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OVIPOSITIONAL BEHAVIOR OF *ANOPHELES GAMBIAE* AS INFLUENCED BY VARIABLE SUBSTRATES AND CHEMICALS

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OVIPOSITIONAL BEHAVIOR OF *ANOPHELES GAMBIAE* AS INFLUENCED BY VARIABLE SUBSTRATES AND CHEMICALS

By

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ABSTRACT

OVIPOSITIONAL BEHAVIOR OF *ANOPHELES GAMBIAE* AS INFLUENCED BY VARIABLE SUBSTRATES AND CHEMICALS

By

Alicia Marie Bray

Anopheles gambiae is arguably one of the most dangerous insects today due to its effectiveness as a vector of human malaria. In Western Kenya, larvae of this mosquito are commonly found in puddles associated with freshly disturbed soils. Laboratory choice tests were performed with different soil types and synthetic chemicals to determine which stimulated or inhibited oviposition. Some soil samples were found to stimulate oviposition 2 fold over a pure water control while others reduced oviposition significantly. Effects of particular soils varied over time. Geosmin, 2-methylisoborneol, phenol, p-ethylphenol, and o-phenylphenol stimulated greater than 60 % ovipositional events compared to distilled water control, while indole and skatole inhibited greater than 60 % oviposition. Further testing needs to be done to determine if they are biologically active in the field. Visual observations of *An. gambiae* females were performed within a 21 x 2.5 x 12 cm Plexiglas arena to identify and quantify behavior details of oviposition. Many hypergravid *An. gambiae* laid eggs readily on moist substrates while others took longer to initiate oviposition, but most accomplished egg deposition the night tested. Once begun, most females deposited all their eggs in one sitting with eggs dropping every 5 ± 1 sec. The overall impression was that ovipositional sequence is quite simple and reproducible.

Dedicated with love to my husband and parents for their unwavering support and guidance through all the obstacles that life gives.

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KEY TO ABBREVIATIONS

Abbreviations for mosquito genera

Ae. .....*Aedes*
An. .....*Anopheles*
Cx. .....*Culex*
Oc. .....*Ocherotatus*
Tx. .....*Toxorhynchites*

CHAPTER I:

LITERATURE REVIEW

Anopheles gambiae biology

The holometabolous life cycle of *An. gambiae* can be thought of as beginning when a gravid female deposits eggs in or near small temporary water puddles with little or no emergent vegetation. Sometimes these habitats are no larger than a footprint (Clements 1992, Blackwell and Johnson 2000). Within 24 h, eggs hatch into larvae that reside mainly at the air/water interface of these pools, where they feed on bacteria, protozoa, and algae by creating a current with their labial brushes drawing food into their mouth (Clements 1992). Larvae undergo four instars during 5-7 d at about 28-30°C before becoming mobile pupa. Pupae normally transform into adults within 24 h; most males in a cohort emerge before females. Adults acquire nutrients from nectar and females feed upon human blood (Clements 1992, Lane and Crosskey 1993). Female *An. gambiae* do not store sufficient resources during larval development to produce eggs, therefore, mated females require one or two human blood meals to obtain the resources (especially protein) necessary for oogenesis. Once the blood meal has been obtained, females normally stay within or around a human dwelling for 1-2 d before foraging for an ovipositional site. Males do not require exogenous protein for sperm production, and therefore, do not blood-feed.

There is selection pressure on *Anopheles* mosquitoes to be effective in finding the resources suitable for larval survival. Puddles in which larvae are

found to develop can evaporate within 7 d, therefore, larvae must develop quickly in these temporary pools.

Various types of microorganisms, mainly of soil origin, populate pools of water that are ovipositional substrates for *An. gambiae* (Blackwell and Johnson 2000). Bacteria and other microorganisms can influence the ovipositional behavior of this mosquito (Walker and Merritt 1993). In addition, some chemo-attractants have been shown to influence oviposition of mosquitoes (Blackwell and Johnson 2000). *An. gambiae* females are thought to use volatile chemicals as one of the cues ultimately triggering oviposition in appropriate habitats (Beehler et al. 1994, Bentley et al. 1981).

Landmarks in history of mosquitoes and other arthropods

Recorded history has continually noted the biting of humans by hematophagous Diptera, including mosquitoes (Service 1978). However, it was not until the late 19th century that mosquitoes were implicated in disease transmission. Theobald Smith (1859-1934) and F. L. Kilbourne (1858-1936) submitted the first documentation of an arthropod vectoring a pathogen in 1891. They discovered that *Babesia bigemina,* causing Texas cattle fever, was transmitted by the cattle tick, *Boophilus annulatus.* Josiah Nott (1804-1873), who believed that mosquitoes were vectoring the yellow fever virus to humans, published speculative evidence of this relationship as early as 1848. Nott's work was advanced by Carlos Finlay (1833-1915) who provided some evidence that *Aedes aegypti* was the potential vector of the virus. This connection was finally proven by Walter Reed (1851-1902) in 1900. At the same time this work with *Ae.*

aegypti was being conducted; Sir Patrick Manson (1844-1922) concretely associated mosquitoes with pathogens in 1877 when he was able to infect *Culex* mosquitoes with filarial worms, *Wuchereria bancrofti.* This set precedence for evaluating other arthropods as potential vectors. In 1898, Sir Ronald Ross (1857-1932) documented the role of mosquitoes in transmission of avian malaria from diseased to healthy birds in India. Giovani Grassi (1854-1925) established the cycle of malarial parasites in anopheline mosquitoes in the same year. In 1902, H. Graham proved that mosquitoes could vector the causative agent of the dengue.

Pathogens and their vectors have molded human actions for centuries and imparted dread difficult for modern society to appreciate fully. One of the most significant cultural upheavals linked to an insect vector was the Black Death in the 14th century. The causative agent, *Yersinia pestis,* is transmitted to a new host by fleas. Before 1348, people had a strong appreciation for the church and their station in life. Once the disease suddenly appeared, it afflicted and killed "good" and "bad" people alike, including the priests and doctors thought to be above 'smite' from God (McClelland 1992). By 1352 at least 25 million people (Mullen and Durden 2002), one third of the overall European population had died; some countries like England lost half to two thirds of their population (McClelland 1992). This devastation caused survivors to question the validity of the Catholic Church, helping to pave the way for the Protestant Reformation. Social structure changed markedly in the aftermath of the Black Death. Due to the decrease in population, but continuing high need for laborers, peasants could no longer be

forced to pay tithes to the Landlord. Instead, they began receiving wages. Although the severity of this epidemic has not been repeated, there are still documented cases of plague throughout the world in rodents and humans (McClelland 1992).

The mosquito-vectored diseases have also molded human history. The fate of the United States could have been altered if the French army had not been severely stricken with yellow fever in Haiti in 1802. The reduction in military forced Napoleon to withdraw from his New World expansion (Mullen and Durden 2002). Yellow fever also influenced a very large economic venture of the United States, the Panama Canal. After thousands of workers digging the canal died from yellow fever, few potential employees were willing to risk disease. The canal project was in danger of cancellation until wide-scale mosquito control was implemented to protect the workers (Garrett 1994).

Mosquitoes are still responsible for 1 of 17 deaths in the world today. Moreover, mosquitoes transmit pathogens to more than 700 million people annually (Fradin 1998). The most deadly mosquito-borne pathogen that affects humans today is malaria; it is estimated to cause as many as three million deaths annually (Fradin 1998), mainly in developing countries. Beyond inflicting a heavy death toll, malaria reduces the productivity of workers in sub-lethal cases and strains already stressed health care facilities.

Chemical influences on mosquito behavior

Female mosquitoes use a variety of chemical cues to guide acquisition of needed resources. The most well-known and widely researched chemical

influencing mosquito behavior is carbon dioxide (CO_2). It was first shown to be a stimulatory agent for mosquitoes by Rudolfs (1922). Receptors for CO_2 are located on the antennae (Willis and Roth, 1952), so the females are able to follow a plume toward a potential host (Geier et al. 1999). This response is found in a variety of species including *Ae. aegypti, Ae. vexans, Culex tarsalis, Ae. nigromaculis, An. franciscanus*, and *An. walkeri* (Brown et al. 1951, Reeves 1953, McIver and McElligott 1989. *An. gambiae* does not respond as positively to a plume of CO_2 as other species of mosquitoes but responds more strongly to a trap baited with both CO_2 plus other human host-odors (Gibson et al. 1997). That same year, Takken et al., also reported that *An. gambiae* preferred plumes of CO_2 combined with acetone (a chemical isolated from expired air, Willemse and Takken 1994) than CO_2 alone.

The chemical cues eliciting particular behavior can also depend on the female's physiological state (Davis and Bowen 1994). For example, the antennae of host-seeking *A. aegypti* are very sensitive to lactic acid, while such sensitivity is not seen in a non-host-seeking female. Lactic acid has been shown to be a mosquito attractant for *A. aegypti* by Acree et al. (1968). Neuronal sensitivity, as measured by electrophysiological techniques, was used by Davis and Bowen (1994) to detect potential oviposition attractants for some mosquitoes. *An. albimanus* responded strongly to o-cresol, ethyl propionate, and 4-methyl cyclohexanol while *An. stephensi* was sensitive to cresols and 2-butoxyethanol. Sensitivity to these chemicals was greatest when females were gravid.

Electroantennogram (EAG) studies have been employed in the search for ovipositional attractants for *Toxorhynchites moctezuma* and *T. amboinensis*. Collins and Blackwell (1998) found that ether extracts of known ovipositional sites were stimulatory to females but not males of both species. Seven compounds commonly found in water with decaying organic matter were also tested by EAG because they were thought stimulatory to other mosquito species. Seven compounds: 4-methylcyclohexanol, indole, 3-methylindole, phenol, m-cresol, o-cresol, and p-cresol, elicited significant EAGs in females of both species; thresholds ranged from 1 X 10-4 μg to 1 μg per filter paper in a Pasteur pipette comprising the EAG cartridge. Collins and Blackwell (2002) followed this EAG study with behavioral tests confirming that EAG active chemicals and water extracts elicited significantly more oviposition than did distilled water. Behavioral studies were performed on a related species, *T. splendens,* that prefers to oviposit in water in which a cohort of *A. aegypti* had been reared and removed (Benzon et al. 1988). These studies showed that predatory mosquitoes not only were able to detect volatiles exuded from their prey but, also from the sites in which their prey had been.

