INVESTIGATIONS OF THE AFRICAN MALARIA MOSQUITO (*ANOPHELES GAMBIAE S.L.*, DIPTERA: CULICIDAE): OVIPOSITIONAL BEHAVIOR AND TOXICITY OF AVERMECTINS

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ABSTRACT:

INVESTIGATIONS OF THE AFRICAN MALARIA MOSQUITO (*ANOPHELES GAMBIAE S.L.*, DIPTERA: CULICIDAE): OVIPOSITIONAL BEHAVIOR AND TOXICITY OF BLOOD-BORNE INSECTICIDES

By

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Anopheles gambiae sensu stricto is a major malaria vector in Sub-Saharan Africa. High malaria morbidity and mortality rates are experienced by humans, mainly children under the age of 5, living in endemic regions. Studiesof the basic biology of this mosquito, including oviposition, provide a background for assessing which attributes might be exploited for suppressing *An. gambiae s.s.* populations. Ovipositional periodicity of groups assessed using a modified battery-powered wall clock revealed bimodal egg deposition. Oviposition by caged *An. gambiae s.s.*groups is most probable in early to mid scotophase, or early photophase. Confined *An. gambiae s.s.* individuals oviposit in a single *ca.* 2–4 h continuous bout per 24 h. However, some oviposition can occur at any hour during 24 h, especially if females were previously deprived of ovipositional substrate. Many females sit on a dark, wet horizontal substrate while ovipositing. However, vertical resting sites are adequate perches from which oviposition can occur if they provide high humidity and the paramount dark and wet ovipositional cues for *An. gambiae s.s.* oviposition. Under such conditions, laboratory tests revealed that *An. gambiae s.s.* were as likely to rain eggs down from a vertical perch, as to oviposit while sitting horizontally on a substrate of moist mud or open water. These studies confirm remarkable ovipositional flexibility by *An. gambiae s.s.*, and I conclude that oviposition is not a suitable target for *An. gambiae s.s.* management.

Host choice appears to be an attribute that is relatively restricted for *An. gambiae sensu lato*. Despite increasing selection pressure imposed by ITN use in Western Kenya, most blood meals from *An. gambiae s.s.* were imbibed from human hosts, whereas sibling species, *An. arabiensis,* blood fed upon cattle. Prior to 1998 *An. gambiae s.s.* comprised between > 70% of the total *An. gambiae s.l.* population in Kisian village. In the present study, >50% of the *An. gambiae s.l.* collected were identified as *An. arabiensis*, revealing a shift in the dominant vector species. Ivermectin, a cattle dewormer, is highly lethal to both *An. gambiae s.s.* and *An. arabiensis.* When cattle are treated with ivermectin, ninety percent of *An. gambiae s.s*. that fed upon them within 2 wk of treatment failed to survive >10 d post blood meal. No eggs were deposited by *An. gambiae s.s.* fed on ivermectin-treated cattle within 10 d of treatment. According to the label, commercial formulations of ivermectin cannot be used on pregnant or lactating animals. Laboratory tests of eprinomectin, a commercially available dewormer for pregnant and lactating cattle, revealed equivalent toxicity to *An. arabiensis* at low concentrations (LC_{50} of 8.5 ppb). These results suggest that cattle treated with ivermectin or eprinomectin in the prescribed range of low dosages as parasiticides have blood toxic to zoophilic malaria vectors. Regionally-coordinated, seasonal treatment of cattle could suppress *An. arabiensis* populations, thereby reducing malaria transmission.

Dedicated with love to:

Brad, who has supported me

Oliver and Corrina, who have inspired me

My parents, who have encouraged me

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GLOSSARY OF ABBREVIATIONS

Ae. *– Aedes*

An. *– Anopheles*

AVM – avermectin $B1_a$

Cx. **–** *Culex*

D. *– Drosophila*

d – day

GABA - γ-aminobutyric acid

h - hour

IRS – Indoor residual spraying

ITN – Insecticide-treated bed net

P. *– Plasmodium*

sensu lato – wide sense

sensu stricto – narrow sense

wk - week

GENERAL INTRODUCTION:
BIOLOGY, ECOLOGY, AND BEHAVIOR OF THE SIBLING SPECIES, *ANOPHELES GAMBIAE S.S.* AND *ANOPHELES ARABIENSIS* AS THEY PERTAIN TO HUMAN MALARIA TRANSMISSION

Impact of Malaria in Africa

Human malaria is the scourge of the tropics, and is devastating to human health, economic growth, and regional development. Each year, malaria causes 2.7 million deaths world-wide, though 90% is experienced by people, particularly children, living in Sub-Saharan Africa (Breman *et al.* 2001, Molyneux 2004). Countries at risk for severe malaria are often impoverished, and experience very slow, or negative economic growth. Between 1965 and 1990, growth of per capita income in countries at low or no risk for malaria was 5 times higher than in malarious countries (Gallup and Sachs 2001). In areas where malaria abounds, development of human society is impeded by medical costs, premature mortality, low worker productivity, absenteeism, and reduced savings and investment (Sachs and Malaney 2002).

Malaria transmission

The malaria parasites *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae* are transmitted between humans by *Anopheles* mosquito vectors. *P. falciparum* causes the majority of life-threatening and deadly malaria cases throughout the tropics. Newly eclosed mosquitoes are only able to transmit malaria after feeding on human blood containing mature male and female gametocytes. Male and female gametocytes unite in the gut of the mosquito and the parasite begins a complex cycle of multiplication and development. Mature parasites (sporozoites) that are infective to humans eventually migrate to the mosquito's salivary

glands, but the time required for parasite maturation depends on the *Plasmodium* species and the external temperature. The two most prevalent parasites, *P. vivax* and *P. falciparum*, require approximately 8-13 days to complete this cycle within the mosquito if external temperatures are 27ºC (Pampana 1969). Lower external temperatures lead to slower parasite maturation (Figure 1.1). Infected *Anopheles* vectors only transmit malaria if they outlive the required time for parasite maturation (Zucker 1996).

