.001$) (Fig 5.2). However, there were no significant differences in the response of mosquitoes to DEET after pre-exposure between any of the three treatments.

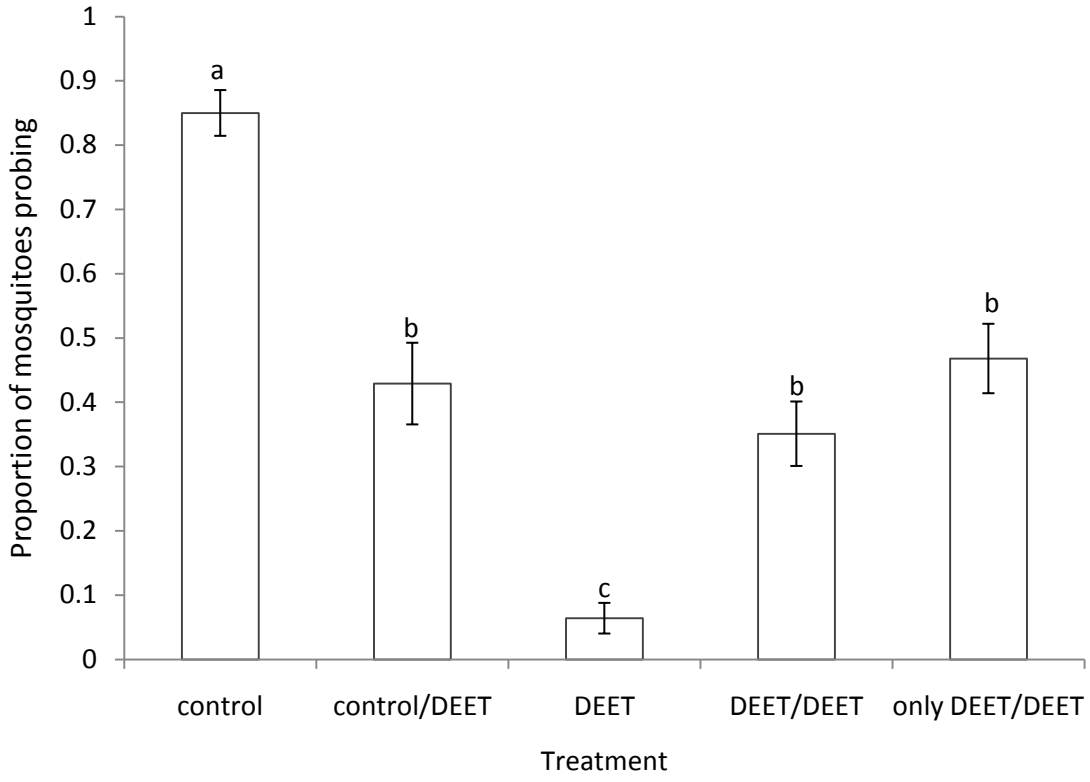


Fig 5.2 Proportion of *Aedes aegypti* female mosquitoes probing in response to a control (0.5 ml ethanol) arm, an arm treated with DEET after being tested with a control arm 3h previously (control/DEET), DEET on an arm with no pre-exposure (0.5 ml, 20% in ethanol) (DEET), an arm treated with DEET after being tested with DEET 3h previously (DEET/DEET), and an arm treated with DEET after being tested with a section of DEET-impregnated tights 3h previously (only DEET/DEET). Means are ± SEM. Means with different letters are significantly different from each other ($p<0.05$).

5.3.3 Experiment 3: Pre-exposure to DEET with an artificial heat source

In cages tested with the Hemotek® artificial heating device in place of an arm, there was no significant difference between mosquitoes tested with the Hemotek® control (Heat) and those retested with the control 3h after pre-exposure to the control (Heat/Heat) (Fig 5.3). However, mosquitoes tested with DEET on a Hemotek® reservoir 3h after pre-exposure to the control (Heat/DEET) had significantly less mosquitoes probing than those tested with DEET without any pre-exposure (DEET) ($p=0.001$) or DEET 3h after pre-exposure to DEET (DEET/DEET) ($p<0.001$). Mosquitoes probed significantly more in response to DEET 3h after pre-exposure to DEET (DEET/DEET) than to DEET without pre-exposure (DEET) ($p=0.016$), though the proportion probing was still lower than the responses to the control (Heat) ($p<0.001$) and the control 3 h after pre-exposure to the control (Heat/Heat) ($p=0.011$).

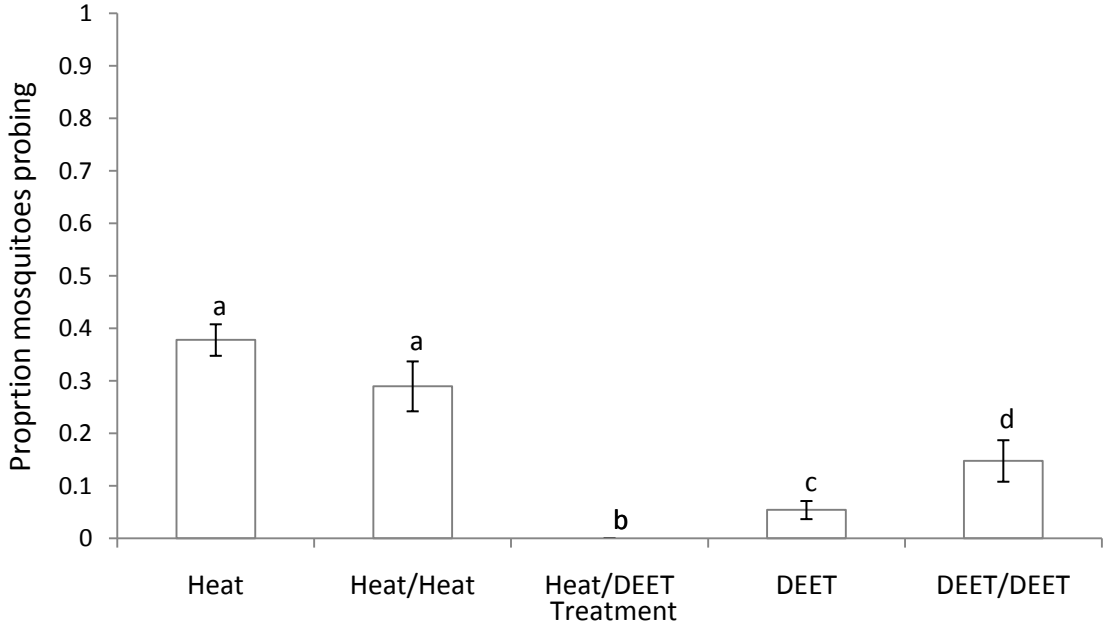


Fig 5.3 Proportion of female *Aedes aegypti* mosquitoes probing in response to a control Hemotek® reservoir (0.5 ml redistilled hexane) (Heat), a control after being tested with a control 3h previously (Heat/Heat), a Hemotek® treated with DEET (0.5ml, 20% in redistilled hexane) after being tested with a control 3h previously (Heat/DEET), a DEET treated Hemotek with no pre-exposure (DEET), and a Hemotek treated with DEET after being tested with DEET 3h previously (DEET/DEET). Means are ± SEM. Means with different letters are significantly different from each other ($p<0.05$).

5.3.4 **EAG**

There were no significant differences in EAG responses to DEET between any of the DEET-insensitive mosquitoes (ins) tested from the groups which had been treated at 0-3h with control/DEET and DEET/DEET, or at 0h with DEET (Fig 5.4). The DEET-sensitive (sens) mosquitoes collected after treatment with DEET/DEET had a significantly greater response to DEET than the three groups of DEET-insensitive mosquitoes tested ($p=0.001$, $p=0.019$, $p<0.001$ respectively). The response to DEET of the control group, using females of unknown sensitivity (sens + ins), was not significantly different from the DEET-sensitive mosquitoes from the DEET/DEET group, or the DEET-insensitive mosquitoes from the DEET group, but was significantly greater than the response of the DEET-insensitive control/DEET ($p=0.01$) and DEET/DEET ($p=0.006$) groups.

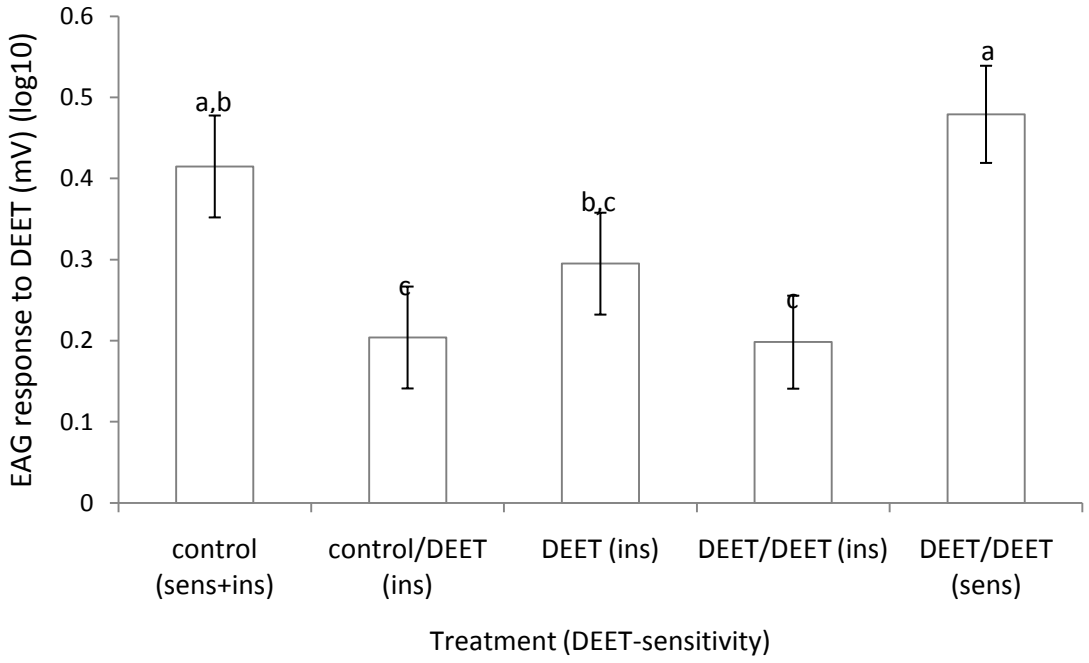


Fig 5.4 EAG responses of female *Aedes aegypti* showing DEET-sensitivity (sens) or DEET-insensitivity (ins) in experiment 1. Mosquitoes of unknown DEET-sensitivity (sens +ins) were collected from cages tested with a control arm (control). DEET-insensitive females were collected from cages tested first with a control arm and then DEET on an arm (control/DEET), tested with DEET on an arm (DEET), and tested with DEET on an arm then retested with DEET on an arm (DEET/DEET). DEET-sensitive mosquitoes were also collected from cages tested with DEET on an arm and then retested with DEET on an arm (DEET/DEET). Means are ± SEM. Means with different letters are significantly different from each other ($p<0.05$).

5.4 Discussion

In selecting for heritable DEET-insensitivity, it would have been useful to test cages of mosquitoes multiple times to check that non-responders were not false negatives. However, retesting mosquitoes with DEET on an human arm 3h after pre-exposure to DEET on an arm led to an increased proportion of the insects probing (Fig 5.1, 5.2, Appendix 5), with half of the previously DEET-sensitive mosquitoes becoming insensitive to DEET. This is likely to be caused by a non-genetic effect. It could not be caused by incomplete penetrance of the heritable DEET-insensitivity trait. Only ~10% of the culture population were phenotypically insensitive, therefore, assuming 60% penetrance, only ~16.6% would have the DEET-insensitive genotype overall, which would not explain the 50% of previously DEET-sensitive mosquitoes displaying insensitivity.