Ochlerotatus triseriatus responds to a suite of ovipositional attractants. Gravid *Oc. triseriatus* oviposited more frequently in water previously containing larvae of their own species than in distilled water. To a lesser extent, they preferred water that had contained *Ae. atropalpus* larvae as opposed to distilled water (Bentley 1976). This effect was lost when a choice test was performed between water with the mosquito eggs against distilled water alone, showing that

the larvae and not another life stage were possibly producing a chemical attractant. This positive effect of the larval exudates increased by 90% when McDaniel et al. (1976) added certain colors to the ovipositional resource. Females oviposited more in amber rather than green or clear vessels containing distilled water. However, the presence of larvae was less influential in amber vessels; only 66% of the eggs were laid in containers containing larvae compared to 34% eggs in water alone.

McDaniel et al. (1979) attempted to determine the origin of the attractants from larvae. By displacing the gut contents of the larvae with kaolin and evaluating their attractiveness, these workers determined that the chemical(s) involved in attraction was a true pheromone associated with larval guts and feces. However, some activity was obtained from the remaining parts of the body. The results of a series of experiments with *Ae. aegypti* led Benzon and Apperson (1988) to challenge the interpretations that the larvae produced a pheromone. *Ae. aegypti*, like *Oc. triseriatus*, preferentially oviposit in water containing conspecific larvae (Soman and Ruben 1970). Benzon and Apperson (1988) were able to discount the effect of the larvae by replicating the same experiments but removing the bacteria from the water before the experiment and limiting the growth of bacteria through the duration of the test. By suppressing bacteria, the attractiveness of the larvae was eliminated; but, attraction remained in treatments with unrestricted bacterial growth.

The types of bacteria present in the ovipositional resource can greatly influence *Ae. aegypti* and *Ae. albopictus* (Pavlovich and Rockett 2000). When

they presented eight different bacteria, *Pseudomonas aeruginosa, Escherichia coli, Bacillus cereus, Bacillus subtilis, Bacillus thuringiensis, Staphylococcus epidermis,* and *Enterobacter aerogenes* in water to *Ae. albopictus,* the females preferred *E. coli* most and *E. aerogenes* least. This effect was enhanced when combined with a red background.

In addition to chemicals from bacteria and to a lesser extent larvae, there can also be chemicals in the water itself that attract and stimulate mosquitoes to oviposit. P-cresol was isolated as a trace component in water containing decayed paper birch and proved to stimulate *Oc. triseriatus* to oviposit (Bentley et al. 1979). Other compounds structurally related to p-cresol were tested: p-ethylphenol, 2, 4-dimethylphenol, and 4-methylcyclohexanol were characterized as attractants, and o-cresol, m-cresol, and 2, 3-dimethylphenol as stimulants (Bentley et al. 1981). The attractant status was assigned if the effect was found for screened ovipositional sites restricted from tactile probing, while the stimulant was only active in the unrestricted open site. Further work focused on 4-methylcylcohexanol. Both the *cis* and *trans* forms were attractants (Bentley 1982).

All of these behaviorally active chemicals are thought to originate from metabolic activity of microorganisms (Stahl and Parkin 1996). Although it has already been shown that the presence of bacteria can significantly attract gravid females to ovipositional sties (Pavlovich and Rockett 2000), further work must be done to isolate and identify the active chemicals and determine if specific microorganisms produce them. Ovipositional sites can vary in attractiveness to

mosquitoes depending on the chemical blends (Bentley and Day 1989). That these signatures are highly influenced by microorganisms is an intriguing possibility worthy of further research.

Cx. quinquefasciatus exhibits some similarities and differences to the other mosquito species already discussed. This mosquito usually lays its eggs on water containing high organic matter associated with decomposing grass, wood, or sewage. Decomposition of these associated materials releases a variety of volatile compounds. Grass infusions are significantly more attractive to gravid females than distilled water (Gjullin et al. 1965). The responsible compounds were isolated by Millar et al. (1992). Fractionation by liquid chromatography led to the isolation of phenol, 4-methylphenol, 4-ethylphenol, indole, and 3-methylindole. These chemicals were then tested further for potential synergistic effects when combined with color (Beehler et al. 1993). Using India ink as the substrate imparting color, the effect of the chemicals alone and the effect of the dye alone proved significant relative to distilled water control; but, the combination of the two treatments was synergistic. No significant differences were found between treatments with all five chemicals and 3-methylindole alone. This one compound at sufficient concentration explained most of the effect (Beehler 1994).

In addition to the attractants from breeding areas, gravid females of *Cx. quinquefasciatus* are attracted to a volatile pheromone (Laurence and Pickett 1985). This compound was identified as erythro-6-acetoxy-5-hexadecanolide and found to be released by portions of egg rafts above the water surface.

Combining this ovipositional pheromone with attractive chemicals from the breeding site yielded an additive effect (Blackwell et al. 1993). Blackwell et al. (1993) also tested each chemical individually by EAG. All the chemicals in question were EAG active; response of females was greater than that of males.

Based on research knowledge from the Culicidae, it is reasonable to propose that chemicals also influence *An. gambiae* and *An. funestus* ovipositional site selection; however, studies that address this question are very limited. McCrae (1984) found that water from a known natural breeding site of *An. gambiae* was more attractive to the gravid females than tap or distilled water. Minukawa and Mutero's work corroborated this as reported in Takken and Knols (1999); more eggs were deposited on a treatment with water and mud than clean water alone. Blackwell and Johnson (2000) conducted an EAG study on whole water samples, ether extracts of water samples, and individual chemicals. Female *An. gambiae* were more sensitive to all of the 10 whole water samples and ether extracts thereof than were male mosquitoes. Individual chemicals that were known attractants to other mosquito species had varying EAG activities on *An. gambiae.* The order of activity (most to least) was 3-methylindole and indole (at 1ng) > p-cresol (10ng), > phenol (< 0.1 μg), > o-cresol (< 1 μg), > and m-cresol and 4-methylcyclohexanol (< 10 μg for each) per 25 X 8mm filter paper inside the EAG cartridge.

Salinity of water was evaluated for potential stimulation or repellency of *An. gambiae* for oviposition by Causey et al. (1943). They determined that females oviposit most on fresh water but, in some tests, 33% of the eggs were

laid in slightly brackish water at 1% salinity. Very few eggs were laid and no larvae hatched from treatments with salinity greater than 5%. This study established that *An. gambiae* in Brazil do not prefer low saline solutions and that high saline concentrations are deterrent. Although some attention had been paid to chemical cues influencing *An. gambiae* oviposition, it is not yet clear that the most important compounds encountered under natural habitats have been uncovered. The approach to date has been markedly arbitrary and strongly influenced by compounds discovered active for other mosquitoes.

Behavioral studies

The behavior of vectors foraging for acceptable hosts or ovipositional sites is an important part of their biology. Mosquitoes oviposit in a variety of areas where water accumulates, but some species have adapted to a particular type of habitat. Breeding sites can vary from simply moist soil, small puddles in the ground, tires, or large permanent water pools. *Cx. pipiens molestus* and *Ae. aegypti* tend to select aqueous sites well shaded or completely covered by vegetation (Kennedy 1942). This behavior is stimulated by water and associated cues, but in most cases, only at particular times in the diel-cycle. The onset of darkness or sunrise is the peak time of oviposition for species like *Ae. aegypti* or *Taeniorhynchus fuscopennatus* (Gillett et al. 1961, Haddow et al. 1958). This rhythm can be disrupted or even eliminated by manipulating the light: dark cycle (Gillett et al. 1961, Haddow et al. 1979).

Within *Anopheles,* there are many similarities as well as notable variation in mating, blood-feeding, and oviposition behavior. *An. punctulatus* tends to rest

for up to one h after a blood meal and then flies in search of a nearby suitable resting habitat to digest the meal. These resting sites are moist sheltered areas that tend to be close to the ground (Roberts and O'Sullivan 1948). Roberts and O'Sullivan (1948) also determined that males were present in these habitats, suggesting that such sites may also serve for breeding. Oviposition was thought to occur in swamps near human settlements; however, this was only inferred by the presence of gravid females. Russell and Ramachandra (1942) observed *An. culicifacies* mating in swarms during the evening hours. Mating pairs exited the swarm. About 48-72 h after blood feeding, oviposition began while hovering over water. *An. maculipennis* also displayed this oviposition 'dance' while scattering eggs over water (Bates 1940). *An. punctipennis* was observed during an act of oviposition by Herms and Freeborn (1921). Their very detailed description included the female jerking her abdomen followed by an egg seen protruding for about 4 sec when another jerk freed the egg while another egg began to protrude. The ovipositional bout continued for 19 min and produced 174 eggs. A severe limitation of this study was that Herms and Freeborn classified this ovipositional scenario as typical for *An. punctipennis*; but their observations were restricted to possibly one individual.

Giglioli (1965) conducted an exceptional study where he was able to view and record by photographs *An. melas* before and during oviposition. Preovipositional behavior was characterized by darting flights from the resting surfaces to the moist substrate and tapping the substrate with the hind tarsi. Oviposition began with the abdomen tilted upward. As the bout commenced, the

abdomen steadily lowered. An egg was deposited each 10-12 sec and clumps ranged from 6-92 eggs. The females in this study laid eggs in three patterns; random scatter and clumps of eggs were the most common, while egg lines were rare.

The behavior of *An. gambiae* and *An. funestus* has also been extensively studied with a view toward developing more effective control strategies. It is common knowledge that females of these species tend to be very anthropocentric; not only do they feed primarily on humans (Clements, 1992), but they also use human dwellings for daytime resting. Due to the weather patterns in Sub-Saharan Africa, the abundance of *An. gambiae* and *An. funestus* tends to be seasonal and to peak during the two rainy seasons during November-December and April-May (VanSomeren et al. 1958). Flight activity by *An. gambiae* is reported to have two peaks; an ovipositional peak occurs at dusk, and a biting peak occurs just before dawn (Haddow and Ssenkubuge 1962, Jones et al. 1972b). Due to this bimodal activity, the females can apparently feed the same night that they oviposit. McCrae (1983) described the flight periods of *An. gambiae* and obtained slightly different results for the ovipositional flight. He reported that oviposition activity slowly increased after dusk, peaked between midnight and 1 AM, and slowly decreased until dawn. This very different pattern between the two studies might be accounted for by the difference in the source of the females. In the former study, the females came from a laboratory Kisumu strain, while the latter study used wild-caught blood-fed females. I suspect that the time the females blood-fed would be the most

significant factor explaining the difference in peak oviposition behavior. Light seems to inhibit flight activity but can be excitatory if it is displayed during the 'normal' peak of activity (Jones et al. 1972a). When comparing flight activity of sibling species in the *An. gambiae* complex, Jones et al. (1974) showed that the sibling species a, b, *An. melas,* and *An. merus* show slight differences in peak flight activity. The peak differences were consistent for both females and males within one given sibling species, suggesting that allochrony might be a mating barrier in natural conditions. Jones et al. (1978) determined flight activity is also influenced by physiological state. Once virgin females were inseminated, their flight shifted to later in the scotophase. Blood feeding diminished activity for 2-3 d until oviposition. This pause in activity between blood feeding and oviposition is consistent with that documented by Gillies (1953) for *An. gambiae* and *An. funestus.*

Substantial attention has been paid to the individual factors that influence ovipositional behavior for *An. gambiae.* McCrae (1984) concluded water color and water type influenced ovipositional site selection and behavior. Wild-caught females oviposited more on a black substrate than grey or white. This preference increased if the background on which the ovipositional dish was placed contrasted with that of the treatment. He also observed on a few occasions that the females ovipositing on a white background were in a settled position throughout the bout while females ovipositing on the black background were more inclined to lay their eggs in flight. He argued that oviposition in flight is optimal since that was observed to occur on the more acceptable substrate.