The primary vectors of malaria in Sub-Saharan Africa are the mosquitoes of the *Anopheles gambiae* Giles *sensu lato* complex (*An. gambiae s.l.*), and *An. funestus* Giles. *Anopheles gambiae sensu lato* (Latin for "wide sense") refers to a complex of morphologically indistinguishable species. This complex includes *An. gambiae sensu stricto* (Latin for "narrow sense", and hereafter referred to as *An. gambiae*), *An. arabiensis* Patton, *An. quadriannulatus*, *An. bwambae*, and the salt water species *An. merus* and *An. melas*. The sibling species *An. gambiae* and *An. arabiensis*, reputed to be two of the most efficient vectors of malaria in the world (Coetzee *et al.* 2000), are the focus of my research. Malaria transmission is dependent upon the prevalence of competent vector species (Omumbo *et al.* 1998) and the availability of suitable aquatic vector breeding sites (Holstein 1954). As larval habitat availability increases at the onset of the rainy season, populations of these species grow rapidly (Joshi *et al.* 1975). Mean indoor resting density of *An. gambiae* and *An. arabiensis* may reach as high as 223 females per house, and average human bite rate can reach over 170 bites per human per night in some regions of Sub-Saharan Africa (Molineaux and Gramiccia 1980). Human biting rates can be attributed not only to high vector abundance, but also accessibility of human hosts. For example, mosquitoes easily pass in and out of homes with open eaves (Figure 1.2), and have nightly access to sleeping humans.

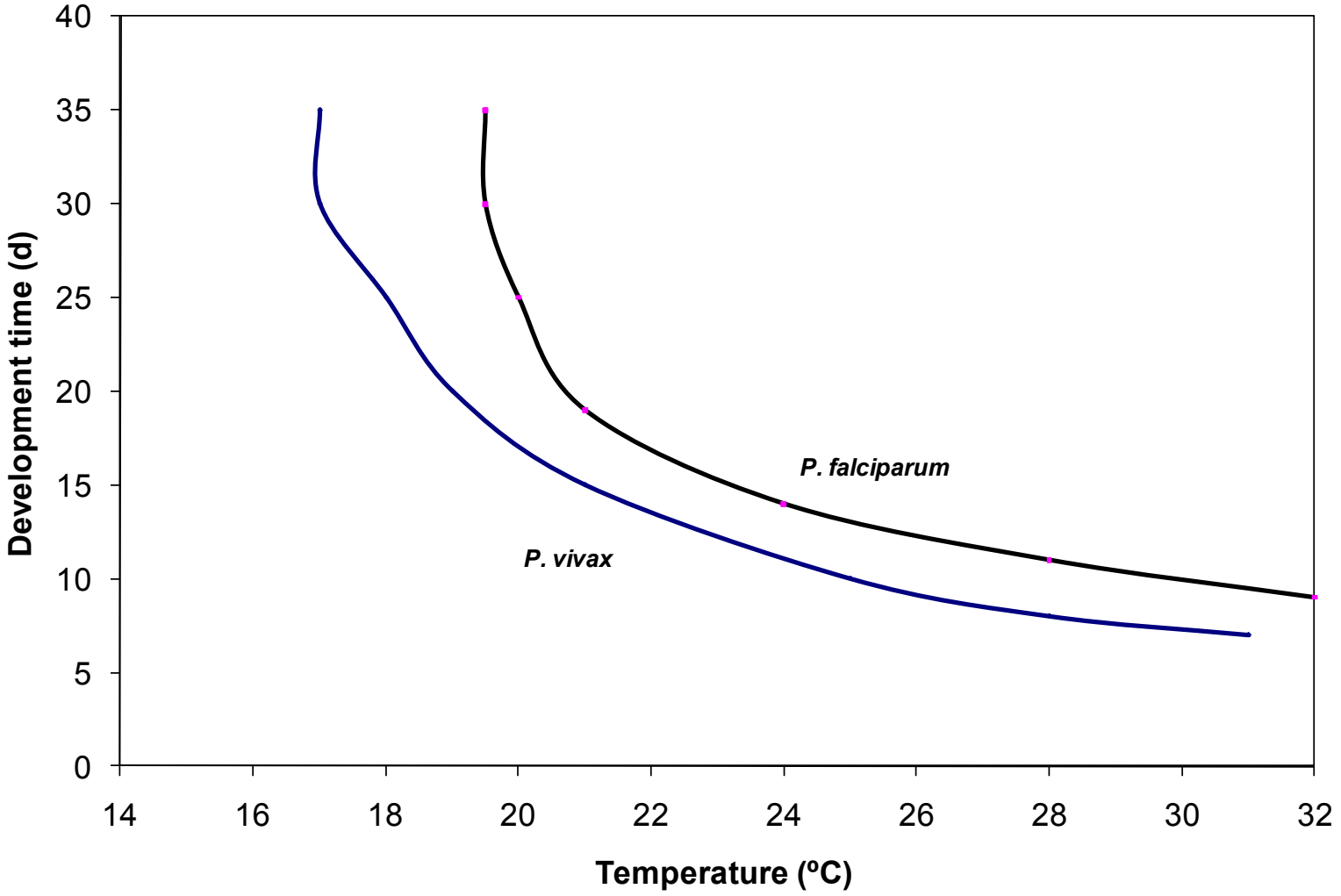

Figure 1.1. Parasite maturation time within the mosquito vector *vs*. external temperature. Adapted from Patz and Olson (2006).

Figure 1.2. Eave-opening in traditional housing of the Luo tribe in Kisian, Kenya. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Although the proportion of *An. gambiae* complex mosquitoes containing mature sporozoites varies only between 1.9 and 11.8 % in Western Africa (Molineaux and Gramiccia 1980), the high human biting rate significantly elevates the risk of malaria infection during the rainy season. Vectorial capacity is positively correlated with both biting frequency (Garrett-Jones 1964b) and especially vector life-span (Gary and Foster 2001). Some *An. gambiae* and *An. arabiensis* females live for 50 days or more (Gary and Foster 2001). Longer-lived females take more blood meals during their lifetime, which increases the probability of acquiring and spreading malaria parasites.