The observed decrease in sensitivity to DEET on a second exposure by previously sensitive mosquitoes suggests they may have 'learned' to associate the DEET odour with the presence of a host arm (or heat source), and were able to 'overcome' the repellent effect. Whilst learning behaviour in *Drosophila* has been previously established (Waddell and Quinn, 2001), the idea of learning behaviour in mosquitoes remains controversial. Some studies have found no evidence for it in host-location (Rawlings and Curtis, 1982; Arredondo-Jimenez *et al*., 1992) or after pre-exposure to positive or negative treatments such as human breath or vibration (Alonso *et al*., 2003). However, in oviposition experiments, mosquitoes are more likely to breed in water containing chemicals that were present during their larval stages, showing 'site fidelity' with no genetic basis (McCall and Eaton, 2001; McCall *et al*., 2001). This suggests that some form of 'imprinting' occurs at an early stage, with the mosquitoes learning the volatiles associated with suitable oviposition sites. Interestingly, mosquitoes reared in repellent-treated water appear to be conditioned against oviposition-site deterrence and do not respond to repellents such as citronella in their choice of oviposition site (Kaur *et al*., 2003). Unfortunately whether mosquitoes ignored these repellents in other situations, or just with regards to oviposition, was not examined, but it does demonstrate that pre-exposure to repellents may make them less effective, or make the mosquitoes insensitive to them. Other investigations have shown

that mosquitoes have a preference for returning to hosts fed on previously (Kelly and Thompson, 2000). These preferences were not passed on to their offspring and were therefore concluded to be due to learned behaviour (Mwandawiro *et al*., 2000), which maximised feeding success while minimising risk. In our study, learning to ignore DEET gave no reward other than the ability to move towards a human arm/heat source. However, the mosquitoes did not face any negative penalties from the repellent or odour in the first test, and were therefore possibly more likely not be repelled by the presence of DEET in the retest. Both *Cx. quinquefasciatus* mosquitoes (Tomberlin *et al.*, 2006) and the parasitic wasp, *M. croceipes* (Lewis and Takasu, 1990), can learn to associate a novel odour not present in nature with a food source, and adapt their host-seeking preferences accordingly. Thus, it might appear that the mosquitoes in our study learned to associate the presence of DEET with the odour of a human arm, even with no reward, and were more likely to probe on a second exposure (Fig 5.1). However, in our study the increase in response after pre-exposure to DEET occurred even when the initial presentation of DEET was not accompanied by an attractant in the form of heat or host-volatiles from a human arm (Figs 5.2, 5.3). This is in contrast to experiments with the haematophagous triatomine bug *Rhodnius prolixus*, which have shown that heat with no attractive odour present is not a sufficient reward to cause associative learning (Abramson *et al.*, 2005). Mosquitoes and triatomines may respond to similar cues when seeking human hosts (Cruz-López *et al.*, 2001), therefore, the difference in mosquito behaviour seen after exposure to DEET in addition to heat could be a response to the pre-exposure to DEET rather than to the presence of a heat source. Thus, the change in the behaviour of *Ae. aegypti* on retesting with DEET seems to be a direct response to a single exposure to the compound, rather than being due to the associative learning otherwise found in host-seeking insects (Lewis and Takasu, 1990; Abramson *et al.*, 2005; Tomberlin *et al.*, 2006; Aldana *et al.*, 2008). The increased DEET-insensitivity possibly results from habituation, whereby there is a decrease in response to a stimulus after repeated exposure (Papaj and Prokopy, 1989).

In both preliminary experiments and experiment 2, mosquitoes showed an increase in probing when DEET was present after being previously exposed to a control arm, compared to mosquitoes tested with DEET for the first time (Fig 5.2, Appendix 5). This difference was not seen in response to the

control arm in experiment 1 or to heat in experiment 3 (Figs 5.1, 5.3). It is unclear why the mosquitoes do not show consistent alteration of their behaviour after exposure to a control arm. It is possible that mosquitoes should show an increase in response to a DEET-covered arm after pre-exposure to a control arm, and that the lack of this behaviour in experiment 1 was an anomaly. The Hemotek® trial, without the attractant of a human arm, may not have been sufficient stimulus to induce the change in response (Fig 5.3). If mosquitoes are changing their response after pre-exposure to a control arm, it is likely to be due to a different cause than mosquitoes adapting their behaviour after pre-exposure to only DEET, as there were no human volatiles present during the latter test. The change in response after exposure to a control arm may therefore be due to the stimulation of receptors which increase the mosquito's host-seeking behaviour, causing olfactory sensitisation (Papaj and Prokopy, 1989), and therefore make them more likely to attempt to feed.

The phenomenon of insects changing their response to a compound after pre-exposure or conditioning has been investigated with EAG studies in *D. melanogaster*, *M. croceipes, A. mellifera and P. terraenovae* (Bhagavan and Smith, 1997; Stortkuhl *et al.*, 1999; Devaud *et al.*, 2001; Park *et al.*, 2001; Barbarossa *et al.*, 2007). For one of the *D. melanogaster* trials and in the study on *M. croceipes* no changes in EAG responses were found, even though the insects were exhibiting changed behavioural responses (Devaud *et al.*, 2001; Park *et al.*, 2001). This was suggested to be caused by a difference in the volume of glomeruli in *D. melanogaster*, with synapse loss in the insects which had been exposed to higher concentrations of the chemical for a week causing the behavioural change (Devaud *et al.*, 2001). In a separate trial on *D. melanogaster*, with a shorter, 1 min pre-exposure to a chemical, work on *A. mellifera*, with a 3 s pre-exposure, and in *P. terraenovae*, with multiple 2 s exposures, a decrease in EAG response to the compounds was observed (Bhagavan and Smith, 1997; Stortkuhl *et al.*, 1999; Barbarossa *et al.*, 2007). For *D. melanogaster*, the insects were no longer behaviourally repelled by a repellent they had been pre-exposed to. However, this EAG decrease only lasted for a brief time, with EAG responses returning to half the normal level in four minutes. In our study the mosquitoes no longer repelled by DEET showed a lower EAG response to the repellent (Fig 5.4), reflecting the results of the latter studies (Bhagavan and Smith, 1997; Stortkuhl *et al.*, 1999;

Barbarossa *et al.*, 2007). This could be due to the brevity of the exposure in our study, two minutes, as compared to the one week of pre-exposure in the *D. melanogaster* study where no change in EAG responses was seen (Devaud *et al.*, 2001). After a week's exposure to a chemical it is likely a different change is causing the altered behaviour, having had time to affect the loss of synapses, as compared to the changes induced in the peripheral olfactory system after brief exposure (Bhagavan and Smith, 1997; Stortkuhl *et al.*, 1999; Barbarossa *et al.*, 2007). Stortkuhl *et al*. (1999) found the change in response after pre-exposure to a repellent did not occur in *D. melanogaster* with a mutant Trp calcium channel. Trp channels are found in the outer membranes of sensory cells, and are activated by sensory stimuli to mediate calcium entry, which is an essential component of cellular response. In *D. melanogaster* it was suggested that Trp plays a role in the differentiation of antennal neurons, and thus *trp* mutants were unable to adapt after pre-exposure as they did not have a fully developed olfactory system. In *P. terraenovae*, repeated olfactory stimulation with the repellent 1-hexanol at low doses led to all individuals ceasing to respond to the chemical, which the authors concluded to be due to non-associative learning processes, such as habituation, of the odour (Barbarossa *et al.*, 2007). At higher doses, such as 10%, approximately half of flies no longer responded to the chemical after repeated exposure. This is similar to the level of behavioural DEET-insensitivity found in our study on re-exposure, and suggests that the same mechanism may be responsible for the behavioural change after exposure to DEET. However, in *D. melanogaster, P. terraenovae* and vertebrates the habituation causing a change in response only lasts for a few minutes to half an hour (Leinders-Zufall *et al.*, 1999; Stortkuhl *et al.*, 1999; Barbarossa *et al.*, 2007), after which the responses return to normal, showing dishabituation (which is a key characteristic of habituation) (Papaj and Prokopy, 1989; Bernhard and van der Kooy, 2000). This is in contrast to the altered behavioural and EAG responses to DEET in our study on *Ae. aegypti*, which have been shown to last for at least 3h. It is possible that habituation varies between species, and lasts longer in *Ae. aegypti* than in *D. melanogaster or P. terraenovae* (Stortkuhl *et al.*, 1999; Barbarossa *et al.*, 2007). However, in the nematode *Caenorhabditis elegans*, two separate causes of decreased response to an odour were shown. At low concentrations of the odour, habituation occurred, with the responses then returning to normal, and, at high concentrations of the odour, adaptation occurred, with responses not returning to normal (Bernhard and van der Kooy, 2000). The cause of the adaptation was thought to be sensory or receptor fatigue.

As, 3h after exposure to DEET, behavioural and EAG responses to DEET were still decreased in our study, decreased receptor activity or effector fatigue could be the cause (Bernhard and van der Kooy, 2000). It would be interesting to explore if behavioural and EAG responses to DEET remained decreased over a longer period of time, or after multiple repeated exposures.

If, as shown here, mosquitoes change their response to a repellent after pre-exposure to a treatment, then insects should not be tested multiple times in behavioural bioassays. Methods which retest the same mosquitoes are commonly used, and these studies could be affected by the adaptive behaviour shown in our study (Curtis *et al.*, 1987; Geier and Boeckh, 1999; Tawatsin *et al.*, 2001; Frances *et al.*, 2005; WHOPES, 2009). Also, the identification of new repellents and attractants could be less effective if insects' responses may not be due to innate preference, but repeated exposure (McCall and Kelly, 2002).

5.5 Conclusion

Mosquitoes repeatedly tested with DEET in a repellency bioassay have altered responses to the chemical, with less being repelled after pre-exposure. Mosquitoes should therefore only be tested once, and not retested to identify individuals which may not have been responding originally.

Chapter 6. **General Discussion**

The aim of this study was to investigate DEET-insensitivity in *Aedes aegypti* mosquitoes through behavioural assays, genetic crosses and electrophysiology. Although previous studies in *Drosophila melanogaster* and *Ae. aegypti* have identified individuals insensitive to DEET they have not examined the underlying causes (Becker, 1970; Rutledge *et al.*, 1994; Reeder *et al.*, 2001).

6.1 Heritable DEET-insensitivity

While insensitivity to DEET has been reported previously in the mosquito *Ae. aegypti* (Rutledge *et al.*, 1978; Rutledge *et al.*, 1994), this study was the first to select for the trait in mosquitoes. The bidirectional selection led to the unique resource of two lines of mosquitoes, the s and i lines, with differential sensitivity to DEET. The proportion of mosquitoes insensitive in the i line rose with successive generations, showing that the trait could be selected for. The rapid increase to 55% of the i line showing insensitivity to DEET in the first two generations may have been influenced by the high initial background level of insensitivity (13%) in the culture. This natural level of insensitivity is likely to have been influenced by the lower genetic variation found in the artificial environment of a laboratory culture, which can lead to unusually high frequencies of rare alleles (Mukhopadhyay *et al.*, 1997). However, even if the trait may be rarer in field populations, this result in the laboratory has important implications for the use of DEET in the field. Insensitivity to DEET confers a reproductive advantage when the repellent is used, as female mosquitoes are able to target hosts normally protected by DEET to gain the blood meal they require to reproduce. DEET is used by tourists travelling abroad in low enough numbers to make selection for insensitivity unlikely, as mosquitoes can easily find alternative hosts. However, due to the threat of West Nile virus, approximately 30% of people in the US use DEET on a daily basis (Osimitz and Murphy, 1997). These people are likely to be in the areas with the highest mosquito populations, leading to locations where all available human hosts may be wearing the repellent. Whilst opportunistic mosquito species can also feed on other animals, the extensive use of DEET by humans could lead to increased selection for DEET-insensitivity in these areas. There have

been no studies investigating the development of resistance in the field, either to DEET or other repellents, and this information is important for the use of personal protection methods in the future.

Many new ways of utilising DEET are also being investigated. For example, bed nets treated with a combination of DEET and propoxur is a promising alterative in areas where pyrethroid resistance makes ITNs less effective (Zaim and Guillet, 2002; Pennetier *et al.*, 2005; Bonnet *et al.*, 2009). As well as having repellent properties, DEET itself may also have direct insecticidal effects. Studies have shown that mosquitoes coming into contact with DEET-impregnated materials and aerosol sprays showed increased knock down and mortality (Xue *et al.*, 2003; Licciardi *et al.*, 2006; N'Guessan *et al.*, 2008). This could lead to resistance to DEET developing faster than if DEET only acted as a repellent. The spread of insecticide resistance is due both to the evolution of distinct resistance mechanisms in geographically separate areas, and the migration of mosquitoes by natural and human-mediated transfer (Pasteur and Raymond, 1996). When moving to insecticide-treated areas, migrating resistant mosquitoes will be more likely to survive and reproduce than any native susceptible mosquitoes, ensuring the spread of the trait. A similar pattern could be seen with DEET insensitivity if insensitive mosquitoes were in habitats where they gained a reproductive advantage over DEET-sensitive mosquitoes.

As the DEET-insensitivity described in this study was found to be a genetically-determined dominant characteristic, only one allele of the insensitive gene needs to be inherited from insensitive parents in order for the offspring to express the phenotype, making it likely to spread quickly through the population. The fact that there is incomplete penetrance of the trait, with only 55% of insensitive females displaying the insensitive phenotype, means that the presence of DEET-insensitivity in a native population could frequently be underestimated. Thus, it will be important to have as much information on the causes of this trait, and ways of monitoring it in wild populations, as possible. For example, it would be useful to examine the DEET-sensitive and insensitive mosquito lines formed here with molecular techniques to determine sequence differences between them. This in turn could lead

to an assay to identify unique markers for DEET-insensitivity, to monitor field populations. Unfortunately, there was not time to pursue this further within the scope of this PhD.