McCrae (1984) concluded oviposition in the settled position was due to the light color to be sub-optimal and not normal. He had difficulty corroborating that the light color was sub-optimal in another series of experiments comparing turbid, distilled, tap, and swamp water. The turbid water received more eggs than the other treatments, however it appeared to be the most pale in color. His explanation was that there was some characteristic in the turbid water that overrode the selection of a dark substrate.

Hocking and MacInnes (1948) saw *An. gambiae* and *An. funestus* in the act of oviposition on a few occasions. *An. gambiae* most commonly oviposited while sitting on water; the eggs dropped onto the water surface from a raised abdomen. Alternatively, the females settled on the side of the bowl about 1 inch above the water and eggs rolled onto the water surface. One "restless" female frequently changed positions during the bout from the water surface to the side of the bowl. This observation was the only time they visualized a female using her left hind leg to dislodge eggs from the abdomen tip. Only three *An. funestus* were observed ovipositing in their study. One settled with her fore legs on the bowl and her middle legs on the water surface. The other two females settled on the bowl above the water line. In all three occasions the eggs fell onto the water. For both mosquito species, the eggs fell at regular intervals for all the ovipositional bouts.

Objectives of this research

The objectives this project were to: 1) determine if an adult female *An. gambiae* differentiates between potential ovipositional sites on the basis of substrate types

and associated chemical cues, and 2) document and quantify pre-ovipositional and egg depositional behaviors.

CHAPTER II

SOIL AND CHEMICAL INFLUENCES ON MOSQUITO BEHAVIOR

Introduction

Various physical, chemical, visual, tactile, and biological factors and stimuli influence mosquito ovipositional site selection, among the species that have been studied (Bentley and Day 1989, Clements 1999, McCall 2002). Presence of water is undoubtedly a key factor, but the mechanisms by which gravid females sense it are poorly known (Kennedy 1942). Dark vs. light-colored substrates tend to be favored in laboratory and field bioassays regardless of the species. Other important stimuli for olfaction and gustation, in relation to volatile and nonvolatile chemicals are aromatic compounds, or undetermined factors in habitat water, underlying substrates, or prepared infusions. The classes of chemicals included in bioassays and electroantennogram studies of oviposition include: carboxylic acids, ketones, phenoles, and indoles (Clements 1999, McCall 2002). For example, Collins and Blackwell (1998) determined that certain ether extracts of water from known ovipositional sites stimulated oviposition by gravid females of *Toxorhynchites moctezuma* and *Tx. amboinensis*. The compounds 4-methylcyclohexanol, indole, 3-methylindole (skatole), phenol, m-cresol, o-cresol, and p-cresol, elicited significant EAGs in females of both species; thresholds ranged from 1×10^{-4} μg to 1μg per filter paper in a Pasture pipette comprising the EAG cartridge. Collins and Blackwell (2002) followed this EAG study with behavioral tests that confirmed EAG active chemicals and water

extracts elicited significantly more ovipositional events than a distilled water control.

Studies show that gravid mosquitoes respond differentially to water in which conspecific larvae were present, to water containing prey, or water from natural habitats (Allan and Kline 1998; Bentley et al. 1976; Benzon and Apperson 1988, Benzon et al. 1988, Ahmadi and McClelland 1983; Wilton 1968).

Aside from direct examination of chemicals in solution, mosquito oviposition has been studied in response to undefined infusions made by mixing various organic materials with water (Hazard et al.1967; Gubler 1971; Reiter et al. 1991; Lampman and Novak 1996a; Lampman and Novak 1996b; Trexler et al. 1998). Indeed, the response of female mosquitoes to oviposition-related chemical cues might properly be viewed as responses to semiochemicals emanating from such infusions, in that the chemical cues may be volatile attractants, as well as contact stimulants. Others have addressed the likelihood that these volatiles arise from microbes, perhaps from microbial metabolic activity and from microbially-mediated decompositional processes. Accordingly, studies in which microbes (in particular, bacteria) are incorporated into ovipositional substrates have in some cases revealed that gravid mosquitoes are more responsive to them than substrates with no microorganisms in choice tests (Millar et al. 1992; reviewed in Clements 1999; and see review in Trexler et al. 2003). For example, Maw (1970) reported that pseudomonad bacteria were attractive to *Cx. resutans* Theobald, while Ikeshoji et al. (1975) showed that *Pseudomonas aeruginosa* elicited more oviposition by both *Ae. aegypti* L. and *Cx. pipiens molestus* Froskål than did a

chemical (decanoic acid) in solution in the substrate. Other studies examined the bacterial strains in natural habitats or in larval-rearing medium and related their presence to oviposition responses with variable results (Hazard et al. 1967, Benzon and Apperson 1988, Hasselschwert and Rocket 1988, Pavlovich and Rocket 2000, Rocket 1987, Wallace 1996).

Despite the importance of *An. gambiae* as a human malaria vector in SubSaharan Africa (Foster and Walker 2002), its ovipositional behavior is comparatively poorly known. Oviposition is reported to occur at night, especially immediately after sunset (Haddow and Ssenkubuge 1962, Jones et al. 1972b, McCrae 1983). McCrae (1984) determined that wild-caught female *An. gambiae* oviposited more on a black than gray or white substrate. This bias was enhanced if the background on which the ovipositional dish was placed contrasted with that of the treatment color. McCrae (1984) also showed that gravid *An. gambiae* laid more eggs onto muddy water than on tap or distilled water. Blackwell and Johnson (2000) conducted an EAG study on whole-water samples from larval habitats, ether extracts of those water samples, and with certain chemicals identified by others as stimulants. Antennal sensilla of females were more sensitive than were those of males to water samples and ether extracts. Individual chemicals that were known attractants to other mosquito species elicited variable EAG activity with *An. gambiae*. The order of activity (greatest to least) was 3-methylindole and indole (at 1 ng) > p-cresol (10ng), > phenol (< 0.1 μg), > o-cresol (< 1 μg), > and m-cresol and 4-methylcyclohexanol (< 10 μg for each). These fragmentary studies suggest that factors associated

with larval habitats, including chemical factors, influence ovipositional site selection by *An. gambiae*. A more complete understanding of the ovipositional behavior and factors affecting it may provide a basis for new monitoring and management strategies for this malaria vector species.

As part of a research program examining oviposition of *An. gambiae*, we focused here on chemical stimulants and responses of gravid females to varied water and soil substrates. The choice of substrates and chemicals used in experiments was dictated by the studies reviewed above, and by our on-going field studies of larval *An. gambiae* habitats in Western Kenya (Gimnig et al. 2001, 2002). The objectives were to determine if: (1) chemicals previously shown to stimulate oviposition by other mosquitoes did so for *An. gambiae* and (2) whether soils differ in ability to stimulate oviposition.

Materials and methods

Mosquitoes

Colonies of *An. gambiae* KISUMU strain and G3 strain were obtained from the Center for Vector Biology Research and Control, Kenya Medical Research Institute (Kisumu, Kenya), and the University of Notre Dame, Center for Tropical Disease Research and Teaching, respectively. Mosquitoes were reared and maintained as described by Benedict (1997): Adults were maintained in 60 X 60 X 60 cm screened cages at 29 ± 3° C and 80% relative humidity under a 12:12 h (L: D) photoperiod. Females were blood-fed twice a week and a 10% clover honey solution was available at all times. Eggs were collected on wet filter paper (Whatman #1) over a 9 cm diam. Petri dish and hatched on moist paper.

Approximately 200 hatched larvae were washed into a 27 x 19.5 x 9.5 cm clear Nalgene tub containing 2 cm of deionized distilled water. The water was not changed or aerated; it was kept at 29 ± 2° C. Ground food mixture (60% wheat flour, 25% bakers yeast, 10% defibrinated beef blood, and 5% non-fat dried milk), sufficient for one day, was dropped onto the surface of the water in each container every day.

General methods for all choice test experiments

Experiments conducted in Kenya were performed in 30 x 30 x 30 cm metal-framed cages with a removable wood floor and covered by white mosquito netting. The cages were draped with damp cloth towels to increase humidity. During testing, cages were placed on a laboratory bench in a room with unregulated temperature and admitting natural sky light.

Experiments conducted at Michigan State University were performed in 30 x 30 x 30 cm metal-screened cages with a metal floor (Bioquip, Rancho Dominguez, CA) held in a Precision Low Temperature incubator (Model 815) maintained at 28±3° C. A Phillips 15 watt fluorescent bulb providing light at *ca.* 300 lux drove the 12:12 h (L: D) photoperiod. During scotophase, light at *ca.* 18 lux was provided by a small tungsten night-light.

Unless otherwise indicated, choice tests were performed using one ovipositional dish per treatment paired with a distilled water control within a cage housing *ca.* 100 gravid *An. gambiae.* Ovipositional dishes were prepared by filling a 9 cm Petri dish with cotton and distilled water topped with an 11 cm Whatman #1 filter paper as per Figure 1. Eggs were counted visually on the filter

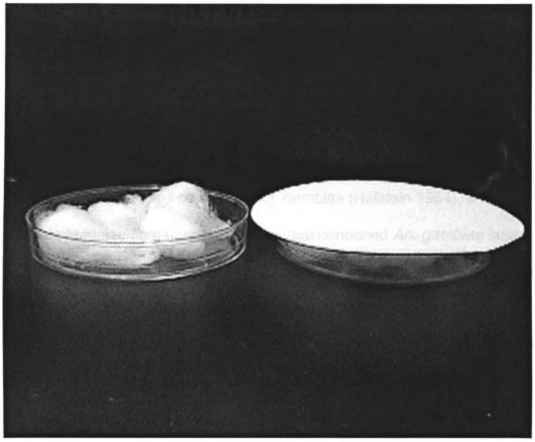

Figure 1. Ovipositional dish filled with cotton balls and distilled water unless otherwise indicated.

papers under a standard dissecting microscope when needed due to high egg density.

Soil and water Experiments

Types of water (Kenya)

During May 2001, ovipositional responses were quantified to water from: 1) a spring, 2) a semi-permanent body of water well-populated with plants and inhabited by *Anopheles* other than *An. gambiae* (Holstein 1954), and 3) small puddles in maize-field drainage ditches that contained *An. gambiae* larvae.