Biology of the mosquito vectors

Populations of *An. gambiae* and *An. arabiensis* are widely distributed throughout Sub-Saharan Africa, and often occur in sympatry. They are morphologically indistinguishable, but can be identified to species level using polymerase chain reaction (Paskewitz and Collins 1990). *An. gambiae* and *An. arabiensis* were considered a single species until 1962, when their distinct behaviors and physiologies led to the recognition of the *An. gambiae* complex (White 1974).

Of these two sibling species, *An. gambiae* predominates in more humid environments (White 1974). In Western Kenya, *An. gambiae* larvae are found in ground pools, irrigation ditches, puddles, tire tracks and hoof prints (Minakawa *et al.* 1999; Mutuku *et al.* 2006) shortly after the onset of the rainy season. Larval development is completed within 5-12 days depending on the temperature (Bayoh and Lindsay 2003), as well as density of larvae per habitat (Gimnig *et al.* 2002). Adult *An. gambiae* are well-known for high endophily (indoor resting behavior) and anthropophagy (White and Rosen 1973, White 1974). *An. gambiae* will preferentially feed on humans, and the human blood index (the proportion of blood meals taken from a human host; Garrett-Jones 1964a) of this species remains at greater than 80 %,

even in the presence of alternative hosts (White 1974). Peak indoor biting time for *An. gambiae* females occurs between 0100 and 0600 h, though bites can occur anytime between 2000 and 0600 h (Githeko *et al.* 1996). In Western Kenya, freshly blood-fed *An. gambiae* are more than twice as likely to rest indoors than outdoors (Odiere *et al.* 2007) while processing their blood meal, although this mosquito is frequently found resting inside houses regardless of physiological state (White 1974).

While *An. arabiensis* often shares larval habitats and ovipositional sites with its sibling species, *An. gambiae* (White and Rosen 1973, Minakawa *et al.* 1999), they can also utilize slow-flowing riverine habitats for egg deposition during the dry season, when transient ground pools disappear (Dukeen and Omer 1986). The ability of *An. arabiensis* to use these year-round habitats for reproduction, along with their higher body water content (Gray and Bradley 2005), allows this mosquito to inhabit drier geographic regions, and predominate during periods of low rainfall. Therefore, the relative proportions of *An. gambiae* and *An. arabiensis* vary due to: 1) microclimatic differences in a region, 2) monthly seasonal rainfall, and 3) yearly changes in average relative humidity (Highton *et al.* 1979).

An. arabiensis is also anthropophagic, but will opportunistically feed on other hosts. In Kenya, it was reported that only 39% of *An. arabiensis* fed on humans, but 59% took blood meals from cattle (Highton *et al.* 1979). In Ethiopia, however, 51% of outdoor resting, and 66% of indoor resting *An. arabiensis* contained human blood (Tirados *et al.* 2006). At this same location, ca. 25% *An. arabiensis* contained mixed cattle-human blood meals. The resting behavior of *An. arabiensis* combines endophily and exophily (outdoor resting behavior). It has been reported that populations of *An. arabiensis* in Northern Sudan are almost entirely endophilic (Dukeen and Omer 1986), but populations from Southern Ethiopia are five times more likely to rest outdoors than indoors (Tirados *et al.* 2006). Behavioral differences between *An. arabiensis* populations are likely linked to genetic variability

(Coluzzi *et al.* 1979), and indoor vector control measures, such as indoor residual spraying (IRS), exert heavy selection pressure for exophily in *An. arabiensis* populations (White 1974).

Reduction in malaria transmission by these important vector species relies upon a thorough understanding of the vector's life history. Life history research identifies behavioral and physiological traits that can be exploited for vector control. Once potential vector control methods are identified, their efficacy and environmental impacts must be thoroughly investigated before widespread implementation in the field. The following dissertation includes research on the basic ovipositional biology of the mosquito, *An. gambiae*, as well as analysis of the efficacy of a novel vector control measure for the sibling species pair, with the ultimate goal of reducing malaria transmission and saving human lives.

PART I:

STUDIES ON THE OVIPOSITIONAL BIOLOGY OF

ANOPHELES GAMBIAE S.S.

CHAPTER 1:

OVIPOSITIONAL BIOLOGY OF *ANOPHELES GAMBIAE S.S.*

Mosquitoes use a diverse, sometimes species-specific combination of physical, chemical, and temporal cues to find a suitable ovipositional substrate. While ovipositional strategies vary, the outcome is identical across mosquito taxa; females provide their offspring with access to aquatic habitats. Mosquito larvae must ultimately find themselves in these aquatic habitats, containing required food resources and suitable water quality, in order to maximize their fitness. Egg placement into these larval habitats represents one way by which the mother improves the likelihood that some of her offspring survive. Thus, ovipositing females may be sensitive to chemical and physical cues correlated with the quality of a larval habitat. Larvae of *An. gambiae* are commonly found in small, sunlit pools that are typically the result of human activity (i.e. tire tracks, cattle hoof prints) (Gimnig *et al.* 2001). These habitats are transient, and typically devoid of vegetation. Attributes of these larval habitats that serve as ovipositional stimulants to *An. gambiae* have been, and continue to be investigated.

Chemical cues

Most species of mosquito spend their immature stages in aquatic habitats rich in decaying organic matter. The volatile chemicals 4-methylcyclohexanol, indole, 3-methylindol, phenol, m-cresol, o-cresol, and p-cresol are all associated with such habitats (Collins and Blackwell 1998). Metabolism of organic matter by microorganisms in the water is thought to produce these volatiles (Stahl and Parkin 1996). Oviposition by gravid females of some mosquito species appears to be stimulated by water containing one or more of these chemicals (Bentley *et al.* 1981, Millar *et al.* 1992, Davis and Bowen 1994, Collins and

Blackwell 1998). In one case, the key bacteria and associated volatiles that stimulate oviposition have been identified (Ponnusamy *et al.* 2008).