6.2 The mechanism of DEET

The debate over the mechanism of how DEET acts as a repellent has never been conclusively resolved. Suggestions that DEET may act by blocking the receptors of attractive compounds, such as lactic acid (Davis and Sokolove, 1976) or 1-octen-3-ol (Ditzen *et al.*, 2008), have been countered by evidence of receptors responding directly to DEET (Davis and Rebert, 1972; Boeckh *et al.*, 1996; Syed and Leal, 2008). In the present study, on *Ae. aegypti,* an olfactory receptor neuron (ORN) which responds to DEET in a dose-dependent fashion was located and fully characterised. We also found that the responses of *Ae. aegypti* to 1-octen-3-ol were not affected by the presence of DEET, except when differences were explained by an artefact of one method used, with DEET possibly reducing the amount of 1-octen-3-ol passing over the antennae due to a fixative effect. A recent investigation into the action of DEET upon *Ae. aegypti* recombinant olfactory receptors (ORs) has further elucidated the matter (Bohbot and Dickens, 2010). AaOR2 was found to respond directly to DEET, and AaOR8 was found to respond to 1-octen-3-ol and to be inhibited by DEET. The inhibition of 1-octen-3-ol was not seen in our study, probably because we used a higher concentration of 1-octen-3-ol, which may not have been inhibited. This suggests DEET acts in two separate ways upon the peripheral olfactory system: by directly causing a repellent effect and by affecting the response to an attractive compound. It may be that the two pathways must be activated together for the mosquito to be repelled. An interesting experiment would be to look at the repellency of DEET in mosquitoes with knockouts of each of these receptors, to examine what the individual effects are, and if one pathway is more important than the other.

6.3 The DEET-sensitive ORN in *Ae. aegypti*

In previous studies, DEET sensitive neurons were identified localised to the short blunt sensilla in *Ae. aegypti* (Davis and Rebert, 1972; Boeckh *et al.*, 1996) and the short sharp sensilla in *Culex quinquefasciatus* (Syed and Leal, 2008). However, these studies did not fully distinguish between the different morphological or functional types of sensilla, thus not fully identifying the relevant sensillum and ORN. In the present study on *Ae. aegypti* we further elucidated the location of the DEET-sensitive ORN as the A neuron in the short blunt type II sensilla, in functional subtype 4 (sbtII 4). This more precise identification of the ORN involved will allow for more effective testing of DEET in single sensillum recordings (SSR) in the future, as it will be possible to target a more specific sensillum type. The DEET-sensitive neuron identified in *Cx. quinquefasciatus* by Syed and Leal (2008) also responded to thujone, 1,8-cineole and linalool, all plant compounds to which mosquitoes show a behavioural response (Bowen, 1992; Park *et al.*, 2005; Traboulsi *et al.*, 2005; Muller *et al.*, 2009). The DEET-sensitive neuron in our study on *Ae. aegypti* also responded to these plant compounds, despite being found in a different morphological type of sensilla. DEET may be recognised by an equivalent olfactory receptor in the two species, which also responds to this set of plant-derived compounds. In *Ae. aegypti*, the recombinant receptor AaOR2 was found to respond directly to DEET (Bohbot and Dickens, 2010), and it may be this OR in sbtII 4 that recognises DEET. As there is no probable reason for mosquitoes to have an OR able to recognise DEET, an entirely artificial compound, it seems likely that DEET is being recognised by a receptor that also responds to different compounds. It is known that *D. melanogaster* feeding behaviour is inhibited by DEET, with DEET stimulating the gustatory receptor neurons normally associated with other, aversive compounds (Curtis, 1992). A similar phenomenon could be occurring in the *Ae. aegypti* ORN, as while there are no obvious similarities between the structures of DEET and thujone, 1,8-cineole or linalool (Fig 4.10), these compounds have been shown to work as repellents (Park *et al.*, 2005; Traboulsi *et al.*, 2005; Muller *et al.*, 2009) or attractants (Bowen, 1992). It is, therefore, possible that the structural characteristic which is responsible for repellency or behavioural triggers across these chemicals is similar, and DEET is able to elicit the same effect. Tested here with SSR, mosquitoes showed a comparable level of response in sbtII 4 to DEET and the plant-derived compounds at the same concentration. For DEET to be effective

as a repellent, it is used at much greater concentrations (10-80%) (Fradin, 1998) than the plant-derived repellents 1,8-cineole and linalool (2-4%) (Park *et al.*, 2005; Traboulsi *et al.*, 2005; Muller *et al.*, 2009). Given the efficacy of DEET compared to the plant-derived compounds, it is possible that DEET acts in multiple ways upon an insect's sensory system. It may cause a repellent effect through the peripheral olfactory system, block attractive volatile signals (Bohbot and Dickens, 2010), and have insecticidal properties (N'Guessan *et al.*, 2008).

The s and i mosquitoes in our study had a differential response to (±)-linalool as well as to DEET. Behaviourally DEET-insensitive mosquitoes did not respond to either DEET or (±)-linalool in SSR, but did respond normally to the other compounds tested. As there was no change in response to (-)-linalool, it is likely that the change in response to (±)-linalool is due to differential detection of (+)-linalool. This indicates there has been a very specific change in the olfactory recognition of DEET and (+)-linalool. This could be caused by a change in the ORs that recognise these compounds, either in the conformation of the ORs or in the number expressed, with the latter causing changed behavioural responses to odours in the moth *Trichoplusia ni* (Domingue *et al.*, 2009). For this to apply to our study, DEET and (+)-linalool would have to be recognised by a different OR from all of the other compounds tested, or the OR would have to have undergone a conformational change which only affected its recognition of these two compounds. For both mammals and insects, individual ORNs have been found to express only one, or a small number, of ORs (Su *et al.*, 2009). In *D. melanogaster*, a single OR was found to define the response profile of most ORNs (Hallem *et al.*, 2004). If this is also the case for mosquitoes, DEET and (+)-linalool could not be recognised by a different OR than the other compounds in this ORN, though there are some ORNs with more than one OR (Fishilevich and Vosshall, 2005; Goldman *et al.*, 2005). For a single OR to have undergone a conformational change and no longer recognise only these two compounds is unlikely, as DEET and (+)-linalool do not have similar chemical structures (Fig 4.10), though the change could relate to a small, specific shared part of the structure as yet unknown. If so, the identification of the shared specific structure could be useful in identifying or designing new repellents.

Another possible explanation for the loss of sensitivity to DEET is a mutation in the gene encoding an odorant-binding protein (OBP) that normally delivers DEET and (+)-linalool to the receptor. OBPs normally bind to a wide range of compounds, so it is likely that DEET and (+)-linalool are recognised by more than one OBP. However, volatiles are bound by some OBPs with greater affinity than others. Thus, if the expression of an OBP which normally binds DEET and (+)-linalool with a high affinity was altered, the same amount of these compounds would no longer be transported to the ORs and the ORs would not respond. A similar change in electrophysiological response to that in our study, with mosquitoes no longer responding to a compound, was shown when OBP1 was silenced in *Anopheles gambiae* (Biessmann *et al.*, 2010).

As i line mosquitoes show behavioural insensitivity to DEET and (±)-linalool, but not (-)-linalool, it seems likely that the difference in detection of DEET and (+)-linalool in sbtII 4 causes the behavioural insensitivity. Other natural repellents and plant-derived compounds were not investigated, but it is important to note that if DEET-insensitivity does become selected for in the wild, there may be cross-insensitivity to other repellents which share the affected olfactory pathway.

6.4 Basis of DEET-insensitivity after pre-exposure

Mosquitoes that were insensitive to DEET in the behavioural assay showed a significantly lower response to DEET in EAG recordings than those sensitive to DEET. This was true both of mosquitoes which had been selected in the s and i lines over generations, and of culture mosquitoes displaying insensitivity to DEET after brief pre-exposure to it. If the insensitive unselected females were removed from the pre-exposure behavioural assay, and the sensitive mosquitoes were then retested with DEET, ∼50% of the previously sensitive females changed their phenotype and became insensitive to DEET. This cannot be explained by a genetic change, as it occurred within three hours. Whilst incomplete penetrance of DEET-insensitivity means that another ∼10% of the unselected culture

have the insensitive genotype, this would not explain the 50% alteration seen. This change in insensitivity after pre-exposure could be caused by a change in the same mechanism responsible for the heritable insensitivity. The idea that the mosquitoes could be learning the odour of DEET, and responding differentially to it upon further testing, is interesting. Mosquitoes have been previously shown to exhibit conditioned or imprinted behaviour, in returning to previous hosts (Kelly and Thompson, 2000) and oviposition sites (McCall and Eaton, 2001; McCall *et al.*, 2001). Mosquitoes have even been shown to be able to overcome the presence of the repellent citronella when they have been reared in citronella-treated water (Kaur *et al.*, 2003). If mosquitoes could learn to ignore DEET and return to bite successfully, it would be far less effective as a repellent. In both *Ae. aegypti* mosquitoes and *Microplitis croceipes*, insects can be conditioned to respond differentially to novel odours in combination with attractants (Lewis and Takasu, 1990; Tomberlin *et al.*, 2006). It is, therefore, possible that mosquitoes could learn to associate the odour of DEET with attractive humans, and overcome the repellent effect. However, in our studies the mosquitoes altered their behaviour after pre-exposure to DEET even when no attractant was present, and there was no incentive for them to do so. It is, therefore, more likely to be a physiological change, rather than a conditioned behavioural change, that leads to the altered behaviour and difference in EAG results. Similar results have been seen in *D. melanogaster*, where flies were no longer repelled by isoamyl acetate or benzaldehyde after a one min pre-exposure to the compounds, even though there was no attractant present at any stage (Stortkuhl *et al.*, 1999). When tested with EAG, *D. melanogaster* that had been pre-exposed showed a lowered response to the compounds, similar to the results seen in our study. *Drosophila melanogaster* mutants with deficient transient receptor protein (Trp) channels no longer showed the adaptive behaviour. Trp channels mediate calcium (Ca^{2+}) entry into sensory cells, in response to sensory stimuli, which is essential to stimulus response. In *D. melanogaster* it was suggested that Trp plays a role in the differentiation of antennal neurons, and thus *trp* mutants were unable to adapt after pre-exposure as they did not have a fully developed olfactory system. It is clear that the components of the olfactory system requiring Trp during development are critical for the ability to adapt responses to an odour. In vertebrates, adaptation to odours induced by pre-exposure is triggered by Ca^{2+} entry into the transduction channels causing changes in the transduction pathways (Kurahashi and Shibuya, 1990). Ca^{2+} mediates the attenuation of adenylate cyclase, which

catalyses the production of cyclic adenosine monophosphate (cAMP), an important molecule in signal transduction. When Ca^{2+} entry is disrupted by inhibiting Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) in salamander ORNs, adaptation in response to sustained pre-exposure no longer occurs (Leinders-Zufall *et al.*, 1999). Therefore, CaMKII's role in attenuating adenylate cyclase may be important to the adaptation process. In the *D. melanogaster trp* mutants (Stortkuhl *et al.*, 1999), the abnormal development of the olfactory system may have resulted in Ca^{2+} no longer entering the transduction channels after exposure to an odour stimuli. A similar mechanism could be responsible for the behavioural change of the mosquitoes in our study. In both *D. melanogaster* and vertebrates this change in response only lasts for a few minutes (Leinders-Zufall *et al.*, 1999; Stortkuhl *et al.*, 1999), likely due to habituation to the odour, whereas in our study the adaptation lasted for at least three hours. It may be more likely that in this study on *Ae. aegypti* there was a change in the peripheral olfactory system which causes longer-lasting DEET-insensitivity after pre-exposure.

The sbtII 4 ORN A was found to no longer respond to DEET in behaviourally selected insensitive females from the i line. If the mosquitoes with altered sensitivity to DEET after pre-exposure have a similar basis for the insensitivity as shown in the selected i line, it would suggest that either their ORs or OBPs are affected. For the conformation of, or number of, ORs to alter after a two minute exposure to a substance seems unlikely, although there is the possibility that the first exposure to DEET inhibited the following responses of the OR. Alternatively, DEET could activate a mechanism which affects the number of OBPs present, either by increased degradation or temporary inhibition of expression. As this change in sensitivity only occurs in half of the sensitive mosquitoes re-exposed to DEET, it might require a certain threshold of DEET to activate the change, with only mosquitoes exposed to the optimal concentration switching their sensitivity. This could mean that females occupying areas of the cage exposed to higher levels of DEET change their sensitivity, whereas those exposed to lower levels of DEET are not affected. It is also possible that the DEET-insensitivity observed in the selected i line, and at a low level in culture mosquitoes, has a completely different mechanism to the insensitivity of mosquitoes adapting their behaviour after pre-exposure. For example, the alteration in sensitivity could be due to decreased receptor activity through receptor

adaptation, which was suggested as the cause for a continued decreased response to an odour after pre-exposure in *Caenorhabditis elegans* (Colbert and Bargmann, 1995; Bernhard and van der Kooy, 2000). An examination of the olfactory receptor cells of the newt *Cynops pyrrhogaster* showed that receptor adaptation to odorants was caused by a modulation of the cAMP gated channel by Ca^{2+} feedback, as Ca^{2+} entering the receptor cell was responsible for the adaptive change in response (Kurahashi and Menini, 1997). Excessive activation of a high affinity receptor for the odour causes downstream targets to instead interact with a low-affinity receptor pathway, leading to the observed adaption (Bernhard and van der Kooy, 2000).