Due to the lack of water in one maize ditch puddle, a pilot choice test was performed on spring water, semi-permanent water, and maize ditch mud. The mud sample was prepared by half-filling a 9 cm Petri dish with soil collected from the site, filling the remainder of the dish with spring water, and stirring to produce a slurry. After placement into a cage of gravid females, ovipositional dishes were topped with a 11 cm filter paper.

Drying mud puddles

In a follow-up study (May 2002), water from three separate puddles (*ca.* 0.5 m^2) containing >25 mosquito larvae present on the grounds of the Kenya Medical Research Institute (KEMRI) in Kisian, Kenya was transferred into a holding container and the exposed mud carefully shoveled into a 50 x 30 cm plastic tub so as to restore the original configuration. The water was then replaced onto the mud. Puddles 1 and 2 were in drainage ditches of a maize field; puddle 3 was in a driveway. The restored puddles were placed in the sun for several days until no standing water remained. The surface mud was placed

into a 9 cm Petri dish with cotton and spring water to produce a slurry then covered with filter paper. As a control treatment, soil was collected from a raised and well-drained site and transformed into a slurry with spring water. A slurry was also made from murram soil known to support fewer *An. gambiae* larvae. A Petri dish containing cotton balls saturated with spring water covered with filter paper served as a negative control. Five replicates were performed for each trial.

Trial 1. This experiment was set up in five separate cages before dusk with a total of three dishes per cage: Spring water, well-drained soil, and mud from reconstituted from puddle 2. Eggs were collected and counted the following morning.

Trial 2. This experiment was set up in five separate cages before dusk with a total of three dishes per cage: Spring water, murram soil, and reconstituted mud from puddle 3. Eggs were collected and counted the following morning.

Trial 3. This experiment was set up in five separate cages before dusk with a total of five dishes per cage: Spring water, murram soil, and separately reconstituted mud from puddles 1, 2, and 3. Eggs were collected and counted each morning for two consecutive d without manipulation of the treatments.

Autoclaved soil

During summer, 2002, I tested ovipositional stimulation of other soils from Michigan on *An. gambiae* (Kisumu strain) at Michigan State University. Soil was collected from a deciduous forest floor in East Lansing, Michigan (42.76196° N

084.46619° W). Choice tests compared egg output on non-autoclaved soil, autoclaved soil, and distilled water as a negative control. Autoclaved soil was prepared by placing soil into a standard biohazard bag loosely closed with a rubberband. This package was placed into an American Sterilization Company UE 650 (Erie, PA) autoclave for 40 min under the dry solution setting. Soil treatments were prepared by half-filling a plastic 9 cm Petri dish with a particular soil and the remainder with distilled water. Soil was then mixed with a sterile glass stirring rod to form a slurry and covered with an 11 cm filer paper. A distilled water control treatment was prepared as per figure 1 above. This test was performed with two dishes of each treatment (total of six) deployed on the floor of the mosquito cage. Tests were conducted over four separate days in 30 x 30 x 30 cm cages with > 30 gravid females for a total of six replicates.

Soil inoculated with actinomycetes

Soil harbors a variety of organisms. Actimomycetes (Class Actinobacteria, Order Actinomycetda) are common soil microorganisms that produce a variety of volatile organic compounds (Stahl and Parkin 1996). I tested whether inoculating sterile soil with isolated actinomycetes elicited greater oviposition than did a distilled water control. Soil was collected from maize ditch sites at KEMRI (Kisian, Kenya). Serial dilutions in distilled water were prepared from 1 g of soil. Sucrose nitrate agar was used to culture potential actinomycete bacteria from 1 mL of the second and third dilutions. Plates were incubated for 2 d at room temperature (*ca.* 25° C) and then held below 0° C during shipment to and storage at Michigan State University until use. Due to strict regulations on

importing foreign soil, Michigan forest soil was autoclaved for testing. Individual colonies of actinomycetes were scraped from the agar plates with a microbiological loop and inoculated into the sterile soil by dragging the loop on the soil surface moistened with distilled water in a 9 cm Petri dish. Inoculated soil in the Petri dish was allowed to incubate for 24 h at room temperature. Inoculated soil Petri dishes were transformed into ovipositional dishes by filling the remaining portion of the Petri dish with distilled water, stirring with a sterile stirring rod and covering the surface with filter paper. A Petri dish with cotton and distilled water covered with filter paper served as a negative control.

Synthetic chemicals

Compounds for choice-tests were geosmin, 2-methylisoborneol, *p*-cresol, phenol, indole, 3-methylindole, *p*-ethylphenol, *m*-phenylphenol, *o*-phenylphenol, putrescine, and cadaverine. All these used were purchased from Sigma-Aldrich and were declared to be >97% pure. Each chemical was dissolved in hexane or water, depending on solubility as described in Table 1, after which 100 µl of solution was applied to 11 cm filter paper. The solvent was allowed to evaporate from the filter paper (approximately 30 sec. with hexane and 15 min. with water). Stock solutions were stored at -20° C for up to 5 d. Controls were made by applying 100 µl of hexane (or water) onto a filter paper and allowing it to evaporate.

Ovipositional dishes were deployed in up to five 30 x 30 x 30 cm cages per day each with *ca.* 100 gravid *An. gambiae* females. After 24 h, dishes were removed and the eggs counted visually, using a standard dissecting microscope

Table 1. Preparation of solutions to be placed on Whatman #1 filter paper for choice-tests with *Anopheles gambiae*. A cage of *ca*. 100 gravid females was used for each ovipositional bioassay, in all cases a binary comparison against distilled water.

Test material	Amount of chemical	Amount of Solvent (ml)
geosmin	50 µl	5 hexane
2-methylisobormeol	50 µl	5 hexane
combination	100 µl geosmin + 20 µl 2-methylisoborneol	5 hexane
p-cresol	50 µl	5 hexane
phenol	50 µg	5 water
indole	50 µg	5 hexane
skatole	50 µg	5 hexane
p-ethylphenol	50 µg	5 hexane
m-phenylphenol	50 µg	5 hexane
o-phenylphenol	50 µg	5 hexane
putrescine	50 µl	5 water
cadaverine	50 µl	5 water

when needed. The number of replicates ranged from 10-25 depending on the amount of compound available unless otherwise specified.

During May 2002, geosmin and 2-methylisoborneol were compared to soil from a known larval *An. gambiae* habitat in Kisian, Kenya. Geosmin was tested at dosages of 5 µg and 50 µg applied to 11 cm filter paper as previously described. Spring water was used as a type of negative control. The positive control was prepared by taking mud from a known larval-habitat and transforming it into a slurry with spring water in a 9 cm Petri dish. The slurry was covered with 11 cm filter paper to collect eggs. 2-methylisoborneol was also compared to larval-habitat soil in the same fashion. Dosages of 2-methylisoborneol tested were 2 µg and 20 µg in one test, and 20 µg and 200 µg in a separate test. Every test had one negative control, two dishes of test chemical at different concentrations, and one positive control for a total of 4 dishes per cage. Ovipositional dishes were deployed in five 30 x 30 x 30 cm cages each with *ca.* 20 gravid *An. gambiae* females. After 24 h, dishes were removed and the eggs counted visually, using a standard dissecting microscope when needed.

Testing of chemicals having earthy odors

Most of the soils that elicited strong oviposition were noted to have a distinct "earthy" smell. Dr. Muraleedharan Nair, a natural product chemist in the Department of Horticulture at Michigan State University made us aware that the chemicals geosmin and 2-methylisoborneol are both well known for their earthy odors and that were included here.

EAG active chemicals

Using the technique of electroantennography, Blackwell and Johnson (2000) showed that *An. gambiae* gravid females were responsive to p-cresol, phenol, indole, and 3-methylindole by electro-antennogram experiments. Therefore, we postulated that these and structurally related compounds would increase oviposition above distilled water control. Solutions are described in Table 1.

Putrescine and cadaverine

Since *An. gambiae* is commonly found in temporary water puddles, it is not unusual to find them in animal hoof prints (Holstein 1954). I speculated that the soils frequented by large animals would be rich in decomposition products of fecal matter. Those habitats might have high populations of bacteria facilitating decomposition and therefore release a large amount of volatile compounds such as putrescine and cadaverine. I postulated that putrescine or cadaverine would stimulate more oviposition than distilled water. Solutions are described in Table 1.

Statistical analysis

Daily egg counts for each treatment were calculated as a percentage of the total count for each cage. Comparisons were made on the percentage values between treatments and controls using paired *t*-tests or ANOVA followed by Tukey's Honestly Significant Difference (HSD) test for mean separations (SAS Institute, 1999) when needed.

Results and Discussion

Soil and water Experiments

Types of water (Kenya)

Oviposition was significantly greater on maize ditch water than water from either the semi-permanent pool or spring water (Figure 2). Oviposition was also greater on maize ditch mud than either water from the semi-permanent pool or spring (Figure 3). These experiments show that the gravid females are able to differentiate between habitats containing their larvae and those atypical for their larvae. As the water from these sites was presented in a common context different from the field, chemical composition (either odor and/or taste) is proposed as the mediator of this differential response. Maize-ditch mud elicited considerably more oviposition than the water treatments. Different or perhaps more concentrated volatile compounds from the mud verses the water treatments might explain this result. Alternately, the darker color of the soil treatment drew more ovipositional events than the white color of the water treatments (Bentley and Day 1989, McCrae 1984).

Drying mud puddles

Trial 1. Oviposition was significantly greater on both a slurry of well-drained soil and mud from Puddle 2 than spring water control (Figure 4). Apparently, there was nothing notably different and superior about the mud remaining at the bottom of an evaporated puddle.

Trial 2. Oviposition was significantly greater on murram soil than spring water and mud from Puddle 3 (Figure 5). This result was surprising since

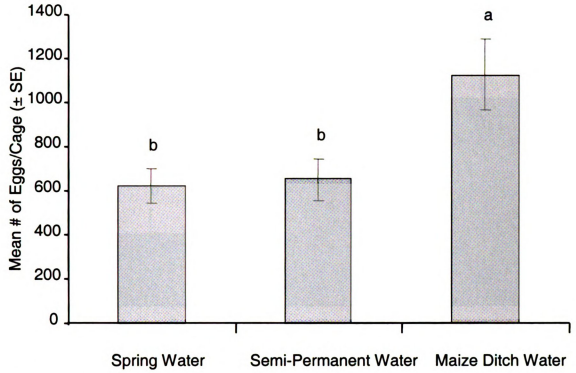

Figure 2. Mean number of eggs laid on spring, semi-permanent, and maize ditch water choice tests. Maize ditch water was significantly different from other treatments (Total eggs = 23,969, n = 15, one-way ANOVA, $P < 0.05$).