Gravid *An. gambiae* oviposit more of their eggs in water from a typical larval habitat, than in tap or distilled water (McCrae 1984, Takken and Knols 1999, Bray 2003, Sumba *et al.* 2004). Chemicals associated with decaying organic matter that are stimulatory to other mosquito species, particularly 3-methylindole and indole, also elicit an electrophysiological response from *An. gambiae* antennae (Blackwell and Johnson 2000). Water containing p-cresol or phenol is favored compared to distilled water (Bray 2003). Interestingly, water containing either 3-methylindole or indole inhibits oviposition by *An. gambiae* in the laboratory. Furthermore, bacterial strains that are isolated from native larval habitats are also inhibitory or neutral to ovipositing females (Huang *et al.* 2006b).

It has been postulated that algal volatiles may be more important than bacterial volatiles for stimulating *An. gambiae* oviposition (Otienoburu *et al.* 2007). Otienoburu *et al.* (2007) demonstrated that water from Lake Victoria, near Kisumu, Kenya, is the most potent stimulus for *An. gambiae* oviposition tested to date. Many species of algae are present in Lake Victoria water (Ochumba and Kibaara 1989), and volatiles from algae are known to influence the behavior of nematodes (Höckelmann 2004). Further research into the stimulatory attributes of algal volatiles may reveal their importance to *An. gambiae* oviposition.

Visual/Physical cues

The presence of moisture is paramount to mosquito oviposition. Many insects have thermo- and hygroreceptors that enable them to respond to temperature and humidity gradients (Tichy and Loftus 1996, Tichy and Kallina 2010). These receptors have been found on the tarsi and antennae of *Ae. aegypti* (Bar-Zeev 1960). In mosquitoes, egg

deposition is stimulated by the presence of water, although egg placement behavior at a water body varies with species. For example, some *Anopheles*, *Coquilletidia*, and *Culex* species oviposit directly into the water, while *Aedes* and *Psorophora* species often oviposit near aquatic habitats, but above the water line (reviewed in Bentley and Day 1989). Early studies of *An. gambiae* revealed that gravid females would oviposit directly into the water while either resting on the water surface (Hocking and MacInnes 1948), or hovering over the water (McCrae 1984). More recent investigations of their ovipositional behavior demonstrated that many *An. gambiae* females deposit their eggs while sitting near a water source. The preponderance of eggs are deposited on moist mud rather than in open water (Miller *et al.* 2007). In fact, Huang *et al.* (2005) demonstrated that some *An. gambiae* will oviposit on sand that feels dry to the human hand, even when standing water is available. Soil moisture is positively correlated with *An. gambiae* egg deposition, yet the presence of standing water does not appear to be necessary for oviposition (Huang *et al.* 2005).

Color also serves as an important stimulatory cue for gravid female mosquitoes foraging for ovipositional resources (Williams 1962, Beehler *et al*. 1993, Li *et al*. 2009). For example, ovipositing *Ae. aegypti* are most sensitive to yellow-green wavelengths (Snow 1971), while *Ae. triseriatus* are maximally stimulated to oviposit by blue wavelengths (Williams 1962). Under natural lighting conditions, more *Cx. quinquefasciatus* deposit their egg rafts in water darkened with India ink (Beehler *et al.* 1993) than in water alone.

McCrae (1984) concluded that egg deposition by *An. gambiae* was also influenced by ovipositional substrate color. Black substrates received more eggs than either white or grey substrates. In another series of experiments, however, he concluded that turbid water, appearing lighter in color, was maximally stimulatory to ovipositing females (McCrae 1984). Huang *et al.* (2007) resolved this contradiction by demonstrating that a black ovipositional substrate is highly stimulatory to gravid *An. gambiae*, but must be placed over a light

background to maximize oviposition. Black ovipositional substrates over a white floor received 5-fold more eggs than did black ovipositional substrates over a black floor (Huang *et al.* 2007). Contrast is likely critical to *An. gambiae* during ovipositional resource location which occurs under night-time low-lighting conditions. Interestingly, Huang *et al.* (2007) report that all treatments in their contrast experiment, including a white ovipositional resource over a white background, received some *An. gambiae* eggs. This indicates a remarkable flexibility by *An. gambiae* in their acceptance of ovipositional resources.

The daily light cycle influences the timing of oviposition among mosquito taxa. Daylight stimulates *Ae. aegypti* to oviposit, and most oviposition occurs just before dusk (Corbet and Chaddee 1990, Chaddee 2010). *Culex* (Suleman and Shirin 1981) and *Anopheles* (Chaddee *et al*. 1993, Chaddee *et al.* 1998, Sumba *et al.* 2004) species oviposit during scotophase, although the peak ovipositional times vary by species. For example, three species within the genus *Anopheles* have distinct peak ovipositional windows; *An. homunculus* deposits the preponderance of their eggs in the middle of the night (Chaddee *et al.* 1998), whereas egg deposition by *An. freeborni* peaks *ca.* 4 h prior to the onset of photophase. Oviposition by *An. albimanus* peaks immediately after the onset of scotophase (Chaddee *et al.* 1993). Mosquito populations rarely exhibit ovipositional windows that are restricted to a couple of hours. Instead, oviposition by individuals in a population is spread over multiple hours of the day or night.

Conflicting data have been reported for the the time of peak oviposition by *An. gambiae* (Causey *et al.* 1942, Haddow and Ssenkubuge 1962, McCrae 1983, Sumba *et al.* 2004). Some studies have demonstrated two ovipositional peaks (Haddow and Ssenkubuge 1962, Sumba *et al.* 2004) for *An. gambiae* populations, while others have demonstrated only one (Causey *et al.* 1942, McCrae 1983). Furthermore, no study has investigated the ovipositional patterns of individual *An. gambiae* to determine whether the observed

bimodality can be attributed to multiple individuals ovipositing at different times, or a single individual splitting oviposition between two different times.