The results reported in this study have important consequences, both in the field and the laboratory. There is the possibility that mosquitoes in the wild may temporarily become more insensitive to DEET after an initial exposure. However, in our study the mosquitoes were given no option other than the DEET-covered arm when they were retested, so it is probable that given the choice they would have chosen a host without the repellent. Thus, the reduction in repellency caused by this pre-exposure may not be as likely in the field, as mosquitoes will still seek out unprotected hosts rather than return to a host wearing DEET, even if they have gained some measure of insensitivity to the repellent. However, people using DEET do get the occasional bite, and it is possible that these bites are the result of mosquitoes with altered DEET-sensitivity after pre-exposure. In laboratory repellency trials, both with DEET and other chemicals, insects are frequently reused multiple times (Curtis *et al.*, 1987; Tawatsin *et al.*, 2001; Frances *et al.*, 2005; WHOPES, 2009). When testing the same insects multiple times with DEET it is likely, based upon the results in our study, that their responses will be different after the first exposure. Depending on the assay method and concentration of DEET used this may not be detectable. For example, DEET has been shown to be effective for up to eight hours where the mosquitoes are retested (Frances *et al.*, 2005), but this may be due to the high concentrations of DEET used compared to our study, or because there are differences in the responses of the different mosquito species tested. In several of our experiments female mosquitoes also showed less sensitivity to DEET after pre-exposure to a human arm. Pre-exposure to an attractant may induce some form of priming in the mosquito, so that, when they are then tested with an arm with DEET on it, they are

more likely to try and probe than naïve flies tested with DEET. If pre-exposure to attractants can also alter a mosquito's responses, then studies testing mosquitoes multiple times conducted with human volunteers and skin odours would be affected (Geier and Boeckh, 1999). Studies where mosquitoes are tested with multiple treatments may therefore have questionable results, and the use of this method in testing repellents especially, but also other volatile chemicals, should be more thoroughly investigated.

6.5 Conclusion

In this study, heritable insensitivity to DEET in *Ae. aegypti* is caused by a difference in the detection of the compound in a specific ORN. This ORN responds differentially to both DEET and the plant-derived repellent (+)-linalool in the strains formed through bidirectional selection for behavioural insensitivity to DEET. The possible selection in the wild for heritable insensitivity to DEET should be investigated and monitored closely because of the increasing further uses for DEET, such as DEET-impregnated bed nets and synergism with carbamates to overcome insecticide resistance. Temporary DEET-insensitivity caused by pre-exposure to the chemical could also have an important impact on laboratory behavioural assay methods, and the interpretation of data gained from studies using the same mosquitoes for multiple trials.

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Appendix 1. Predictions and Explanations of the Genetic Inheritance of DEET-Insensitivity

Calculations performed by John Brookfield, University of Nottingham.

Models of inheritance presented here: Polygenic at: 1) 20%, 2) 40%, 3) 60%, 4) 80% heritability

100% penetrance of trait - 5) Monogenic dominant

6) Monogenic recessive

60% penetrance of trait - 7) Monogenic dominant

8) Monogenic dominant with 8% s line insensitive

9) Monogenic incomplete dominance

10) Monogenic recessive

1.1.1 DEET-insensitivity is polygenic with a heritability of 0.2

It is assumed the liability to be DEET-insensitive is normally distributed. In order for a mosquito to be insensitive to DEET, the level of liability to insensitivity, due to the combination of genetics and environment, must be above a certain threshold. It is possible to work out how far away the mean liability of the population is from the threshold for insensitivity. This distance is shown in stan`dard deviations (X). Individuals above the threshold, displaying insensitivity, have a mean that is i standard deviations above the population mean.

Parental generation: 9 % of females show the threshold trait (preliminary trials, data not shown), therefore the threshold is 1.341 standard deviations (SDs) above the mean (X), and individuals above the threshold are, on average, 1.804 SDs away from the mean (i) (Falconer and Mackay, 1996). X, i and p% values are obtained from Appendix Table A (p379). The strength of selection in female

mosquitoes is therefore 1.804, and with no selection for insensitivity in males, the average selection is 0.902 SDs.

F_1 generation: The response to selection can be calculated by heritability x strength of selection, 0.2 x 0.902 = 0.1804 SDs. The new threshold for insensitivity (X) is calculated: 1.341 (previous threshold) – 0.1804 (response to selection) = 1.161 SDs above the mean of the population (X). 12.29% of the population will be above 1.161 SDs above the mean (Falconer and Mackay, 1996), and thus over the threshold for insensitivity. **Therefore, 12.29% of the F_1 generation will be insensitive.** These insensitive individuals are, on average, 1.656 SDs above the mean (i). Thus the average strength of selection is 1.656 (females) + 0 (males) / 2 = 0.828.

F_2 generation: The response to selection is 0.2 (heritability) x 0.828 (strength of selection) = 0.1656 SDs. The new threshold for insensitivity (X) is 1.161 (previous threshold) – 0.166 (response to selection) = 0.995 SDs above the mean. 15.98% of the population will be above 0.995 SDs above the mean, so **the predicted percentage of DEET-insensitivity in the F_2 generation is 15.98%.**

Calculations proceed in this manner, substituting in the appropriate values, until the F_{10} generation.

Table 1.1a. Polygenic (20% heritability) predictions of DEET-insensitivity as the population is selected.

Generation	Parental	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
% Insensitivity	9	12.29	15.98	19.98	24.09	28.3	32.5	36.58	40.52	44.28	47.8

1.1.2 DEET-insensitivity is polygenic with a heritability of 0.4

Calculations as in 1), with a heritability value of 0.4 instead of 0.2.

Table 1.2a. Polygenic (40% heritability) predictions of DEET-insensitivity as the population is selected.

Generation	Parental	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
% Insensitivity	9	16.35	24.88	33.61	41.85	49.24	55.68	61.23	65.93	69.89	73.27

1.1.3 DEET-insensitivity is polygenic with a heritability of 0.6

Calculations as in 1), with a heritability value of 0.6 instead of 0.2.

Table 1.3a. Polygenic (60% heritability) predictions of DEET-insensitivity as the population is selected.

Generation	Parental	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
% Insensitivity	9	21.18	34.81	47.16	57.23	65.11	71.17	75.87	79.53	82.44	84.76

1.1.4 DEET-insensitivity is polygenic with a heritability of 0.8

Calculations as in 1), with a heritability value of 0.8 instead of 0.2.

Table 1.4a. Polygenic (80% heritability) predictions of DEET-insensitivity as the population is selected.

Generation	Parental	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
% Insensitivity	9	26.80	44.96	58.88	68.75	75.66	80.53	84.1	86.72	88.71	90.28

1.1.5 DEET-insensitivity is monogenic and dominant (one copy of the allele confers the phenotype).

Hardy-Weinburg equation: $p^2 + 2pq + q^2 = 1$

Where q and p are the frequencies of the two alleles for a gene. p^2 and q^2 represent the proportions of the population homozygous for each allele, and $2pq$ represents the proportion of heterozygotes. Here '*I*' is the allele with frequency p, and stands for the DEET-insensitive allele. '*S*' has frequency q, and stands for the sensitive allele. The three possible genotypes are therefore *II*, *IS* and *SS*. As the trait is dominant, both *II* and *IS* will show DEET-insensitivity, and *SS* will be sensitive.

Parental generation: As 9 % of females show DEET-insensitivity (preliminary trials, data not shown), the starting frequency of the trait in the population is 0.09.

The proportion of *SS* homozygotes in the starting population is 1 – 0.09 (*II*) = 0.91.

According to Hardy Weinburg, the frequency of the *S* allele is therefore $\sqrt{}$ = 0.9539.

The frequency of the *I* allele is 1 – 0.9539 =0.0461.

The proportion of *II* homozygotes is $0.0461^2 = 0.0021$.

The proportion of *IS* heterozygotes is the frequency of *I* x frequency of *S* x 2 (as it is possible to have either *IS* or SI). 0.0461 x 0.9539 x 2 = 0.0879.

The frequency of the *I* allele in selected insensitive females = (proportion *II* homozygotes +half of the proportion of heterozygotes) / proportion of females selected = (0.0021 + 0.5 x 0.0879) / 0.09 = 0.5117. This is the proportion of *I* gametes from the selected females used to form the next generation.

The proportion of *I* gametes from unselected males is 0.0461, the frequency of the *I* allele in the population.

F_1 generation: The proportion of *II* homozygotes is 0.5117 (probability of getting *I* allele from mother) x 0.0461 (probability of getting *I* allele from father) = 0.0236.

The proportion of heterozygotes is 0.5117 x (1-0.0461) (if *I* from mother and *S* from father) + 0.0461 x (1-0.5117) (if *S* from mother and *I* from father) = 0.5106.

The proportion of *SS* homozygotes is 1 – (0.0236 + 0.5106) (the proportion of *II* homozygotes + heterozygotes) = 0.4658.

The frequency of insensitivity in the F_1 is 0.0236 (*II* homozygotes) + 0.5106 (heterozygotes) = 0.5342, or 53.42%.

The frequency of the *I* allele in the selected female gametes, is (0.0236 + 0.5 x 0.5106) / 0.5342 = 0.5221.

The frequency of the *I* allele in the male gametes, is 0.0236 + 0.5 x 0.5106 = 0.2789.

F_2 generation: The proportion of *II* homozygotes is 0.5221 (probability of getting *I* allele from mother) x 0.2789 (probability of getting *I* allele from father) = 0.1456.

The proportion of heterozygotes is 0.5221 x (1-0.2789) (if *I* from mother and *S* from father) + 0.2789 x (1-0.5221) (if *S* from mother and *I* from father) = 0.5098.

The proportion of *SS* homozygotes is 1 – (0.1456 + 0.5098) (the proportion of *II* homozygotes + heterozygotes) = 0.3446.

The frequency of insensitivity in the F_2 is 0.1456 (*II* homozygotes) + 0.5098 (heterozygotes) = 0.6554, or 65.54%.

Calculations proceed in this manner, substituting in the appropriate values, until the F_{10} generation.

Table 1.5a. Monogenic dominant predictions of DEET-insensitivity as the population is selected.

Generation	Parental	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
% Insensitivity	9	53.42	65.54	76.69	83.17	87.5	90.44	92.51	94	95.1	95.93

1.1.6 DEET-insensitivity is monogenic and recessive (two copies of the allele are required to show the phenotype).

Parental generation: As 9 % of females show DEET-insensitivity (preliminary trials, data not shown), the starting frequency of the trait in the population is 0.09. According to Hardy-Weinburg as shown in 5), the proportion of *II* in the population is therefore 0.09, as two *I* alleles are required for DEET-insensitivity.

The frequency of the *I* allele in selected females, which must be *II*, and their gametes, is 1.

The frequency of the *I* allele in the unselected males is equal to the frequency of the *I* allele in the population, which is $\overline{\quad}$ = 0.3.

F_1 generation: All mosquitoes will have inherited an *I* allele from the female parent. The proportion of *II* homozygotes is therefore 1 x 0.3 (probability of getting an *I* from mother and father) = 0.3. The proportion of heterozygotes is 1 – 0.3 (proportion of *II*) = 0.7.

The frequency of insensitivity in the F1 is 0.3, or 30%.

The frequency of the *I* allele in the gametes of selected females, which must be *II*, is 1.

The frequency of the *I* allele in the gametes of the unselected males is the frequency of *I* in the population, 0.3 + (0.5 x0.7) = 0.65.

F_2 generation: All mosquitoes will have inherited an *I* allele from the female parent. The proportion of *II* homozygotes is therefore 1 x 0.65 (probability of getting an *I* from mother and father) = 0.65. The proportion of heterozygotes is 1 – 0.65 (proportion of *II*) = 0.35.

The frequency of insensitivity in the F1 is 0.65, or 65%.

Calculations proceed in this manner, substituting in the appropriate values, until the F_{10} generation.

Table 1.6a. Monogenic recessive predictions of DEET-insensitivity as the population is selected.

Generation	Parental	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
% Insensitivity	9	30	65	82.5	91.25	95.63	97.82	98.91	99.45	99.73	99.86

1.1.7 DEET insensitivity is monogenic, dominant, and has a penetrance of 60% (only 60% of DEET-insensitive genotype display the phenotype).

The i line, selecting for DEET-insensitivity

Parental generation: Based on selection for DEET-insensitivity in this study, 13% of unselected mosquitoes display DEET-insensitivity (Section 2.3.2). As there is only 60% penetrance, the proportion of mosquitoes with a genotype conferring insensitivity is actually 0.13 / 0.6 = 0.2167. Both *II* homozygotes and heterozygotes will confer insensitivity as the trait is dominant, so the proportion of *SS* homozygotes is 1 – 0.2167 = 0.7833.

From Hardy Weinburg, as described in 5), the frequency of the *S* allele in the population is $\overline{\qquad}$ = 0.885. The frequency of the *I* allele in the population is therefore 1 – 0.885 = 0.115.

The proportion of *II* homozygotes in the population is 0.115^2 = 0.0132.

The proportion of heterozygotes in the population is the frequency of *I* x frequency of *S* x 2 (as it is possible to have either *IS* or SI) = 0.885 x 0.115 x 2 = 0.2035.

The frequency of the *I* allele in the selected insensitive female gametes is the proportion of *II* + half the proportion of heterozygotes, divided by the proportion of mosquitoes with an insensitive genotype = (0.0132 + 0.5 x 0.2035) / 0.2167 = 0.531.