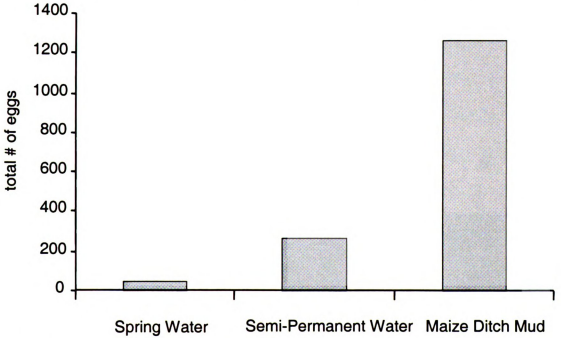

Figure 3. Total number of eggs laid on spring water, semi-permanent water, and maize ditch mud in distilled water slurry choice test. (Unreplicated test,Total eggs = 1,569)

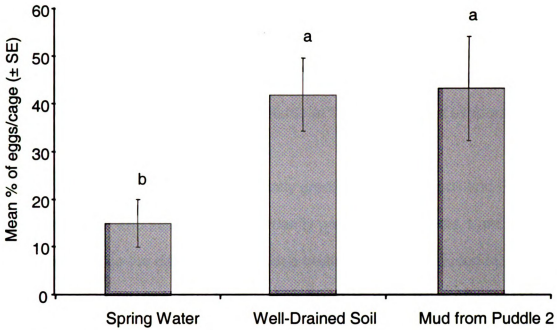

Figure 4. Mean number of eggs laid on spring, well-drained soil, and mud from Puddle 2 choice tests. Well-drained soil and mud from Puddle 2 were significantly different from spring water (Total eggs = 2,413, n = 5, one-way ANOVA, $P < 0.05$).

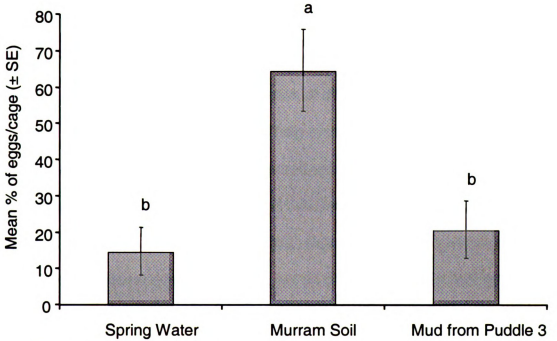

Figure 5. Mean number of eggs laid on spring water, murram soil, and mud from Puddle 3. Murram soil was significantly different from other treatments (Total eggs = 304, n = 5, one-way ANOVA, $P < 0.05$).

murram soil is commonly brought to this area of Kisumu, Kenya to build roads and known to support fewer *An. gambiae* larvae. The observation that there was no significant difference between spring water and mud from Puddle 3 gives further evidence that there is nothing notable at the bottom of an evaporated puddle.

Trial 3. Oviposition was significantly greater on murram soil and Puddle 2 than spring water and those were significantly greater than Puddle 1 and Puddle 3 on day one while the differences between treatments were reduced by day two (Figure 6). On day one, mud from Puddle 2 and murram soil continued to be stimulatory for oviposition consistent with Trial 1 and 2, while mud from Puddles 1 and 3 were found to be inhibitory for oviposition when compared to the other treatments.

Trial 1 and 3 both had significantly greater oviposition on mud from Puddle 2 while Trial 2 and 3 had significantly greater oviposition on murram soil and very little oviposition on Puddle 3. The reduction of differences between treatments from days one and two (Figure 6) might help explain the phenomenon that gravid females prefer to oviposit in freshly disturbed soil (Takken, 1999). Given another day of testing, I speculate the differences between treatments would continue to decrease. This series also reports that the presence of soil and water will generate ovipositional events, however, not all soils are equal in their effect on stimulation of oviposition.

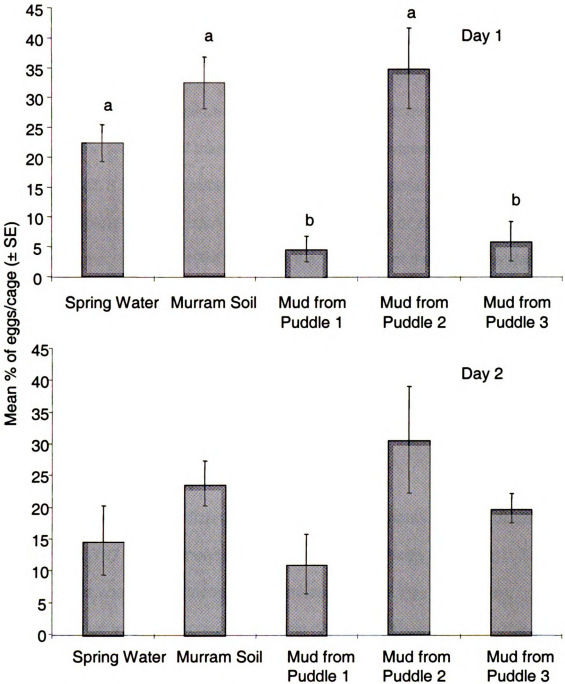

Figure 6. Mean number of eggs laid on spring water, murram soil, Puddle 1, Puddle 2, and Puddle 3 choice test over two evening period. On d 1, Mud from Puddle 1 and 3 were less stimulatory than the other treatments (Total eggs = 1,102, ANOVA, n = 4, $P<0.01$, Tukey mean separation). On d 2 that trend had dissipated such that all the treatments were not significantly different (Total eggs = 3,483, n = 5, ANOVA, $P<0.01$, Tukey mean separation)

Autoclaved soil

Oviposition was significantly greater on non-autoclaved soil and distilled water than autoclaved soil (Figure 7). Living organisms (bacteria or fungi) within the non-autoclaved soil could be the cause for the greater oviposition, or more importantly, the reduction of these organisms inhibited oviposition on the autoclaved soil. It is also possible that females were inhibited by the process chemicals imparted by autoclaving and not the presence or absence of live microorganisms. McCrae (1984) reported that the color of the substrate was important in ovipositional site selection; females prefer dark over light substrates. In this experiment, the non-autoclaved and autoclaved soils were darker than the distilled water control. Although autoclaved soil was dark in color, it inhibited oviposition. This preliminary test stimulated the below ovipositional choice test experiments.

Soil inoculated with actinomycetes

Oviposition was significantly greater on soil treatments than the distilled water control (797 vs. 393 mean number of eggs respectively (Figure 8)). The actinomycetes used for the inoculation of autoclaved soil may have been releasing a wide variety of volatile chemicals (Stahl and Parkin, 1996). The mere presence of a particular type of microbial community within an ovipositional substrate is hereby shown to enhance oviposition. With further work on identifying the species of microorganisms that produce the highest ovipositional events, a control method for *An. gambiae* in the wild might be developed. Inoculating pans with soil, water, and microorganisms that produce these volatile

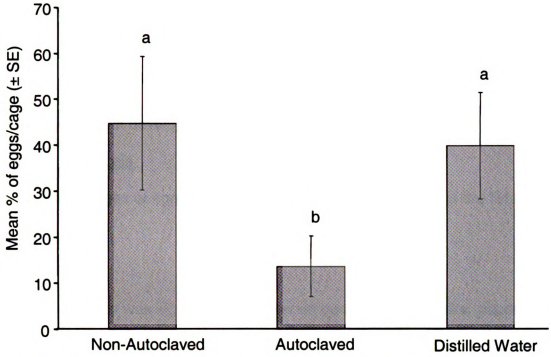

Figure 7. Mean percentage of eggs laid on non-autoclaved, autoclaved, and distilled water choice test. Number of eggs laid on non-autoclaved and distilled water was significantly greater than autoclaved soil (Total eggs = 5,498, n = 6, one-way ANOVA, $P < 0.05$)

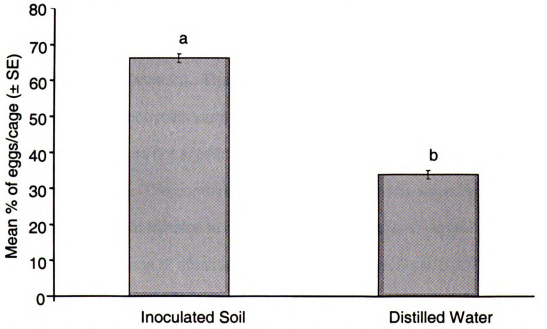

Figure 8. Mean number of eggs laid on inoculated soil with actinomycetes verses distilled water choice tests. Inoculated soil was significantly different from distilled water control (Total eggs = 17,262, n = 15, paired t-test, $P < 0.05$).

attractants could be the first step in developing a gravid trap specifically for *An. gambiae*. The microorganisms reproduce themselves with little input, so the only needed maintenance to the pans would be to remove the larvae every 4 d to ensure they do not develop into adults (Clements 1992).

Synthetic chemicals

Percentages of egg deposition for all chemical choice tests are listed in Table 2.

Earthy odors

Oviposition was significantly greater on geosmin-treated filter paper than on the hexane control. Mean numbers of eggs per treatment were 1,752 and 1,050, respectively (Table 2). 2-Methylisoborneol was significantly more attractive than the hexane control (2,490 vs. 1,150 mean eggs respectively (Table 2)). Finally, combining geosmin and 2-methylisoborneol in hexane also yielded significantly more oviposition than the hexane control (6,250 vs. 3,000 eggs respectively (Table 2)). The mean number of eggs laid was significantly higher when both chemicals were present, even though the percent of eggs mixture treatment was not significantly different from tests performed with the chemicals singularly. The combination of the two chemicals appeared to be stimulating the gravid females to release a greater number of eggs.

The significance of attraction of geosmin and 2-methylisoborneol was eliminated when compared to mud from known larval *An. gambiae* habitats (Figure 9 and 10). The dosage of compounds used in the choice tests were inactive in the presence of the positive control. Apparently, geosmin and 2-

Table 2. Percentage of eggs on Whatman #1 filter paper with various treatments in a binary choice-test against distilled water with *Anopheles gambiae*. A cage of *ca*. 100 gravid females was used for each replicate of a test.