The presence or absence of vegetation influences mosquito oviposition. For some species of mosquito, such as *Wyeomyia mitchelli*, attributes of the vegetation influence oviposition (Frank 1985, reviewed in Clements 2000). In other cases, mosquitoes may oviposit more readily among vegetation due to the low lighting conditions or shade provided (Muirhead Thomson 1940). Some Anophelines, such as *An. melas* and *An. minimus*, deposit their eggs in aquatic habitats among vegetation (Giglioli 1965, Clements 2000, Overgaard 2007). Oviposition among vegetation has also been documented for *An. gambiae* (reviewed in Clements 2000, Minakawa *et al.* 2004, Huang *et al.* 2006a). However, there are no studies that investigate how *An. gambiae* navigate the three-dimensional structures present in their environment during oviposition, and whether any aspects of these structures are stimulatory. The following studies aimed to:

1.) Examine the influence of photoperiod on *An.gambiae* egg deposition, and compare the daily ovipositional patterns of *An. gambiae* populations with those of individuals.
2.) Investigate the ways in which ovipositing *An. gambiae* interact with the three-dimensional structures, including surrogate vegetation, in their environment.

CHAPTER 2:

OVIPOSITIONAL PERIODICITY OF CAGED *ANOPHELES GAMBIAE* INDIVIDUALS

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Abstract

Anopheles gambiae is a major malaria vector in Sub-Saharan Africa. Studies of the basic biology of this mosquito, including oviposition, provide a background for assessing which attributes might be exploited for suppressing *An. gambiae* populations. Here, we report on when during the diel cycle *An. gambiae* individuals deposit eggs as compared to the ovipositional patterns of groups. Battery-powered wall clocks were modified so as to present a unique section of dark and wet ovipositional substrate at hourly intervals over two consecutive 12 h periods. Ovipositional periodicity of mosquito groups (Kisumu laboratory strain or feral females) and individuals was determined by counting the number of eggs present on each section of the ovipositional substrate. Capacity for mid-afternoon oviposition by groups of Kisumu laboratory strain *An. gambiae* was determined by presenting hypergravid females with an ovipositional substrate exclusively between 1200 and 1600 h. On equatorial time, caged laboratory strain *An. gambiae* groups deposited 65% of their total eggs between 1800 and 0 h, and the remaining 35% were spread between 0 and 1000 h. Caged house-collected *An. gambiae* groups deposited 74% of their total eggs between 1800 and 200 h, ceased oviposition for 3 h, and then spread the remaining 26% of their eggs near or after dawn. Ninety-six percent of individual *An. gambiae* females spread their eggs over a continuous 2-4 h period without interruption. In tests of capacity for mid-afternoon oviposition, females given evening access to an ovipositional resource deposited 2% of their total eggs between 1200 and 1700 h. *An. gambiae* females given only access to an

ovipositional resource between 1200 and 1700 h deposited 3 times more eggs during that time period than did females previously given evening access. Confined individual *An. gambiae* oviposit in a single *ca.* 2-4 h continuous bout per 24 h. Oviposition is most probable in early scotophase, mid scotophase, or early photophase. However, some oviposition can occur at any hour during 24 h, especially if females were previously deprived of ovipositional substrate.

Introduction

Diel ovipositional patterns have been studied in various Diptera, including *Drosophila melanogaster* (Allemand 1976a, 1976b, 1977, Fleugel 1978), *D. pseudoobscura* (Fleugel 1984), *Delia antiqua* (Havukkala and Miller 1987), *Chrysomya bezziana* (Spradbery 1979), *Aedes aegypti* (Haddow and Gillett 1957), *Anopheles albimanus* (Chaddee *et al.* 1993), *An. freeborni* (Chaddee *et al.* 1993), *An. albitarsis* (Chaddee 1995) and *An. gambiae* (Causey *et al.* 1942, Haddow and Ssenkubuge 1962, McCrae 1983). All of these species are reported to deposit the preponderance of their forthcoming eggs within a 2-4 h period, but the time of maximal egg deposition varies interspecifically. However, in no case was oviposition reported to occur strictly within that 2-4 hour window. For *An. albimanus* (Chaddee *et al*. 1993) and *D. melanogaster* (Fleugel 1978), ovipositional rhythm was reported to be bimodal with unequal modes; light intensity during photophase was reported to influence the modality of *D. melanogaster* oviposition (Allemand 1977). Fluegel (1978) found that light levels furnished by a 40 W white fluorescent bulb resulted in bimodal egg deposition by *D. melanogaster* individuals. Chadee *et al.* (1993) reported that individual *An. albimanus* laid their entire complement of eggs at once rather than splitting them between two different periods.

Outcomes of research on the ovipositional periodicity of groups of *An. gambiae* held in a common cage (Causey *et al.* 1942, Haddow and Ssenkubuge 1962, McCrae 1983, Sumba *et al.* 2004) have been divergent. Causey *et al.* (1942) suggested that *An. gambiae* was capable of oviposition at any time during the night. However, they observed that five of a total of nine batches of eggs were laid between 2000 and 2300 h. Under equatorial conditions, McCrae (1983) reported wide variation in nocturnal peaks for *An. gambiae* oviposition. He postulated that the time of peak oviposition during a night was related to the time at which the blood meal was taken. However, Sumba *et al.* (2004) were unable to confirm this effect. Haddow and Ssenkubuge (1962) and Sumba *et al.* (2004), reported that oviposition of *An. gambiae* commenced at scotophase (1800 h) and peaked between 1800-2100 h. A second but smaller ovipositional peak was documented by both research teams, but at inconsistent times. In all cases, some oviposition occurred throughout scotophase. In one case (Sumba *et al*. 2004) a feral population deposited about 3% of the total eggs after the onset of photophase. In other cases, it was unclear whether any attention was paid to the possibility of oviposition throughout photophase. Left unknown in all of these studies is whether individual females spread their oviposition across many hours, or whether some individuals deposit all of their eggs early in the night while others deposit all of their eggs near morning.