The frequency of the *I* allele in the male gametes is the same as the frequency of *I* in the population, 0.115.

F_1 generation: The proportion of *II* homozygotes is predicted by the probability of obtaining an *I* allele from the female parent and the male parent = 0.531 x 0.115 = 0.0610.

The proportion of heterozygotes is 0.531 x (1-0.115) (if *I* from mother and *S* from father) + 0.115 x (1-0.531) (if *S* from mother and *I* from father) = 0.5237.

The proportion of *SS* homozygotes is 1 – (0.115 + 0.531) (the proportion of *II* homozygotes + heterozygotes) = 0.4153.

The frequency of insensitivity in the F_1 i line is 0.0610 (*II* homozygotes) + 0.5237 (heterozygotes) x 0.6 (proportion of mosquitoes with an insensitive genotype) = 0.3508 or 35.08%.

The frequency of the *I* allele in the selected female gametes, is (0.0610 + 0.5 x 0.5237) / (0.0610 + 0.5237) = 0.5222.

The frequency of the *I* allele in the male gametes is 0.0610 + 0.5 x 0.5237 = 0.3229.

F_2 generation: The proportion of *II* homozygotes is predicted by the probability of obtaining an *I* allele from the female parent and the male parent = 0.552 x 0.3229 = 0.1783.

The proportion of heterozygotes is 0.5522 x (1-0.3229) (if *I* from mother and *S* from father) + 0.3229 x (1-0.5522) (if *S* from mother and *I* from father) = 0.5185.

The proportion of *SS* homozygotes is 1 – (0.3229 + 0.5522) (the proportion of *II* homozygotes + heterozygotes) = 0.3032.

The frequency of insensitivity in the F_2 i line is (0.1783 (*II* homozygotes) + 0.5185 (heterozygotes)) x 0.6 (proportion of mosquitoes with an insensitive genotype) = 0.4181 or 41.81%.

Calculations proceed in this manner, substituting in the appropriate values, until the F_{10} generation.

The s line, selecting for DEET-sensitivity

Parental generation: 13% of unselected mosquitoes display DEET-insensitivity (Section 2.3.2). With 60% penetrance, 0.13 / 0.6 = 0.2167 of mosquitoes have a genotype conferring insensitivity. Both *II* homozygotes and heterozygotes will confer insensitivity as the trait is dominant, so the proportion of *SS* homozygotes is 1 – 0.2167 = 0.7833. The frequencies of the *I* and *S* alleles are 0.115 and 0.885 respectively, as discussed for the i line.

Females with a DEET-sensitive phenotype will be the *SS* homozygotes and 40% of the *II* and *IS* females. Females selected as sensitive will therefore be:

SS 0.7833 (proportion of *SS* in population) / 0.87 (proportion displaying sensitivity) = 0.9003.

IS 0.2035 (proportion *IS*) x 0.4 / 0.87 = 0.0936.

II 0.0132 (proportion *II*) x 0.4 / 0.87 = 0.0061.

The frequency of the *I* allele in the selected sensitive female gametes is 0.0061 (*II*) + (0.0936 (*IS*) x 0.5) = 0.0529.

The frequency of the *I* allele in the male gametes is the frequency of *I* in the population, 0.115.

F_1 generation: The proportion of *II* homozygotes is predicted by the probability of obtaining an *I* allele from the female parent and the male parent = 0.0529 x 0.115 = 0.0061.

The proportion of heterozygotes is 0.0529 x (1-0.1150) (if *I* from mother and *S* from father) + 0.1150 x (1-0.0529) (if *S* from mother and *I* from father) = 0.1557.

The proportion of *SS* homozygotes is 1 – (0.0061 + 0.1557) (the proportion of *II* homozygotes + heterozygotes) = 0.8382.

The frequency of insensitivity in the F_1 generation of the s line is (0.0061 + 0.1557) x 0.6 = 0.0971, or 9.71%.

The frequency of the *I* allele in the selected sensitive female gametes is 0.4 (proportion of insensitive females showing sensitive phenotype) x (0.0061 (*II*) + 0.5 x 0.1557 (*IS*)) / (1-0.0971) (frequency of insensitivity) = 0.0372.

The frequency of the *I* allele in the male gametes is 0.0061 + (0.5 x 0.1557) = 0.0839.

F_2 generation: The proportion of *II* homozygotes is predicted by the probability of obtaining an *I* allele from the female parent and the male parent = 0.0372 x 0.0839 = 0.0031.

The proportion of heterozygotes is 0.0372 x (1-0.0839) (if *I* from mother and *S* from father) + 0.0839 x (1-0.0372) (if *S* from mother and *I* from father) = 0.1149.

The proportion of *SS* homozygotes is 1 – (0.0031 + 0.1149) (the proportion of *II* homozygotes + heterozygotes) = 0.8820.

The frequency of insensitivity in the F_2 generation of the s line is (0.0031 + 0.1149) x 0.6 = 0.0708, or 7.08%.

Calculations proceed in this manner, substituting in the appropriate values, until the F_{10} generation.

Table 1.7a. 60% penetrance monogenic dominant predictions of DEET-insensitivity as the population is selected for the i and s lines.

Generation	Parental	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
% Insensitivity in the i line	13	35.08	41.81	47.44	50.85	53.14	54.71	55.82	56.63	57.23	57.69
% Insensitivity in the s line	13	9.71	7.08	5.1	3.65	2.59	1.83	1.29	0.91	0.64	0.45

1.1.8 DEET insensitivity is monogenic, dominant, and has a penetrance of 60% (only 60% of DEET-insensitive genotype display the phenotype), with 8% of sensitive mosquitoes showing insensitivity.

The i line, selecting for DEET-insensitivity

Parental generation: 13% with insensitive phenotype. 8% of these are sensitive mosquitoes displaying an insensitive phenotype. 5% of the 13% are therefore genetically insensitive mosquitoes. The

insensitivity of *II* and *IS* is 60%, 52% higher than the 8% insensitivity of sensitive mosquitoes. The proportion of *II* and *IS* is therefore 0.05/0.52 = 0.0962. The proportion of *SS* is 1 – 0.0962 = 0.9038. According to Hardy-Weinburg, the frequency of the *I* allele in the population is 0.0493, and the frequency of the *S* allele in the population is 0.9507.

In the selected females, the proportions of the genotypes are:

II= 0.0493^2 (proportion of *II*) x 0.6 (proportion showing phenotype) / 0.13 (proportion insensitive) =0.0112.

IS= (2 x 0.0493 x 0.9507)(*IS*) x 0.6 / 0.13 = 0.4326.

SS= 0.9507^2 (proportion of *SS*) x 0.08 / 0.13 = 0.5562.

The frequency of the *I* allele in the gametes of selected insensitive females is 0.0112 + (0.5 x 0.4326) = 0.2275.

The frequency of the *I* allele in the gametes of males is the frequency of the *I* allele in the population, 0.0493.

F_1 generation: The proportion of *II* homozygotes is predicted by the probability of obtaining an *I* allele from the female parent and the male parent = 0.2275 x 0.0493 = 0.0112.

The proportion of heterozygotes is 0.2275 x (1-0.0493) (if *I* from mother and *S* from father) + 0.0493 x (1-0.2275) (if *S* from mother and *I* from father) = 0.2544.

The proportion of *SS* homozygotes is 1 – (0.0112 + 0.2544) (the proportion of *II* homozygotes + heterozygotes) = 0.7344.

The proportion of the population that are *II* and showing insensitivity = 0.0112 x 0.6 = 0.0067.

The proportion of the population that are *IS* and showing insensitivity = 0.2544 x 0.6 = 0.1526.

The proportion of the population that are *SS* and showing insensitivity = 0.7344 x 0.08 = 0.0588.

The frequency of the insensitivity phenotype in the F_1 generation of the i line is 0.0067 + 0.1526 + 0.0588 = 0.2181, or 21.81%.

The frequency of the *I* allele in the gametes of selected insensitive females is 0.0067 + (0.5 x 0.1526) / 0.2181 = 0.3807.

The frequency of the *I* allele in the gametes of males is 0.0112 + (0.5 x 0.2544) = 0.1384.

F_2 generation: The proportion of *II* homozygotes is predicted by the probability of obtaining an *I* allele from the female parent and the male parent = 0.3807 x 0.1384 = 0.0527.

The proportion of heterozygotes is 0.3807 x (1-0.1384) (if *I* from mother and *S* from father) + 0.1384 x (1-0.3807) (if *S* from mother and *I* from father) = 0.4138.

The proportion of *SS* homozygotes is 1 – (0.0527 + 0.4138) (the proportion of *II* homozygotes + heterozygotes) = 0.5335.

The proportion of the population that are *II* and showing insensitivity = 0.0527 x 0.6 = 0.0316.

The proportion of the population that are *IS* and showing insensitivity = 0.4138 x 0.6 = 0.2483.

The proportion of the population that are *SS* and showing insensitivity = 0.5335 x 0.08 = 0.0427.

The frequency of the insensitivity phenotype in the F_2 generation of the i line is 0.0316 + 0.2483 + 0.0427 = 0.3225, or 32.25%.

Calculations proceed in this manner, substituting in the appropriate values, until the F_{10} generation.

The s line, selecting for DEET-sensitivity

Parental generation: 13% with insensitive phenotype. 8% of these are sensitive mosquitoes displaying an insensitive phenotype. 5% of the 13% are therefore genetically insensitive mosquitoes. The insensitivity of *II* and *IS* is 60%, 52% higher than the 8% insensitivity of sensitive mosquitoes. The proportion of *II* and *IS* is therefore 0.05/0.52 = 0.0962. The proportion of *SS* is 1 – 0.0962 = 0.9038. According to Hardy-Weinburg, the frequency of the *I* allele in the population is 0.0493, and the frequency of the *S* allele in the population is 0.9507.

The proportions of the genotypes are:

II= 0.0493^2 = 0.0024.

IS= 2 x 0.0493 x 0.9507 = 0.0937.

SS= 0.9507^2 = 0.90939.

The proportion of the population that are *II* and showing sensitivity = 0.0024 x 0.4 = 0.0009.

The proportion of the population that are *IS* and showing sensitivity = 0.0937 x 0.4 = 0.0375.

The proportion of the population that are *SS* and showing sensitivity = 0.9039 x 0.92 = 0.8316.

The proportion of females with the sensitive phenotype in the F_0 generation of the s line is 0.0009 + 0.0375 + 0.8316 = 0.87. The proportion of insensitive females in the F_0 generation of the s line is therefore 1 – 0.87 = 0.13.

The frequency of the *I* allele in the gametes of selected sensitive females is 0.0009 + (0.5 x 0.0375) / 0.87 = 0.0227.

The frequency of the *I* allele in the gametes of males is 0.0024 + (0.5 x 0.0937) = 0.0493.

F_1 generation: The proportion of *II* homozygotes is predicted by the probability of obtaining an *I* allele from the female parent and the male parent = 0.0227 x 0.0493 = 0.0011.

The proportion of heterozygotes is 0.0227 x (1-0.0493) (if *I* from mother and *S* from father) + 0.0493 x (1-0.0227) (if *S* from mother and *I* from father) = 0.0697.

The proportion of *SS* homozygotes is 1 – (0.0011 + 0.0697) (the proportion of *II* homozygotes + heterozygotes) = 0.9292.

The proportion of the population that are *II* and showing sensitivity = 0.0011 x 0.4 = 0.0004.

The proportion of the population that are *IS* and showing sensitivity = 0.0697 x 0.4 = 0.0279.

The proportion of the population that are *SS* and showing sensitivity = 0.9292 x 0.92 = 0.8548.

The proportion of females with the sensitive phenotype in the F_0 generation of the s line is 0.0004 + 0.0279 + 0.8548 = 0.8831.

The proportion of females with the insensitive phenotype in the F_1 generation of the s line is therefore 1 – 0.8831 = 0.1169, or 11.69%.

The frequency of the *I* allele in the gametes of selected insensitive females is 0.0004 + (0.5 x 0.0279) / 0.8831 = 0.0163.

The frequency of the *I* allele in the gametes of males is 0.0011 + (0.5 x 0.0697) = 0.0360.

F_2 generation: The proportion of *II* homozygotes is predicted by the probability of obtaining an *I* allele from the female parent and the male parent = 0.0163 x 0.036 = 0.0006.

The proportion of heterozygotes is 0.0163 x (1-0.036) (if *I* from mother and *S* from father) + 0.036 x (1-0.0163) (if *S* from mother and *I* from father) = 0.0511.

The proportion of *SS* homozygotes is 1 – (0.0006 + 0.0511) (the proportion of *II* homozygotes + heterozygotes) = 0.9483.

The proportion of the population that are *II* and showing sensitivity = 0.0006 x 0.4 = 0.0002.

The proportion of the population that are *IS* and showing sensitivity = 0.0511 x 0.4 = 0.0204.

The proportion of the population that are *SS* and showing sensitivity = 0.9483 x 0.92 = 0.8725.

The proportion of females with the sensitive phenotype in the F_2 generation of the s line is 0.0002 + 0.0204 + 0.8725 = 0.8931.