Test material	Test	Control	± S.E.M	Total eggs	# of replicates	df	t-value	P-value
geosmin	67	33	4.1	23,925	13	24	1.98	<0.0001
2-methylisoborneol	67	33	2	50,826	13	24	2.8	<0.0001
combination	67	33	5.1	91,820	13	24	2.2	<0.0001
p-cresol	59	41	1.6	51,498	10	18	2.1	<0.0001
phenol	63	37	1.5	16,051	15	28	2.04	<0.0001
indole	37	63	1.5	20,333	15	28	2.04	<0.0001
skatole	38	62	1.4	18,730	15	28	2.04	<0.0001
p-ethylphenol	61	39	1.6	10,638	18	34	2.03	<0.0001
m-phenylphenol	54	46	2.7	17,368	15	28	2.04	<0.0001
o-phenylphenol	61	39	1.2	18,158	15	28	2.04	<0.0001
putrescine	48	52	4.5	18,401	15	28	2.04	0.0322
cadaverine	51	49	0.4	17,609	15	28	2.04	0.0662

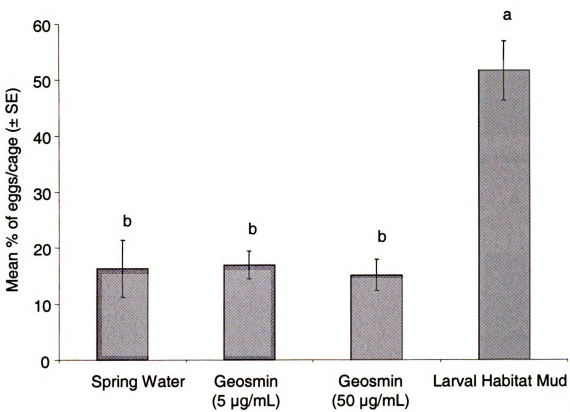

Figure 9. Mean percentage of eggs laid on spring water, 5 µg/mL geosmin, 50 µg/mL geosmin, and *Anopheles gambiae* larval habitat choice tests. Larval habitat mud was significantly more stimulatory than other treatments (Total eggs = 4,348, n = 5, ANOVA, $P<0.05$, Tukey mean separation)

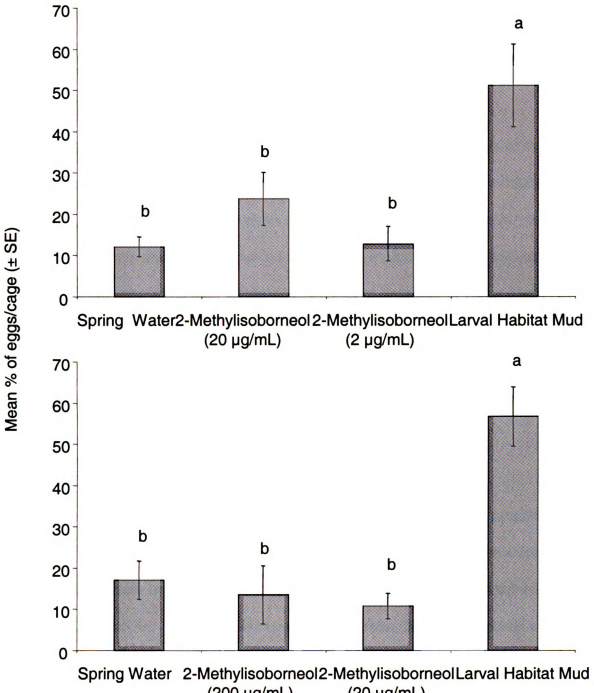

Figure 10. Mean percentage of eggs laid on spring water, concentrations of 2-methylisoborneol, and mud from *Anopheles gambiae* larval habitats. Number of eggs laid on Larval habitat mud was significantly higher than low concentrations of 2-methylisoborneol (Total eggs = 3,642, n = 5, ANOVA, $P<0.05$, Tukey mean separation) and high concentrations of 2-methylisoborneol (Total eggs = 1,702, n = 5, ANOVA, $P<0.05$, Tukey mean separation)

methylisoborneol are able to stimulate more ovipositional events when compared to distilled water, however, they did not compete with an authentic larval site. Perhaps there are other blends of chemicals yet to be identified that strongly influence oviposition attraction.

Geosmin and 2-methylisoborneol, being secondary compounds of many actinomycetes and cyanobacteria (Stahl and Parkin 2000), might be exhibiting an attractive quality to the gravid females when tested separately. However, these isomeric chemicals always co-occur in nature. The majority of the literature on these chemicals concerns their detrimental effects of decreasing water quality for humans (Blevins et al. 1995). They have been reported to be long-range attractants of camels to water (Simons 2003). Further testing is needed to determine if these chemicals are biologically important in any context to *An. gambiae* and at what concentration they are active.

EAG active chemicals

Increased oviposition was shown with p-cresol and phenol treatments over distilled water control (Table 2). Conversely, distilled water treatments were selected over indole and skatole (Table 2). These four chemicals are known to be EAG active for *An. gambiae* (Blackwell and Johnson, 2000). Blackwell and Johnson (2000) reported indole and skatole had the highest level of EAG activity followed by p-cresol and phenol. These behavioral tests show that the p-cresol and phenol are favored ovipositional substrates when compared to distilled water control, while indole and 3-methylindole are inhibitory. When relating the two

studies, the females have higher EAG activity to chemicals that are inhibitory than to the chemicals shown to be stimulatory.

Oviposition was significantly greater on p-ethylphenol, m-phenylphenol, and m-phenylphenol than distilled water control (Table 2). The chemicals structurally similar to phenol (Bentley et al. 1981) elicit greater oviposition when compared to distilled water control. Further testing is required to determine if these chemicals are stimulating greater oviposition due to their own unique structure or if the active site for detection of these chemicals on antenna has plasticity to detect all of these chemicals at the same site. Field tests of these chemicals are recommended.

Putrescine and cadaverine

Comparisons between putrescine and distilled water control resulted in significantly greater ($P < 0.038$) oviposition on putrescine (53 vs. 47 mean percentage of eggs respectively (Table 2)). Although statistically significant, the effect of this chemical was weak compared to other chemicals tested in this study.

Oviposition was not significantly different on cadaverine than distilled water control (51 vs. 49 mean percentage of eggs respectively (Table 2)).

In these experiments, I found that females were able to distinguish between different substrates for oviposition. Different soil origins elicited varied amounts of oviposition when the females were given a choice between them. Unsterilized soil was clearly favored when compared to autoclaved soil. Even when the females were given a choice of different non-sterilized soils, they

preferred to oviposit on certain soil over others. Murram soil and Puddle 2 elicited the most ovipositional events, while Puddle 1 and Puddle 3 elicited fewer. Inoculating sterilized soil with bacteria from productive *An. gambiae* habitats "recharged" the soil with organisms stimulating females to oviposit.

The individual chemicals tested also influenced ovipositional choices. Chemicals elevating oviposition were: geosmin, 2-methylisoborneol, p-cresol, phenol, p-ethylphenol, m-phenylphenol, o-phenylphenol, and to the lesser extent putrescine. Indole and 3-methylindole inhibited oviposition. These results are consistent with the EAG responses of previous studies mentioned earlier (Clements 1999, Collins and Blackwell 1998, McCall 2002). Unfortunalty, the work conducted on these chemicals does not yet give a clear answer to what chemicals, if any, are able to attract *An. gambiae* to a specific location for oviposition. Further work should be conducted on mixtures of chemicals in attempt to determine if blends are more behaviorally active than single compounds. Isolation of volatile compounds within the head-space analysis may give insight to the specific chemicals that may be responsible for behavioral attraction to an ovipositional site.

CHAPTER III

VISUAL OBSERVATIONS OF OVIPOSITION OF *ANOPHELES GAMBIAE*

Introduction

Mosquitoes remain very important to world-wide human health. They are responsible for 1 out of every 17 deaths and they transmit disease to more than 700 million people annually (Fradin 1998). The most deadly of transmitted human pathogens is malaria, which causes up to three million deaths annually (Fradin 1998), mainly in developing countries. Also crippling to society are sub-lethal cases of malaria which severely reduce productivity of workers and put a heavy strain on already stressed health care facilities. *Anopheles gambiae* is the predominant vector of human malaria in Sub-Saharan Africa (Takken and Knols, 1999). This mosquito is a very effective vector due to its highly anthropomorphic behavior of primarily biting humans and resting within the dwellings after having taken a blood meal (Clements 1992, Takken and Knols 1999).

An. gambiae behavior has been the focus of research aimed at understanding and reducing malaria transmission. Haddow and Ssenkubuge (1962) reported *An. gambiae* has two peaks of flight activity. The first occurs shortly after sunset and was attributed to ovipositional site selection and egg deposition. The second peak occurs just before sunrise and was attributed to blood-feeding. This bimodal activity was confirmed by Jones and Gubbins (1978) who further determined that the peak flight activity could be shifted by altering time of blood-feeding. In contrast to the earlier studies, McCrae's 1983 study showed that oviposition was spread across 12 h with the peak at midnight.

McCrae (1984) continued investigating *An. gambiae* oviposition by varying the composition of an ovipositional site. He reported that females preferred to deposit eggs on substrates that were black verses gray or white. This effect increased when the background contrasted to that of the target substrate.

Surprisingly few direct observation of oviposition behavior have been conducted on *An. gambiae*. Hocking and MacInnes (1948) reported that most females oviposited while sitting on the surface of the water or sitting on the side of an ovipositional bowl. In both cases, the eggs either were dropped into the water directly or rolled into the water. Giglioli (1965) conducted an elegant study reporting behavior of *An. merus*, a sibling species of *An. gambiae*. Females always alighted on wet filter paper before deposition and extruded an egg every 10-12 sec. At the beginning of a depositional bout, the abdomen was tilted up at 45°; it slowly lowered during the bout to the final position of being parallel to the substrate surface. Although this study gives insight on how *An. gambiae* might behave, such details have never been established for this highly medically important species.

Questions raised and answered in this study are: 1) Will *An. gambiae* females oviposit in small laboratory arenas amenable to close observations? 2) What are the identifiable and reproducible steps leading up to initiation of oviposition? 3) What are the identifiable and reproducible steps within a bout of egg deposition? 4) Do behaviors change depending upon type of substrate abutting water? 5) From what locations will females in this arena oviposit? 6) Do behaviors change with ovipositional experience?

Materials and Methods

Sources of mosquitoes and physiological states

Wild females

An. gambiae females were collected with hand-held aspirators from houses outside the Kenya Medical Research Institute (KEMRI) compound (Kisian, Kenya) between 7am and noon. The mosquitoes were transported to the laboratory in cardboard containers (8 cm diam. x 8 cm high) and separated by physiological state (blood-fed, half-gravid and gravid) as defined by Hocking and MacInnes (1948). Blood-fed and hypergravid groups were kept in the container with access to water and 10% sugar solution and withheld from an ovipositional resource until they were hyper-gravid (at least one full day after becoming gravid). Gravid females were tested the same night of the day they were collected.