Methods

Mosquitoes

Two sources of mosquitoes were used: 1) The Kisumu laboratory strain of *An. gambiae s.s.* originated from the Kenya Medical Research Institute (KEMRI) of Kisumu, Kenya. It was reared at Michigan State University according to Huang *et al.* (2005). 2) Blood-fed feral females were aspirated from the walls of houses near the KEMRI compound.

As previously reported, *ca.* 90% of the females were *An. gambiae s.s*. and the remainder were *An. arabiensis* as determined by PCR (Huang *et al.* 2005). Cages of mosquitoes were held in an environmental chamber maintained at 28 ± 1º C and 80 ± 10% RH under a LD 12:12 h photoperiod. Indirect light of about 0.17 lx was provided during scotophase by a shaded 4 W tungsten bulb; it was intended to mimic moonlight. Mosquitoes were offered defibrinated horse blood via a membrane feeder 2-3 days before ovipositional tests. These females were held in laboratory cages under high humidity for 24 h before ovipositional tests.

Automated Ovipositional Clock

The method of egg collection may influence the oviposition rhythm of some Diptera (David and Fouillet 1973). Use of a mechanized egg collector may be less disruptive to egg deposition than manually changing an ovipositional resource at hourly or two-hour intervals. Mechanized egg collectors have previously been utilized in the study of ovipositional rhythms for both *D. melanogaster* (Fleugel 1984, David and Fouillet 1973) and *Agrotis segetum* (Byers 1987). In the current study, we developed a new automated mechanical apparatus to sample oviposition over time. This apparatus was used to compare ovipositional periodicity by *An. gambiae* individuals *vs.* groups.

Battery-powered wall clocks (DSA Incorporated, Farmington Hills, MI, U.S.A.) measuring 31 cm in diameter were modified to progressively present a unique section of dark and wet substrate onto which mosquitoes could oviposit over 12 h (Figure 2.1). Each clock was positioned horizontally and its original face and hands were removed. The clock body was filled with moist sand of particle size 250 – 425µm. The sand was topped with Envision® high-capacity brown paper towelling (Georgia Pacific, Camas, WA, U.S.A). This

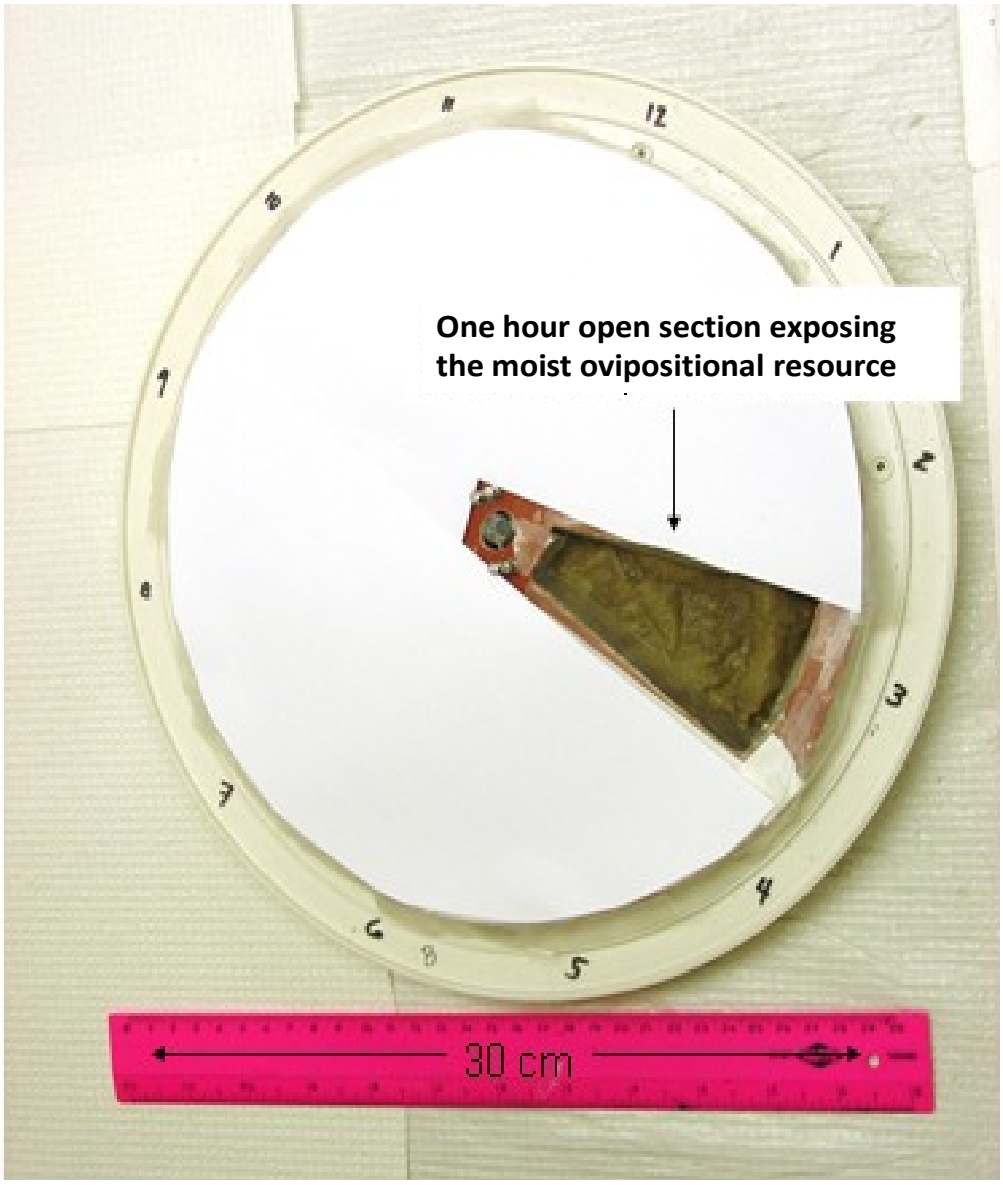

Figure 2.1. Clock apparatus used in automated measurement of *Anopheles gambiae* ovipositional periodicity.

paper towelling appears light when dry, but dark when wet. Thus, it provided the two key stimuli (dark and wet) necessary and sufficient to strongly stimulate *An. gambiae* to oviposit (Huang *et al.* 2005).