The proportion of females with the insensitive phenotype in the F_2 generation of the s line is therefore 1 – 0.8931 = 0.1069, or 10.69%.

Calculations proceed in this manner, substituting in the appropriate values, until the F_{10} generation.

Table 1.8a. 60% penetrance monogenic dominant predictions of DEET-insensitivity, with 8% of sensitive mosquitoes phenotypically insensitive, as the population is selected for the i and s lines.

Generation	Parental	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
% Insensitivity in the i line	13	21.81	32.25	40.08	45.79	49.32	51.84	53.63	54.92	55.92	56.62
% Insensitivity in the s line	13	11.69	10.69	9.95	9.42	9.03	8.74	8.53	8.38	8.27	8.2

1.1.9 DEET insensitivity is due to monogenic, incomplete dominance, with 60% penetrance.

The i line, selecting for DEET-insensitivity

Due to incomplete dominance, the *II* homozygotes show insensitivity 60% of the time, and the heterozygotes show insensitivity 30% of the time. *SS* homozygotes display no insensitivity to DEET.

Parental generation: The 13% insensitive is formed from 0.6 $p(I)^2$ + 0.3 x 2 x $p(I)$ x (1 – $p(I)$) to account for the *II* homozygotes and the *IS* and SI heterozygotes. This equation can be rearranged to give 0.6 x $p(I)$ = 0.13.

Therefore the frequency of the *I* allele, $p(I)$, in the population is 0.21667.

The proportion of *II* homozygotes in the population is found through Hardy-Weinburg as $p(I)^2$ = 0.0469.

The proportion of heterozygotes in the population is 2 x 0.2167 x (1 – 0.2167) = 0.3395.

The proportion of *SS* homozygotes in the population is $(1 – 0.2167)^2$ = 0.6136.

In the selected insensitive females, the proportions of the genotypes are:

II= 0.0469 (proportion of *II*) x 0.6 (proportion showing phenotype) / 0.13 (proportion insensitive) =0.2166.

IS=0.3395 x 0.3 / 0.13 = 0.7834.

The frequency of the *I* allele in the gametes of selected insensitive females is 0.2166 + (0.5 x 0.7834) = 0.6083.

The frequency of the *I* allele in the gametes of males is the frequency of the *I* allele in the population, 0.2167.

F_1 generation: The proportion of *II* homozygotes is predicted by the probability of obtaining an *I* allele from the female parent and the male parent = 0.6083 x 0.2167= 0.1318.

The proportion of heterozygotes is 0.6083 x (1-0.2167) (if *I* from mother and *S* from father) + 0.2167 x (1-0.6083) (if *S* from mother and *I* from father) = 0.5614.

The proportion of *SS* homozygotes is 1 – (0.1318 + 0.5614) (the proportion of *II* homozygotes + heterozygotes) = 0.3068.

The frequency of insensitivity in the F_1 i line is 0.1318 x 0.6 (*II* homozygotes phenotypically insensitive) + 0.5614 x 0.3 (heterozygotes phenotypically insensitive) = 0.2475 or 24.75%.

In the selected insensitive females, the proportions of the genotypes are:

II = 0.1318 x 0.6 (proportion of *II* showing phenotype) / 0.2475 (proportion insensitive) =0.3195.

IS =0.5614 x 0.3 / 0.2475 = 0.6805.

The frequency of the *I* allele in the selected female gametes, is 0.3195 + (0.5 x 0.6805) = 0.6598.

The frequency of the *I* allele in the male gametes is 0.1318 + (0.5 x 0.5614) = 0.4125.

F_2 generation: The proportion of *II* homozygotes is predicted by the probability of obtaining an *I* allele from the female parent and the male parent = 0.6598 x 0.4125 = 0.2721.

The proportion of heterozygotes is 0.6598 x (1-0.4125) (if *I* from mother and *S* from father) + 0.4125 x (1-0.6598) (if *S* from mother and *I* from father) = 0.5280.

The proportion of *SS* homozygotes is 1 – (0.2721 + 0.5280) (the proportion of *II* homozygotes + heterozygotes) = 0.1999.

The frequency of insensitivity in the F_2 i line is 0.2721 x 0.6 (*II* homozygotes insensitive) + 0.5280 x 0.3 (heterozygotes insensitive) = 0.3217 or 32.17%.

Calculations proceed in this manner, substituting in the appropriate values, until the F_{10} generation.

The s line, selecting for DEET-sensitivity

Parental generation: The 13% insensitive is formed from 0.6 p*(I)*^{2} + 0.3 x 2 x p*(I)* x (1 – p*(I)*) to account for the *II* homozygotes and the *IS* and SI heterozygotes. This equation can be rearranged to give 0.6 x p*(I)* = 0.13.

Therefore the frequency of the *I* allele, p*(I)*, in the population is 0.21667.

The proportion of *II* homozygotes in the population is found through Hardy-Weinburg as *p(I)*^{2} = 0.0469.

The proportion of heterozygotes in the population is 2 x 0.2167 x (1 – 0.2167) = 0.3395.

The proportion of *SS* homozygotes in the population is (1 – 0.2167)^2 = 0.6136.

Females with a DEET-sensitive phenotype will be the *SS* homozygotes, 40% of the *II* females, and 70% of the *IS* females. Females selected as sensitive will therefore be:

SS 0.6138 (proportion of *SS* in population) / 0.87 (proportion displaying sensitivity) = 0.7053.

IS 0.3395 (proportion *IS*) x 0.7 / 0.87 = 0.2731.

II 0.0132 (proportion *II*) x 0.4 / 0.87 = 0.0216.

The frequency of the *I* allele in the selected sensitive female gametes is 0.0216 (*II*) + (0.5 x 0.2731 (*IS*)) = 0.1581.

The frequency of the *I* allele in the male gametes is the frequency of *I* in the population, 0.2167.

F_1 generation: The proportion of *II* homozygotes is predicted by the probability of obtaining an *I* allele from the female parent and the male parent = 0.1581 x 0.2167 = 0.0343.

The proportion of heterozygotes is 0.1581x (1-0.2167) (if *I* from mother and *S* from father) + 0.2167 x (1-0.1581) (if *S* from mother and *I* from father) = 0.3063.

The proportion of *SS* homozygotes is 1 – (0.0343 + 0.3063) (the proportion of *II* homozygotes + heterozygotes) = 0.6594.

The frequency of insensitivity in the F_1 generation of the s line is (0.0343 x 0.6) + (0.3063 x 0.3) = 0.1124, or 11.24%.

The frequency of sensitivity is therefore 0.8876, or 88.76%.

In females selected as sensitive the insensitive genotypes will be:

II 0.0343 (proportion *II*) x 0.4 / 0.8876 = 0.0155.

IS 0.3063 (proportion *IS*) x 0.7 / 0.8876 = 0.2416.

The frequency of the *I* allele in the selected sensitive female gametes is 0.0155 +(0.5 x 0.2416) = 0.1363.

The frequency of the *I* allele in the male gametes is 0.0343 + (0.5 x 0.3063) = 0.1874.

F_2 generation: The proportion of *II* homozygotes is predicted by the probability of obtaining an *I* allele from the female parent and the male parent = 0.1363 x 0.1874 = 0.0255.

The proportion of heterozygotes is 0.1363 x (1-0.1874) (if *I* from mother and *S* from father) + 0.1874 x (1-0.1363) (if *S* from mother and *I* from father) = 0.2726.

The proportion of *SS* homozygotes is 1 – (0.0255 + 0.2726) (the proportion of *II* homozygotes + heterozygotes) = 0.7019.

The frequency of insensitivity in the F_2 generation of the s line is (0.0255 x 0.6) + (0.2726 x 0.3) = 0.0971, or 9.71%.

Calculations proceed in this manner, substituting in the appropriate values, until the F_{10} generation.

Table 1.9a. Intermediate dominance predictions of DEET-insensitivity as the population is selected for the i and s lines.

Generation	Parental	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
% Insensitivity in the i line	13	24.75	32.17	38.70	43.75	47.67	50.67	52.96	54.69	56.01	57
% Insensitivity in the s line	13	11.24	9.71	8.36	7.19	6.17	5.29	4.53	3.97	3.31	2.82

1.1.10 DEET insensitivity is monogenic, recessive, and has a penetrance of 60% (only 60% of DEET-insensitive genotype display the phenotype).

The i line, selecting for DEET-insensitivity

Parental generation: As insensitivity is recessive, only homozygous *II* females will have the trait. If only 60% of mosquitoes show the insensitivity, and an initial 13% are insensitive, this means that the proportion of *II* homozygotes in the population is actually 0.13 / 0.6 = 0.21667. The frequency of the *I* allele, according to Hardy-Weinburg, is therefore $\overline{\qquad}$ = 0.4655.

In the gametes of selected insensitive females, which must be *II* homozygotes, the frequency of the *I* allele is 1.

In the gametes of males, the frequency of the *I* allele is equal to the frequency in the population = 0.4655.

F_1 generation: The proportion of *II* homozygotes is predicted by the probability of obtaining an *I* allele from the female parent and the male parent = 1 x 0.4655 = 0.4655.

The proportion of heterozygotes is 1 - 0.4655 (I from mother and *S* from father) = 0.5345.

The frequency of insensitivity in the F_1 generation of the i line is 0.4655 x 0.6 = 0.2793, or 27.93%.

In the gametes of selected insensitive females, which must be *II* homozygotes, the frequency of the *I* allele is 1.

In the gametes of males, the frequency of the *I* allele is 0.4655 = (0.5 x 0.5345) = 0.7327.

F_2 generation: The proportion of *II* homozygotes is predicted by the probability of obtaining an *I* allele from the female parent and the male parent = 1 x 0.7327 = 0.7327.

The proportion of heterozygotes is 1 - 0.7327 (I from mother and *S* from father) = 0.2673.

The frequency of insensitivity in the F_2 generation of the i line is 0.7327 x 0.6 = 0.4396, or 43.96%.

Calculations proceed in this manner, substituting in the appropriate values, until the F_{10} generation.

The s line, selecting for DEET-sensitivity

Parental generation: As insensitivity is recessive, only homozygous *II* females will have the trait. If only 60% of mosquitoes show the insensitivity, and an initial 13% are insensitive, this means that the proportion of *II* homozygotes in the population is actually 0.13 / 0.6 = 0.21667. The frequency of the *I* allele, according to Hardy-Weinburg, is therefore $\sqrt{}$ = 0.4655.

Proportion of *II* = 0.4655^2 = 0.2167.

Proportion of *IS* = 2 x 0.4655 x (1 – 0.4655) = 0.4976.

Proportion of *SS* = $(1 – 0.4655)^2$ = 0.2857.

Proportion insensitive = 0.2167 x 0.6 = 0.13.

Proportion sensitive = 1 – 0.13 = 0.87.

In the selected sensitive females, the proportions of the genotypes are:

II = 0.2167 x 0.4 (proportion of *II* not showing insensitivity) / 0.87 (proportion population sensitive) = 0.0996.

IS = 0.4976 / 0.87 = 0.5720.

SS = 0.2857 / 0.87 = 0.3284.

In the gametes of selected sensitive females the frequency of the *I* allele is 0.0996 + (0.5 x 0.5720) = 0.3856.

In the gametes of males, the frequency of the *I* allele is equal to the frequency in the population = 0.4655.

F_1 generation: The proportion of *II* homozygotes is predicted by the probability of obtaining an *I* allele from the female parent and the male parent = 0.3856 x 0.4655 = 0.1795.

The proportion of heterozygotes is 0.3856 x (1 - 0.4655) (if *I* from mother and *S* from father) + 0.4655 x (1 -0.3856) (if *S* from mother and *I* from father) = 0.4921.

The proportion of *SS* homozygotes is 1 – (0.1795 + 0.4921) (the proportion of *II* homozygotes + heterozygotes) = 0.3284.

The frequency of insensitivity in the F_1 generation of the s line is 0.1795 x 0.6 = 0.1077, or 10.77%.

The frequency of sensitivity is 1 – 0.1077 = 0.8923.

In the selected sensitive females, the proportions of the genotypes are:

II = 0.1795 x 0.4 (proportion of *II* not showing insensitivity) / 0.8923 (proportion population sensitive) = 0.0805.

IS = 0.4921 / 0.8923 = 0.5515.

SS = 0.3284 / 0.8923 = 0.3680.

In the gametes of selected sensitive females the frequency of the *I* allele is 0.0805 + (0.5 x 0.5515) = 0.3562.

In the gametes of males, the frequency of the *I* allele is 0.1795 + (0.5 x 0.4921) = 0.4255.

F_2 generation: The proportion of *II* homozygotes is predicted by the probability of obtaining an *I* allele from the female parent and the male parent = 0.3562 x 0.4255 = 0.1516.

The proportion of heterozygotes is 0.3562 x (1 - 0.4255) (if *I* from mother and *S* from father) + 0.4255 x (1 -0.3562) (if *S* from mother and *I* from father) = 0.4786.

The proportion of *SS* homozygotes is 1 – (0.1516 + 0.4786) (the proportion of *II* homozygotes + heterozygotes) = 0.3698.