Laboratory females

Observations were also performed on individuals from a colony of *An. gambiae* (G3 strain) obtained from William E. Collins through the Malaria Research & Reference Reagent Resource Center (Manassas, VA.). Larvae were reared in clear plastic containers (27 cm X 19.5 cm X 9.5 cm) filled with 2 cm of deionized water at a density of *ca.* 200 larvae per container. The water was not changed or aerated and was maintained at 28 ± 2° C. Approximately 5-25mg of food mixture (60% wheat flour, 25% bakers yeast, 10% defibrinated beef blood, and 5% non-fat dried milk) was dispensed onto the surface of each container every day. Adults were maintained in 60 X 60 X 60 cm cloth-screened

cages at 29 ± 3° C and 80% relative humidity under a 12:12 h (L: D) photoperiod. Females were blood-fed twice a week and a 10% clover honey solution was available at all times. Eggs were deposited on wet, white filter paper (11 cm Whatman #1) over a Petri dish filled with distilled water and cotton balls. Eggs were incubated within moist paper and hatched larvae transferred to new larval pans for colony maintenance.

Behavioral arena

The observational arena was a Plexiglas container 21 X 2.5 X 12 cm provisioned with soil and distilled water as shown in (Figure 11). Unless otherwise stated, a strip of Whatman number 1 filter paper covered the soil so as to aid counting and recovering of eggs turning black *ca.* 1 h after deposition. Experiment 1 used murram soil (see Chapter 2), Experiment 2 used Michigan soil (see Chapter 2), and Experiment 3 used Michigan soil or heat sterilized glass beads to create the slant. The top of the arena was covered with nylon netting.

Experiment 1: Behavioral observations of egg depositional behaviors using wild-caught females

This experiment was performed in a guest room of the Nyanza Club, (Kisumu, Kenya) overlooking Lake Victoria. The apparatus was set up at sundown. Light for the tests were provided by a 4watt tungsten bulb shielded to provide approximately 20 lux at the observational arena. At most, five hyper-gravid mosquitoes were drawn into a hand-held aspirator and gently blown into the observational arena. Mosquito activities in the arena were monitored and recorded with Sony Digital 8 video cameras using night-shot capability. When a

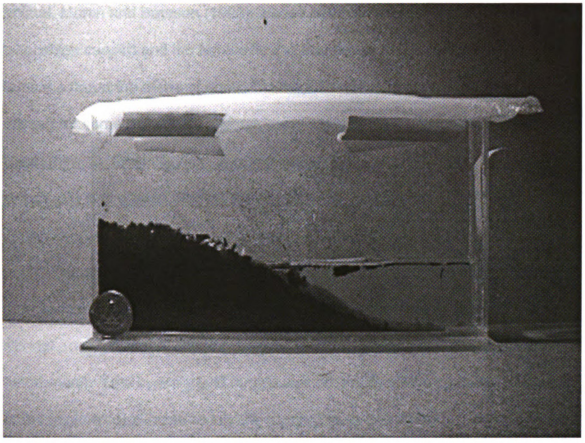

Figure 11. Plexiglas arena (21 x 2.5 x 12 cm) for ovipositional behavioral experiments.

female settled and began depositing eggs, that individual became the focal animal, Martin and Bateson (1993), whose behavior was recorded on video until oviposition ceased and the female flew to another location. Since more than one female entered the arena at once, this meant that initiation of oviposition was missed for some individuals and oviposition by various individuals was ignored.

Experiment 2: Quantification of ovipositional behaviors of G3 females tested individually and offered distilled water and Michigan soil overlaid with white filter paper

Females were drawn from the rearing cage by hand-held aspirator after their second blood-meal and placed into a cardboard container (see above) without an ovipositional site until they were hyper-gravid (i.e. 4-5 d after the blood-meal). One hyper-gravid female was drawn out of the container, the end of the aspirator was inserted into the arena (Figure 11) under the mesh, and the female was allowed to exit the aspirator voluntarily. The light level was similar to that in Experiment 1. Recording began once the female entered the arena. Females were visually observed without instrumentation; types of behavior and times were recorded verbally onto a tape recorder for subsequent transcription. Age and experience of the mosquitoes were not specified.

Experiment 3: Comparison of oviposition behaviors of inexperienced and experienced G3 females tested individually and offered diverse substrates

Laboratory females having oviposited at least once were segregated from those that had never laid eggs. One hyper-gravid female of known ovipositional experience was placed individually into an arena containing one of 4 types of

substrate treatments: recently disturbed Michigan soil with and without a cover of Whatman #1 filter paper, or, glass beads with or without filter paper. Behavior was recorded as per Experiment 2. The data were analyzed using a 2 x 2 x 2 factorial design (experience, substrate type, covered or not) followed by Tukey's Honestly Significant Difference (HSD) test for mean separations (SAS Institute, 1999).

RESULTS AND DISCUSSION

Experiment 1: Behavioral observations of egg depositional behaviors using wild-caught females

After being transferred into the Plexiglas arena, a typical female intermittently flew about the container, landing on the cover and sidewalls. Most females initiated oviposition less than five minutes after entering the arena. Within this small arena, females displayed considerable plasticity in locations from which oviposition could occur. Egg deposition began after females alighted at various locations: five females did so on the glass over water, six on the glass over soil, three on the slant with eggs dropping into the water, five on the slant with eggs dropping onto the soil, nine on the soil, and seven while sitting on the water. There were two instances where falling eggs could not be connected to a given female. In these cases it is possible that a female may have been at the top of the arena out of sight of the video camera, or, possibly in flight. The latter case would support the claim of McCrae (1984) that females are capable of oviposition in flight, however, this behavior was, at best, rare under our conditions. Typically, the female landed at its ovipositional location with its

abdomen projecting upward at *ca.* 45° throughout the bout. This angle of the body is consistent with the beginning stance of *An. merus* (Giglioli 1965), however in contrast to *An. merus*, *An. gambiae* held that angle throughout the bout (Giglioli 1965).

Once an egg protruded from the gonopore, it remained there for approximately 2 sec, perhaps for completion of insemination, then was ejected and dropped. The next egg became visible almost immediately after the previous egg dropped. The egg-dropping interval was 5 sec ±1 S.D. Egg expulsion continued with high regularity until all available eggs were laid or another female disrupted the bout, causing the female to relocate within the arena. This egg-dropping interval is longer than the 2 sec Hocking and MacInnes (1948) reported for *An. gambiae*. The estimate of 5 sec is realistic when the need to inseminate each egg individually is taken into account (Clements 1992). Impressingly, this egg-dropping interval is still faster than other *Anopheles* species: *An. punctipennis* = 6 - 7 sec (Herms and Freeborn 1921), *An. merus* = 10 - 12 sec (Giglioli 1965), and *An. albimanus* = 10 - 15 sec (Rozenboom 1936).

Most females slowly waved their metathoracic legs in a treadmill-type motion throughout most of the ovipositional bout. The only female not observed to wave her legs in the bout was observed for a very short time at the end of her bout. This is inconsistent with the observations of *An. gambiae* reported by Hocking and MacInnes (1948), since, they only noticed this behavior in one of nine females. In addition to leg movement in my study, four females telescoped

their abdomen in small twitching motions, three females walked during deposition, and three females slowly moved their wings up and down (Table 3). All of these behaviors seemed to be associated with egg expulsion, since they occurred just before an egg dropped. Large amounts of abdominal twitching or abdominal contractions were not observed in the current study as reported for *Ae. aegypti* (Curtin and Jones 1961). Four females had eggs cling to the abdomen and that did not fall individually (Table 3). In one of these cases, the female touched her abdomen to the substrate and was able to dislodge the eggs, while in the other cases the female was able to dislodge the eggs via metathoracic leg waving. Most females ended the ovipositional bout by flying to a different location within the arena where they remained motionless.

Experiment 2: Quantification of ovipositional behaviors of G3 females tested individually and offered distilled water and Michigan soil with white filter paper

Ovipositional behaviors of females from the G3 laboratory culture were nearly identical to those of wild-caught females. The locations of deposition were: five on glass over water, three on glass over soil, two on the slant with eggs dropping into water, three on the slant with eggs dropping onto the soil, seven on soil, and five on water. No females were observed to oviposit during flight (Table 4). Most of the females first landed on the arena cover and then located a position to oviposit with their 2nd or 3rd landing (Table 4). Only two individuals landed four times before ovipositing. For all the females ovipositing, mean initiation time was 123 ± 42 S.D. sec.

Table 3. Behaviors of wild-caught (n=37) an laboratory (n=24) *Anopheles gambiae* females during continuous monitoring of egg deposition in a 21 x 2.5 x 12 cm Plexiglas observational arena

	Wild-caught females		Laboratory females	
Category of behavior	Frequency[1]	Mean total duration per video sample (sec)	Frequency[1]	Mean total duration per video sample (sec)
Proboscis probing	37/37	6 ± 4	24/24	223 ± 73
Metathoracic leg waving	36/37	378 ± 84	24/24	648 ± 97
Abdominal telescoping	4/37	132 ± 52	15/24	9 ± 3
Walking	3/37	27 ± 15	3/24	42 ± 14
Wing movement	2/37	72 ± 13	3/24	14 ± 9
Eggs clinging to abdomen	4/37	n/a	2/24	n/a
Abdomen touching	1/37	2 ± 1	3/24	1 ± 1
Bout ended by flying	36/37	n/a	24/24	n/a

[1]. Indicated behavior occurred at least once during the sample

Table 4. Location of landings for individual laboratory *Anopheles gambiae* females directly observed in a Plexiglas arena (21 x 2.5 x 12 cm) containing Michigan soil during ovipositional site selection

Location of Landing	1st	2nd	3rd	4th
Top	20	1	0	0
Glass over soil	0	3	2	0
Glass over water	0	4	3	0
Soil	3	6	4	0
Water	1	4	1	1
Slant	0	4	3	1

Metathoracic leg waving bouts were longer in the laboratory strain than the wild females, 648 ± 97 and 378 ± 84 S.D. sec respectively. Proboscis probing, where the mosquito was seen taping the substrate, was also greater in the G3 strain then the wild females, 223 ± 73 and 6 ± 4 S.D. sec respectively (table 3). These differences could be explained most simply by the fact that the mosquitoes in Experiment 2 but not Experiment 1 could be observed for the entire duration on the ovipositional bout and therefore more data were able to be collected or that it is a byproduct of laboratory rearing. Although more females exhibited abdominal telescoping in the G3 strain than the wild females (15 of 24 females and 4 of 37 females, respectively) the G3 females did not sustain this behavior as long as did the wild females (9 ± 3 and 132 ± 52 S.D. sec respectively).

Many eggs were not deposited in water directly. More than half of the eggs in all the experiments were laid on moist soil within 10 cm from the standing water. This would imply that the larva have some method of reaching the water once they have hatched on the mud, or that the area would flood with water before the eggs desiccated. Koenradt et al. (2003) and Miller et al. (unpublished) have evidence that larvae can displace by tail-lashing or caterpillaring and are prone to move down a slope, allowing larvae that hatched away from the water a method to reach the puddle.