A new and removable clock face was fashioned from a circular piece of thin plastic, from which had been cut a section equivalent to one h on the clock (Figure 2.1). The opening and perimeter of the face-plate were lined with Parafilm ® (Pechiney Plastic Packaging, Menasha, WI, U.S.A.) flaps to prevent mosquitoes from depositing eggs on any unexposed section of the substrate. The clock face was mounted on the hour-hand driver, so that the opening in the face plate made one revolution every 12 h. The clock face was covered with white paper so as to maximize contrast between background *vs.* the actual ovipositional site (Huang *et al.* 2005)

Prior to insertion into the clock apparatus, the paper towel substrate was divided by pencil marks into 12 equal wedge-shaped sections. After being exposed to gravid female mosquitoes for 12 h, a clock was removed from the cage of mosquitoes and another was immediately inserted so as to extend the study over a full 24 h. The face of an exposed clock was carefully removed and the paper toweling bearing eggs was carefully peeled off the sand for egg counting under a dissecting microscope. Clock sections open at the beginning and end of a given measurement were exposed to females for a total of 1 h. However, it took 1 h for each intervening section to fully open and another 1 h for each to fully close. Thus, some of the eggs on each intervening section could have been laid over a span of 2 h.

Experimental Series 1 - Automated measurement of caged mosquito groups

Clocks were presented in white BugDorm-2 insect rearing cages (Mega View Science Education Services Co., Taiwan) measuring 60 x 60 x 60 cm and containing approximately 500 laboratory-reared females of the Kisumu strain varying in reproductive stages. The light

cycle in the environmental chamber was set at 12:12 LD, to approximate the natural light cycle found in Kisumu, Kenya. A small tungsten bulb continued to burn in the laboratory at night so as to provide the equivalent light from the night sky. Light levels during scotophase were slightly less than full moonlight (10^{-3} W m^{-2}) (Gibson 1995).

Ovipositional clocks were also presented to groups of house-collected gravid females as described above. In this experiment, the BugDorm-2 cages housing approximately 100 females were placed just inside a screened porch of a house in Kisumu, Kenya. Egg recording sessions for both house-collected and laboratory-reared groups were replicated 8 and 6 times respectively, using a different set of females for each test. Each recording session began at 1700 h, one h prior to the onset of scotophase, and continued for 24 h. At 0500 h, a clock apparatus containing fresh paper towelling was exchanged for the loaded clock. The numbers of eggs laid within each hourly period were counted under a dissecting microscope and incorporated into frequency histograms. The proportion of eggs deposited by laboratory-strain *An. gambiae* at 1800, 2100, 0, 0400, and 0500 h were arcsine-square-root transformed prior to analysis of variance. Tukey's test of significant differences (HSD) was used to separate means.

Experiment 2 - Automated measurement of caged individuals

The bottom of the enclosure for these tests was the clock apparatus over which sat a 12 cm high cylindrical wire frame. Nylon netting (18 intersections/cm) was placed over the frame and secured by a drawstring. Six 2 cm diameter wet cotton balls (Kendall, Mansfield, MA) were placed on the roof of the cage as a source of moisture. Blood meals were offered to females between 1200 and 1700 h three to four days prior to use. Three or four days after a blood meal, an individual female was gently transferred to the clock cage by aspirator before scotophase. After the female had been exposed to the ovipositional resource for 4-5 h,

the female was removed from the cage and fresh paper toweling was substituted for the previously exposed paper toweling within the clock. Then, the female was carefully reinserted. The exchange of the ovipositional resource was repeated 11 h later. The numbers of eggs laid within each hourly period were counted and incorporated into a histogram. Correlation analysis (SAS software version 9.1) was used to test for a correlation between the length of the preoviposition interval (defined as the time interval between a female's first exposure to the clock and the time oviposition was initiated) and the length of the oviposition interval (time interval during which oviposition occurred). It was also used to test for a correlation between the length of the preoviposition interval and the total number of eggs deposited per female. A Levene's test, as modified by Brown and Forsythe (1974), was used to compare the variances of the preoviposition and oviposition intervals. The ovipositional periodicity was measured for a total of 56 individual females, all of the Kisumu laboratory strain.

The terms gravid and hypergravid as used by Sumba *et al.* (2004) refer to the condition of the female mosquito when they are presented with an ovipositional resource three and four days, respectively, after obtaining a blood meal. Differences in oviposition by gravid *vs.* hypergravid females were examined by comparing the mean numbers of eggs oviposited per female per h of each respective group using a paired t-test (SAS software version 9.1). After each trial, females were dissected under a dissecting microscope to check for residual eggs.

Experiment 3 - Assessment of capacity for mid-afternoon oviposition

Engorged females were randomly selected from newly blood-fed cages of mosquitoes and placed in groups of 20 into 8 cages made from 15 cm high and 19 cm in diameter white cardboard cartons. The top of the cage was covered with white netting (8 intersections/cm)

and a sleeve of the same netting was fitted to a 10.5 cm hole cut in the side for mosquito and ovipositional resource insertion. Females were provided with a constant source of 10% honey solution and six wet cotton balls (Kendall, Mansfield, MA) were placed on the top of the cage to provide extra moisture. Two days after blood-feeding, an ovipositional resource was provided to half of the cages approximately 2 h before the lights were turned off to record egg deposition during scotophase. The ovipositional resource was a 100 x 35 mm clear plastic Petri dish containing 20 mL of distilled water, placed over a circular piece of black paper. At 1200 h the following day, the loaded ovipositional resources were replaced with new Petri dishes containing fresh filtered water. Four ovipositional resources were also introduced into the 4 cages from which an ovipositional resource had been withheld. These resources, identical to those previously mentioned, were used to record oviposition by gravid females during photophase. After the initial introduction, ovipositional resources were changed hourly from 1200 -1600 h in the latter half of the cages and all exposed ovipositional resources were examined for the presence of eggs. Using a small brush, eggs present were brushed into lines on a piece of white paper and counted.