The frequency of insensitivity in the F_2 generation of the s line is 0.1516 x 0.6 = 0.0909, or 9.09%.

Calculations proceed in this manner, substituting in the appropriate values, until the F_{10} generation.

Table 1.10a. 60% penetrance monogenic recessive predictions of DEET-insensitivity as the population is selected for the i and s lines.

Generation	**Parental**	**F_1**	**F_2**	**F_3**	**F_4**	**F_5**	**F_6**	**F_7**	**F_8**	**F_9**	**F_{10}**
% Insensitivity in the i line	13	27.93	43.96	51.98	55.99	58	59	59.5	59.75	59.88	59.94
% Insensitivity in the s line	13	10.77	9.09	7.74	6.63	5.73	4.98	4.36	3.84	3.41	3.03

Appendix 2. Collection of experimental mosquitoes from the culture cage

To determine if mosquitoes should be selected at random from the culture cage for repellency bioassays, or from those already known to respond to human odour, a trial was carried out to test differences between the responses of mosquitoes in a repellency assay chosen according to their location in the culture cage when a human was present in front of the cage (human odours attract normally responding mosquitoes to the front of the cage). It was important when determining the DEET-sensitivity of mosquitoes that all females were actively host seeking. If a female was not actively host seeking, it might be counted as sensitive to DEET even if it was insensitive.

Methods

Each trial consisted of 10 test cages, with 10 mosquitoes in each cage. Five test cages contained mosquitoes taken from the back and sides of culture cages (BS) and thus not considered to be actively responding to human odour, and five test cages contained mosquitoes actively moving to the front of the culture cage (F) when a human was present. Cages were tested with a control arm (methods as in Section 2.2.3) to measure the number of mosquitoes probing. In order to test more than 100 mosquitoes, the experiment was repeated several times over 2-3 days. Three hundred F mosquitoes and 300 BS mosquitoes were tested. The responses of mosquitoes from the two locations were compared using a Student's t-test in Genstat® (12th edition).

Results and discussion

Significantly more mosquitoes that were collected from the front of the culture cage probed during the trial compared to those collected from the back and sides of the culture cage ($p<0.001$) (Fig 2.1a). This indicates mosquitoes shown to be responding to human odour prior to the experiment are more likely to probe during the experiment. Mosquitoes taken from the back and sides of the cage have a ~40% chance of not responding to an attractant. Therefore when tested with DEET, these mosquitoes might appear sensitive even if they are not. Mosquitoes for the repellency bioassay were therefore taken from the front of the culture cage.

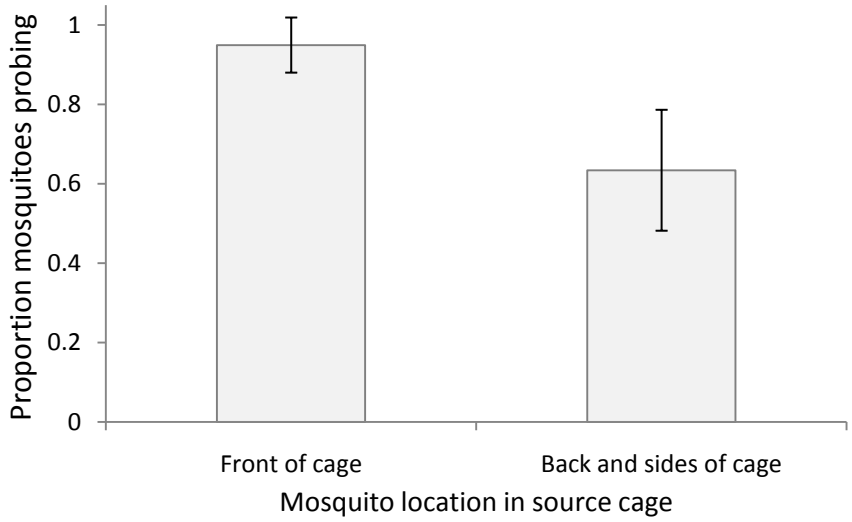

Fig 2.1a Proportion of *Aedes aegypti* females probing in response to a human arm when taken from the front, or back and sides of the colony cage. Means are ± SEM.

Appendix 3. Repellency bioassay methods

Repellency trials in which the volunteer's arm is inserted into the cage are standard (World Health Organisation, 1996; Frances *et al.*, 1998; Tawatsin *et al.*, 2001), but a method in which there is a barrier between the arm and the cage (Chou *et al.*, 1997; Dogan and Rossignol, 1999) would be preferable for this study as it would not require direct contact, with the likelihood of being bitten, with the mosquitoes.

Methods

Mosquitoes from the unselected culture and the i line of a preliminary selection experiment (data not shown) were tested with two different repellency trial methods to see if the results were comparable. Arm-on-cage and arm-in-cage methods were compared. For the arm-in cage method, the arm was inserted into the cage through a netting sleeve with a nitrile glove covering the hand. The mosquitoes landing on and probing a DEET-covered arm were counted as insensitive to DEET. All other procedures remained the same as in the arm-on-cage method (Section 2.2.3). For both methods, cages of mosquitoes were tested with either a control arm (0.5 ml ethanol) or DEET (0.5 ml, 20%) on an arm.

The number of mosquitoes successfully probing was analysed using regression analysis in a generalised linear model (GLM) in Genstat® (12th edition), modelling binomial proportions with a logit transformation using replicates as blocks. This was used to obtain predicted means and SEMs. Differences were deemed to be significant when the difference between means was greater than the LSD.

Results

When the arm was inserted into the cage, both the unselected and i line probed significantly less in response to a DEET-covered arm than to a control arm ($p<0.001$) (Fig 3.2a A). There was no difference between the proportion of mosquitoes probing for the i line compared to the unselected culture in response to a DEET covered arm.

When the arm was held over a mesh outside the cage, both the unselected and i line probed significantly less in response to a DEET-covered arm than to a control arm ($p<0.001$) (Fig 3.2a B). Significantly more mosquitoes from the i line probed in response to a DEET covered arm than mosquitoes from the unselected culture ($P=0.012$).

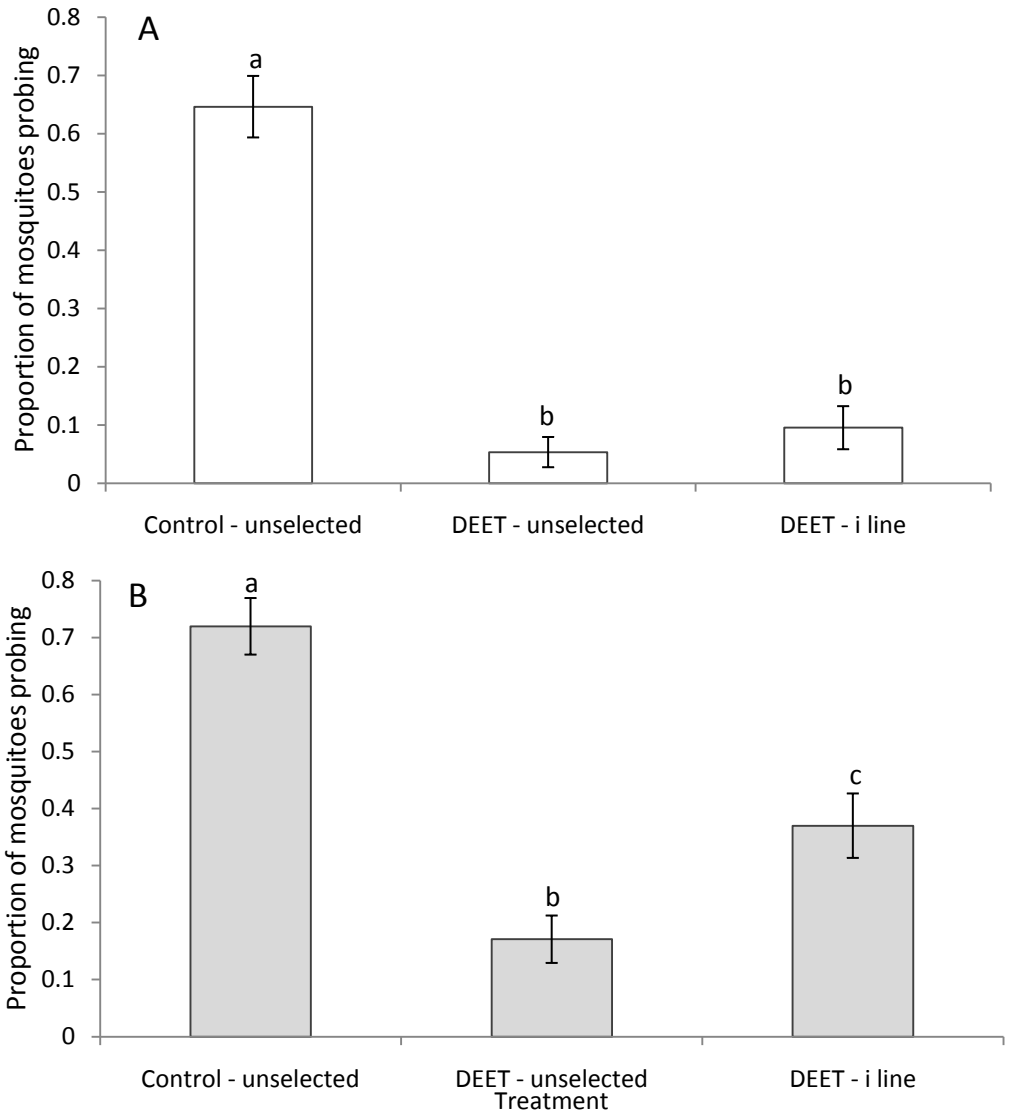

Fig 3.2a Proportion of unselected culture or i line *Aedes aegypti* females probing in response to a control arm (hexane, 0.5 ml) or DEET (20%, 0.5 ml) covered arm when tested with A) the arm inserted into the cage through a netting sleeve and B) the arm held outside the cage over a mesh through which volatiles could pass. Means are ± SEM.

Discussion

In our study the arm-on-cage method was adapted from previous repellency studies and a WHOPES method (World Health Organisation, 1996; Chou *et al.*, 1997; Dogan and Rossignol, 1999). This method was ideal for measuring the repellent effects of DEET without allowing direct contact with and probing of the volunteer's arm. Both methods showed a reduction in attraction compared to the controls when there was DEET present for both the unselected culture and the preliminary i line. However, the arm-on-cage method showed a difference in response to DEET between the culture and the i line, with the i line more insensitive to DEET, that was not seen in the arm-in-cage method. This may have been due to the difference in the way volatiles would have dispersed during the experiment. For the arm-in-cage method, the mosquitoes were much closer to the repellent-covered arm, which may have led to a higher effective concentration and build-up of DEET. At higher concentrations, i line mosquitoes which would normally be insensitive to DEET may have shown a sensitive phenotype. As DEET also works as a contact repellent, this additional element would have been present in the arm-in cage trials. In the arm-on-cage method, the volatiles are being drawn through the mesh rather than being in the same immediate area as the mosquitoes. This may have caused a spatial repellency effect, with the mosquitoes using long distance volatile detection. There may be separate mechanisms working in the detection of volatiles by olfactory receptors at range, and the close contact landing and probing behaviour. At close range the mechanisms which cause the DEET-insensitivity seen in this study may be overridden by a different mode of action of DEET involved with contact. As both methods show DEET to be repellent, and the arm-on-cage method shows additional sensitivity in detecting differences in individual mosquito's responses to DEET, the arm-on-cage method was selected as a suitable comparative assay for the response of mosquitoes to the repellent (Chou *et al.*, 1997; Dogan and Rossignol, 1999).

Appendix 4. Air entrainment of DEET on tights

In order to avoid chemicals being applied directly to the skin, they could be applied to a section of tights drawn over the arm. To determine if this method was suitable in reducing the amount of chemical in contact with the skin, air entrainments of the tights on the arm were performed.

Methods

Two layers of tights (Boots brand 97% nylon, 3% LYCRA®, small/medium, nude, Denier 10, 12cm long when unstretched) were worn on the arm, with the treated layer on top. When the treatment was applied to the tights, it was allowed 2 mins to evaporate. A concentration of 1% DEET was chosen for this experiment so as not to overload the gas chromatogram.

Air entrainment methods followed those described in Section 3.2.5. An arm was inserted up to the elbow into a multi-purpose cooking bag (250 x 380 mm, Sainsbury's). Charcoal filtered air was pushed in at the top of the bag at 1200 ml min^{-1} and pulled through a glass tube containing 50 mg porapak at the bottom of the bag at 1000 ml min^{-1}. The arm was entrained for 10 mins.

Treatments were:

1) Arm wearing tights with 0.5 ml hexane spotted evenly across them (Control).

2) Arm with 0.5 ml DEET (1%) in ethanol applied (DEET on arm).

3) Arm wearing tights with 0.5 ml DEET (1%) in hexane spotted across them (DEET on tights).

4) Arm after removing tights with 0.5 ml DEET (1%) after 2 mins (Arm after tights).

5) Arm washed with odourless soap (Simple©) and water after removing tights with 0.5 ml (1%) DEET after 2 mins (Washed arm).