In contrast to Hocking and MacInnes (1948) who reported *An. gambiae* ovipositional bouts are often incomplete and spread out over space and time, females in the current study deposited all their eggs before ending the bout, if left

undisturbed. This is supported by Herms and Freeborn (1921) who reported *An. punctipennis* deposited eggs continually for 19 min.

Experiment 3: Comparison of oviposition behaviors of inexperienced and experienced G3 females tested individually and offered diverse substrates

Experience of the females had the greatest treatment effect in this experiment. Experienced females initiated oviposition with a frequency of 46% compared to 33% for inexperienced females (Table 5 and 6). Moreover, experienced females initiated deposition significantly faster than inexperienced females (179 vs. 230 sec respectively, $P< 0.0001$) (Figure 12). Experienced females deposited significantly more eggs than inexperienced females (126 vs. 108 eggs, respectively), an outcome reported by Clements (1992) to be a general pattern. Proboscis probing prior to deposition and during deposition was significantly greater in inexperienced females than experienced females (prior to deposition 85 vs. 62 sec respectively, $P< 0.0001$: during deposition 173 vs. 149 sec respectively, $P< 0.0001$).

As expected from earlier studies (McCrae 1984, Pavlovich and Rockett 2000) the type of substrate can influence oviposition of mosquitoes. In this study, females oviposited significantly faster on soil than glass beads (187 vs. 221 sec respectively, $P< 0.0001$) (Figure 12). This trend was consistent across states of experience of the female; experienced females oviposited sooner on soil than glass beads (159 vs. 197 sec, respectively), and inexperienced females oviposited sooner on soil than glass beads (214 vs. 245 sec, respectively).

Table 5. Individual experienced or first time laboratory female *Anopheles gambiae* observed inside a 21 x 2.5 x 12 cm Plexiglas ovipositional arena with Michigan soil or glass beads with filter paper

Behavior during oviposition	Soil			
	Inexperienced		Experienced	
	Freq. for group[1]	Mean elapsed time (min) ± S.E.M. (sec)	Freq. for group[1]	Mean elapsed time (min) ± S.E.M. (sec)
Initiated oviposition	15/38	n/a	15/25	n/a
Proboscis probing	15/15	4.2 ± 35	15/15	3.5 ± 43
Metathoracic leg waving	15/15	10.4 ± 143	15/15	10.0 ± 252
Abdominal telescoping	7/15	1.2 ± 31	3/15	0.9 ± 12
Walking	1/15	0.5 ± 0	2/15	0.4 ± 42
Wing movement	2/15	0.3 ± 27	1/15	0.4 ± 0
Eggs clinging to abdonen	3/15	n/a	1/15	n/a
Abdomen touching	5/15	0.02 ± 14	0/15	n/a
Bout ended by flying	15/15	n/a	15/15	n/a
	Glass beads			
Initiated oviposition	15/49	n/a	15/32	n/a
Proboscis probing	15/15	5.6 ± 44	15/15	4.2 ± 33
Metathoracic leg waving	15/15	11.02 ± 133	15/15	10.6 ± 103
Abdominal telescoping	3/15	0.9 ± 21	0/15	n/a
Walking	3/15	1.3 ± 15	1/15	0.8 ± 0
Wing movement	0/15	n/a	3/15	0.06 ± 24
Eggs clinging to abdonen	0/15	n/a	1/15	n/a
Abdomen touching	2/15	0.02 ± 03	0/15	n/a
Bout ended by flying	15/15	n/a	15/15	n/a

1. Indicated behavior occurred at least once during the sample

Table 6. Individual experienced or first time laboratory female *Anopheles gambiae* observed inside a 21 x 2.5 x 12 cm Plexiglas ovipositional arena with Michigan soil or glass beads without filter paper

Behavior during oviposition	Soil			
	Inexperienced		Experienced	
	Freq. for group[1]	Mean elapsed time (min) ± S.E.M. (sec)	Freq. for group[1]	Mean elapsed time (min) ± S.E.M. (sec)
Initiated oviposition	15/42	n/a	15/33	n/a
Proboscis probing	15/15	2.7 ± 12	15/15	1.2 ± 22
Metathoracic leg waving	15/15	9.9 ± 144	15/15	11.7 ± 121
Abdominal telescoping	2/15	0.8 ± 16	1/15	0.3 ± 0
Walking	4/15	0.8 ± 23	1/15	0.25 ± 0
Wing movement	2/15	0.27 ± 17	0/15	n/a
Eggs clinging to abdonen	1/15	n/a	0/15	n/a
Abdomen touching	0/15	n/a	0/15	n/a
Bout ended by flying	15/15	n/a	15/15	n/a
	Glass beads			
Initiated oviposition	15/50	n/a	15/45	n/a
Proboscis probing	15/15	4.8 ± 131	15/15	5.3 ± 96
Metathoracic leg waving	15/15	10.1 ± 261	15/15	10.35 ± 246
Abdominal telescoping	3/15	0.6 ± 29	2/15	0.8 ± 15
Walking	2/15	0.7 ± 38	0/15	n/a
Wing movement	0/15	n/a	0/15	n/a
Eggs clinging to abdonen	1/15	n/a	0/15	n/a
Abdomen touching	0/15	n/a	1/15	0.02 ± 0
Bout ended by flying	15/15	n/a	15/15	n/a

[1]. Indicated behavior occurred at least once during the sample

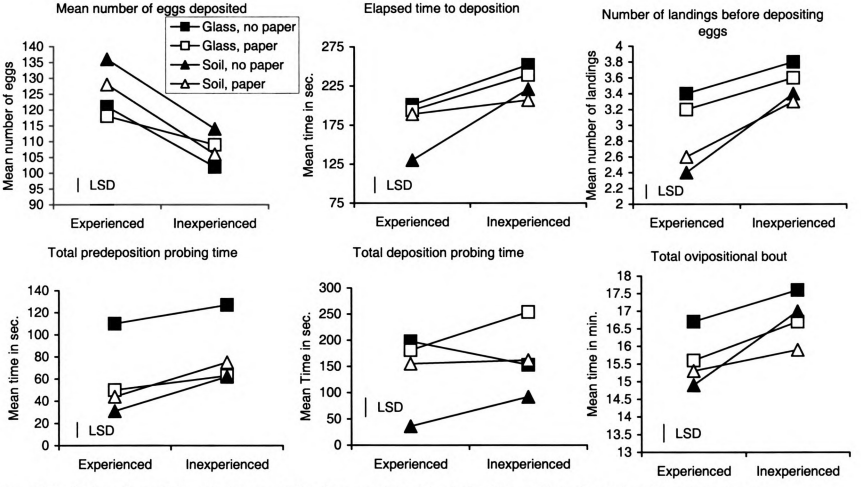

Figure 12. Oviposition of *Anopheles gambiae* females in a 21 x 2.5 x 12 cm Plexiglas ovipositional arena as ifluenced by substrate type (Michigan soil or glass beads), presence or absence of filter paper over the substrate, and female ovipositional experience.

The only results that were influenced by the filter paper treatment were proboscis probing prior to oviposition and proboscis probing during deposition (Figure 12). Females probed the substrate with the proboscis significantly more frequently on substrates without than with filter paper (85 and 62 sec, respectively, $P< 0.0001$). Analyzing total proboscis probing time during deposition revealed the opposite trend; there was significantly greater probing on substrates with filter paper than without paper (199 and 123 sec, respectively). Comparing the effects of treatments for both probing prior to deposition (preprobing) and total proboscis probing during the entire ovipositional, there was a significant interaction effect between the paper treatment and experience of the females and between the paper treatment and substrate (preprobing $P=0.0026$ and $P< 0.001$ respectively, total probing $P=0.0003$ and $P< 0.001$) (Figure 12). As stated earlier, the experience of the female and type of substrate are highly significant with respect to probing time. Due to the highly significant effect of these two parameters, I suggest their effect plays a greater role in proboscis probing time than the presence or absence of paper.

It is interesting that the location of oviposition within the arena for Experiment 3 was similar to that in Experiment 2, with the exception of the treatments with glass beads without filter paper. Experienced and inexperienced females did not deposit eggs while sitting directly on the bear glass beads. This inhibition was not seen on the treatments of glass beads with filter paper since the paper wicked moisture over the beads and therefore maintained an acceptable ovipositional site.

In general, the overall ovipositional sequence did not change due to experience of the female or the difference in substrate type (Table 5 and 6). The actual performance of behaviors by females did not change greatly with any of the eight treatment combinations. The differences noted were mainly durations of behaviors, e.g., the duration of proboscis probing. The most inefficient group, i.e. laying the fewest eggs, using more time and energy to locate an appropriate site, and probing a high amount, was the inexperienced females with filter paper covering the glass beads. Conversely, the most efficient treatment combination was experienced females with free access to soil. Finally, this experiment also demonstrates that even a very poor ovipositional site can elicit deposition when no other resource is available.

APPENDIX

APPENDIX 1

Record of Deposition of Voucher Specimens

Appendix 1

Record of Deposition of Voucher Specimens*

The specimens listed on the following sheet(s) have been deposited in the named museum(s) as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the Voucher No. have been attached or included in fluid-preserved specimens.

Voucher No.: 2003-06

Title of thesis or dissertation (or other research projects):

Ovipositional Behavior of *Anopheles gambiae* as Influenced by Variable Substrates and Chemicals

Museum(s) where deposited and abbreviations for table on following sheets:

Entomology Museum, Michigan State University (MSU)

Investigator's Name(s) (typed)
Alicia Marie Bray

Date 12/11/03

*Reference: Yoshimoto, C. M. 1978. Voucher Specimens for Entomology in North America.
Bull. Entomol. Soc. Amer. 24: 141-42.

Deposit as follows:

Original: Include as Appendix 1 in ribbon copy of thesis or dissertation.

Copies: Include as Appendix 1 in copies of thesis or dissertation.
Museum(s) files.
Research project files.

This form is available from and the Voucher No. is assigned by the Curator, Michigan State University Entomology Museum.

Appendix 1

Table 7. Voucher Specimen Data

Page 1 of 1 Pages

Species or other taxon	Label data for specimens collected or used and deposited	Number of: Eggs	Larvae	Nymphs	Pupae	Adults ♀	Adults ♂	Other	Museum where deposited
Anopheles gambiae (G3 strain)	Michigan State University laboratory colony December 11, 2003					10	10		MSU

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

Alicia Marie Bray

Date 12/11/2003

Voucher No. 2003-06

Received the above listed specimens for deposit in the Michigan State University Entomology Museum.

Curator 12 Dec 2003

Date

LITERATURE CITED

LITERATURE CITED

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