The porapak was eluted with 1.5 ml redistilled diethyl ether, and the sample was concentrated to 50 µl. A 4 µl sample was run on a gas chromatogram containing an HP1 column (see Section 3.2.5). The amount of DEET present in each sample was calculated using an external standard quantification method.

The mean amount of DEET collected in the 10 minute entrainment for each treatment was compared using a one-way ANOVA in Genstat® (12^{th} edition). Differences were judged significant when the difference between means was greater than the LSD.

Results and Discussion

There was significantly less DEET on the arm after removing DEET-covered tights than when DEET was applied directly to the arm, or compared to the amount on the tights ($p<0.001$) (Fig 4.1a, 4.2a). The amount of chemical remaining on the arm after the treated tights were removed was significantly higher than the control ($p<0.005$), thus some residual DEET may remain. The entrainments show that there was no difference between the amount of DEET present on the human arm when it had been washed following wearing tights with DEET on, and the control of hexane, thus washing the arm completely removes the treatment. The decrease in the amount of chemical left on the arm when using tights was, therefore, useful for experiments, but the small amount of chemical that does remain made it advisable to wash the arm between treatments, or to test increasing concentrations.

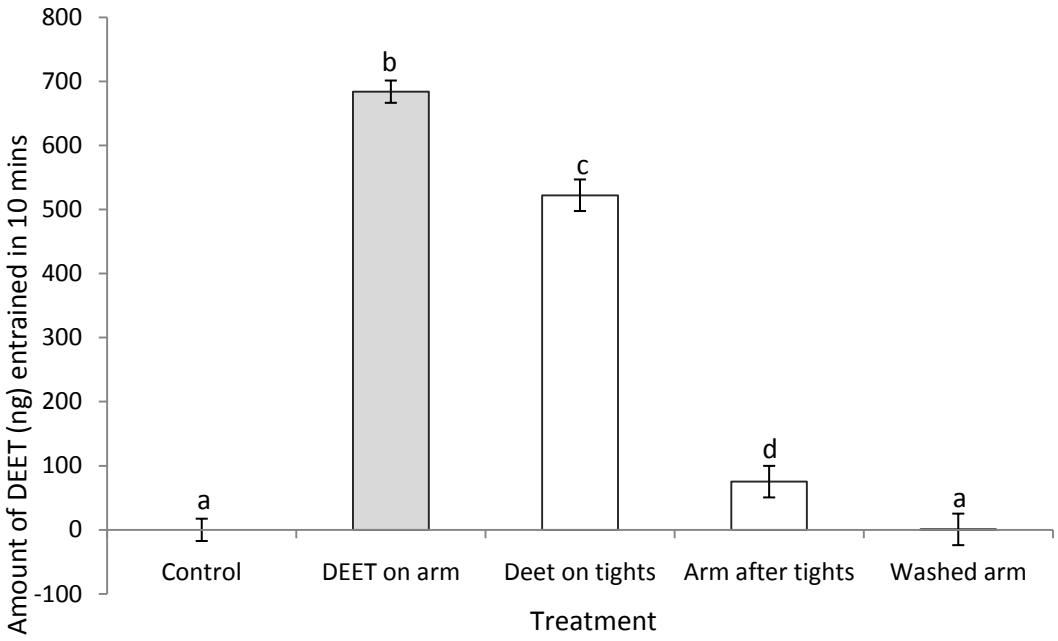

Fig 4.1a The average amount of DEET (ng) entrained from an arm in 10 mins when wearing tights with 0.5 ml hexane spotted evenly across them (Control), without tights with 0.5 ml DEET (1%) in ethanol (DEET on arm), wearing tights with 0.5 ml DEET (1%) in hexane spotted across them (DEET on tights), after removing tights with 0.5 ml DEET (1%) after 2 mins (Arm after tights), and arm washed after removing tights with 0.5 ml DEET (1%) after 2 mins (Washed arm). Means are ± SEM. Means with different letters are significantly different from each other ($p<0.05$).

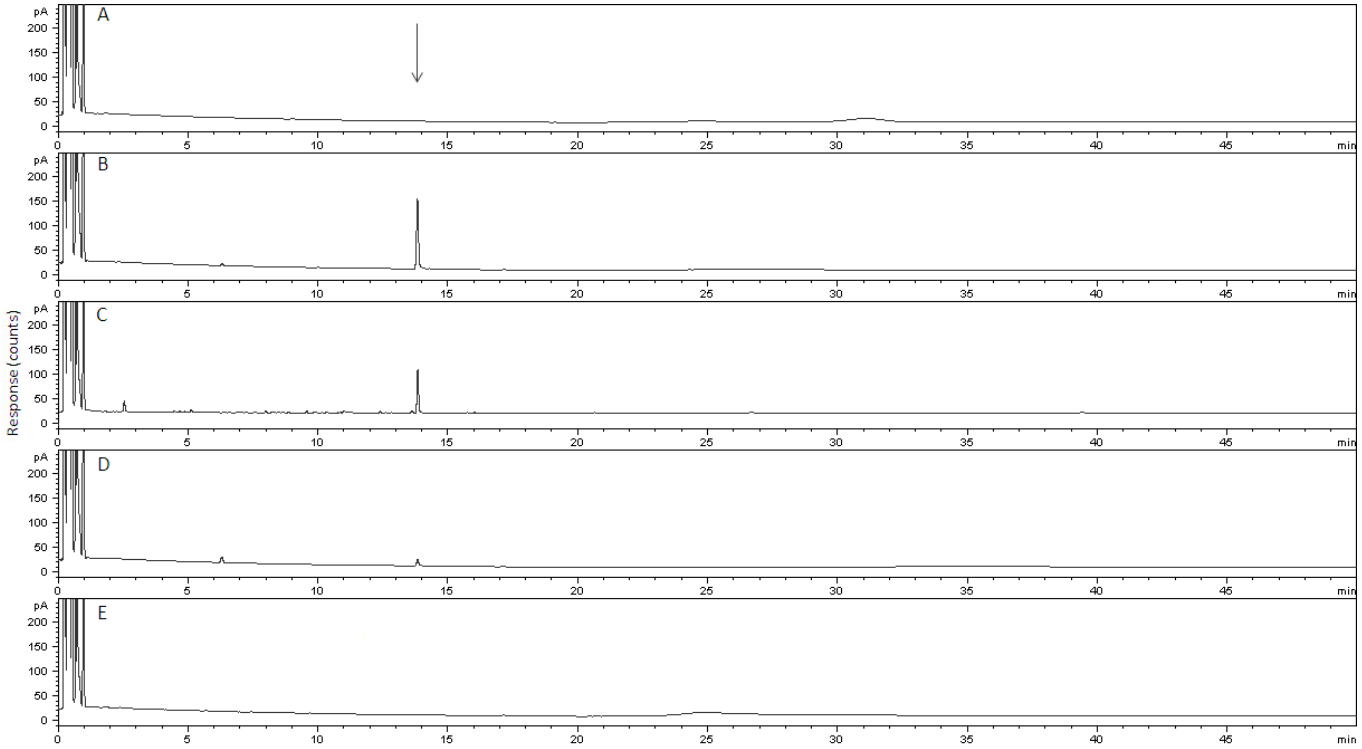

Fig 4.2a Typical gas chromatograms of 10 min air entrainment extracts from A) an arm when wearing tights with 0.5 ml hexane spotted evenly across them (Control), B) without tights with 0.5 ml DEET (1%) in ethanol (DEET on arm), C) wearing tights with 0.5 ml DEET (1%) in hexane spotted across them (DEET on tights), D) after removing tights with 0.5 ml DEET (1%) after 2 mins (Arm after tights), and E) arm washed after removing tights with 0.5 ml DEET (1%) after 2 mins (Washed arm).

Appendix 5. DEET pre-exposure bioassay methods

Methods

For a repellency trial in which mosquitoes were pre-exposed to DEET, arm-on-cage and arm-in-cage methods were compared. The arm-in-cage method was the same as that described in Appendix 3. All other procedures remained the same as the arm-on-cage method (Section 2.2.3). For both methods, cages of mosquitoes were tested with either a control arm (0.5 ml ethanol) or DEET (0.5 ml, 20%) on an arm at 0h, and 3h later the same mosquitoes were retested with DEET on an arm (Cages 1 + 2, Table 5.1a). After the 3h mark, cages of mosquitoes not previously exposed were tested with a control or DEET arm (Cages 3 + 4, Table 5.1a). Five cages of 10 mosquitoes were tested with each treatment for the arm-in-cage method, and 10 cages of 10 mosquitoes were tested with each treatment for the arm-on-cage method.

Table 5.1a. Treatments for experiment 1. N=5 for arm-in-cage. N=10 for arm-on-cage.

Cage	Tested at 0h	Tested/retested at 3h
1	control	DEET
2	DEET	DEET
3		control
4		DEET

The number of mosquitoes successfully probing was analysed using regression analysis in a generalised linear model (GLM) in Genstat® (12th edition), modelling binomial proportions with a logit transformation using replicates as blocks. This was used to obtain predicted means and SEMs. Differences were deemed to be significant when the difference between means was greater than the LSD.

Results

There were no significant differences between the arm-on-cage and arm-in cage methods in response to the control arm (control), the control arm retested with DEET on an arm 3h later (control/DEET), or DEET on an arm (DEET) (Fig 5.1a). Significantly more mosquitoes probed in the arm-in-cage method than in the arm-on-cage method in response to DEET after pre-exposure to DEET (DEET/DEET) ($p=0.006$). In both the arm-on-cage and arm-in-cage experiments mosquitoes were significantly more insensitive to DEET when tested with DEET on an arm 3h after pre-exposure to either a control arm (control/DEET) ($p<0.001$, $p=0.017$ respectively) or DEET on an arm (DEET/DEET) ($p<0.001$, $p=0.003$ respectively).

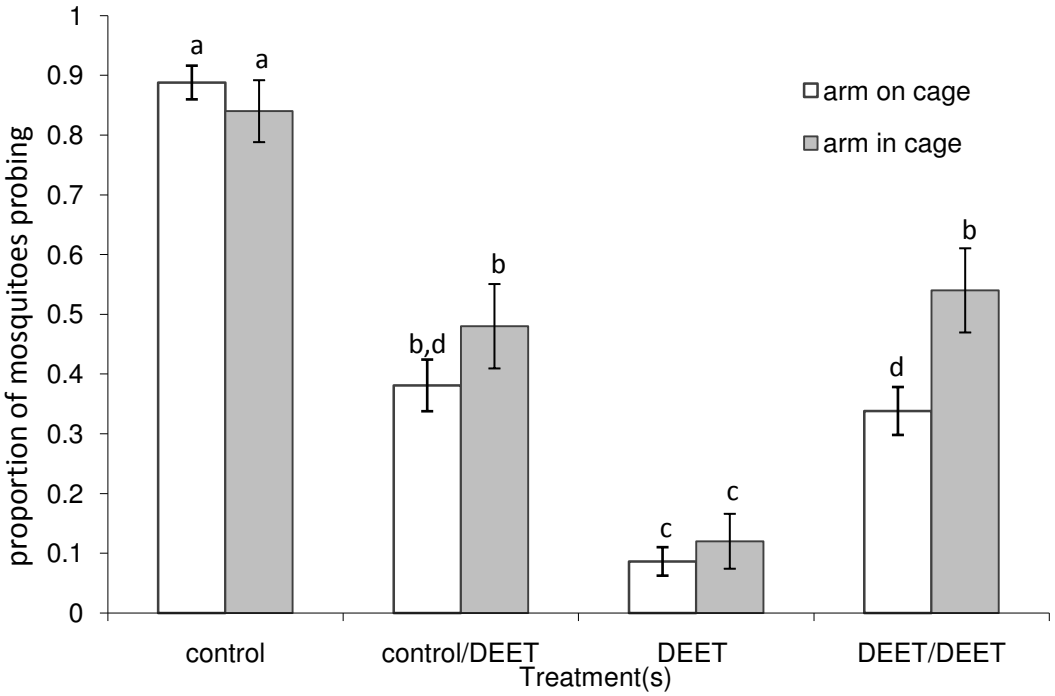

Fig 5.1a Proportion of female *Ae. aegypti* mosquitoes probing in response to a control (ethanol) arm, an arm treated with DEET after being tested with a control arm 3h previous (control/DEET), DEET on an arm (DEET), and an arm treated with DEET after being tested with DEET 3h previous (DEET/DEET). These treatments were tested with the treated arm either in the cage or on top of the cage with a layer of mesh between the arm and mosquitoes. Means are ± SEM. Means with different letters are significantly different from each other ($p<0.05$).

Discussion

More previously sensitive mosquitoes changed their behaviour to insensitive in the arm-in-cage method than in the arm-on-cage method, which may be due to the proximity of volatiles and heat from the arm. Once mosquitoes were pre-exposed, the threshold of attractant required to change a mosquito from sensitive to insensitive may have been reached more frequently with the arm in the cage as opposed to outside it. As both methods showed a significant difference in response between mosquitoes tested for the first time with DEET and those being retested, further experiments were conducted with the arm-on-cage method in order to avoid direct probing of the arm.