Results

Experimental Series 1 – Bimodal oviposition by caged groups

An. gambiae of the Kisumu laboratory strain revealed two ovipositional pulses (Figure 2.2). The first occurred from 1800 to 0 h, peaked at 2100 to 2200 h, and accounted for 65% of the total eggs deposited. A second but smaller pulse occurred between 0 and 1000 h, and peaked at 400 h. It is notable that some oviposition by females in groups occurred throughout scotophase. Moreover, a few eggs were deposited in the early hours of photophase. The proportion of eggs deposited by the laboratory strain during the

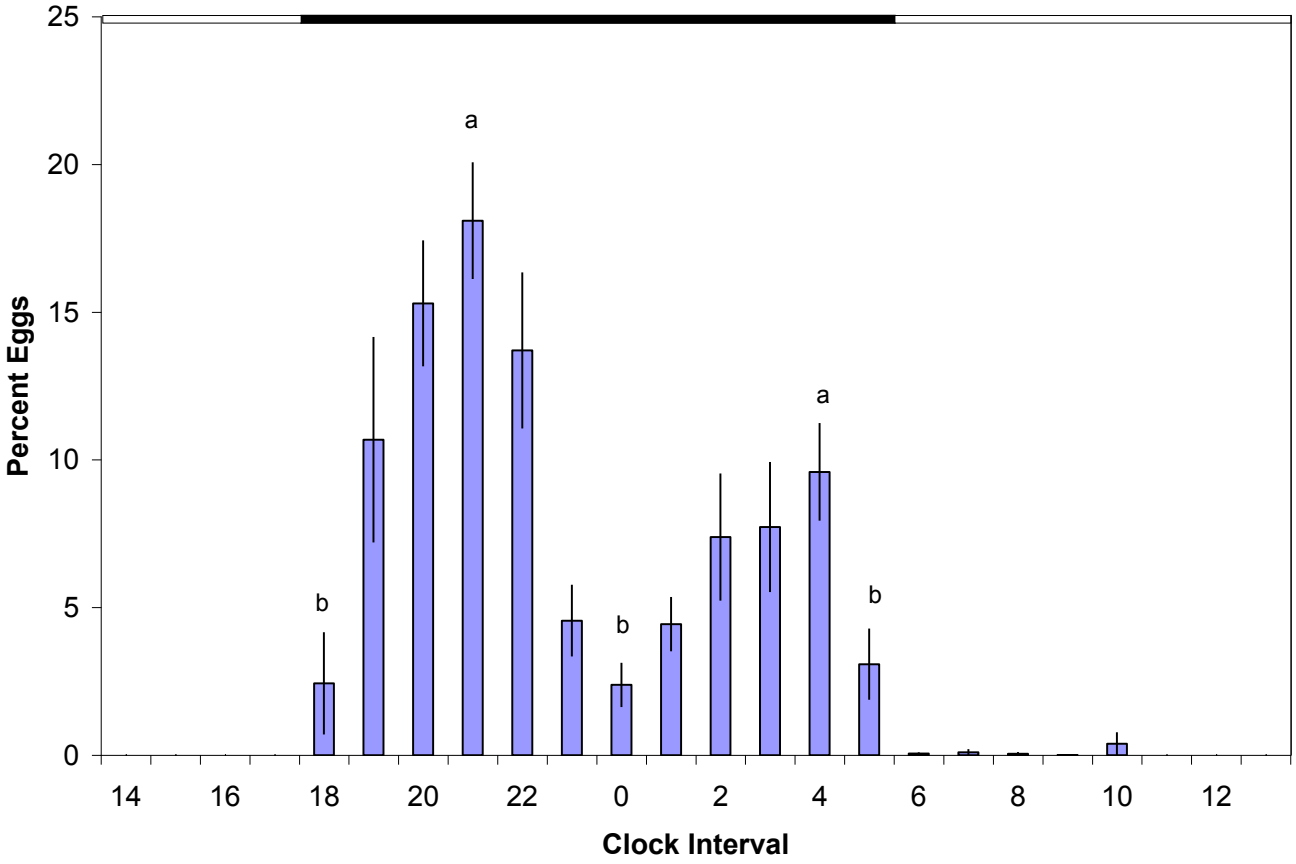

Figure 2.2. Ovipositional periodicity of laboratory strain *Anopheles gambiae* groups. Mean percent of eggs oviposited per hour by a caged laboratory strain group (500 females per replicate; total eggs = 18, 303). Bars bearing a common letter are not significantly different (Tukey's HSD, $\alpha = 0.05$). *An. gambiae* exposure to a 12:12 LD photoperiod during testing is represented by the alternating light and dark horizontal bar above the figure.

first peak (2100 h) was significantly greater than the proportion of eggs deposited at 0 h when oviposition greatly diminished ($p < 0.0001$; Tukey's HSD test). The proportion of eggs deposited during the second peak was not significantly different from the proportion deposited during the first peak at 2100 h ($p = 0.3$). The valley between these two peaks was marginally significant ($p = 0.054$). Two discrete pulses of oviposition were recorded for house-collected *An. gambiae* groups (Figure 2.3). The first began at dusk, peaked at 1900 h, and ceased after 0100 h. Seventy-four percent of the total eggs were laid between 1800 and 0200 h. The second pulse commenced near dawn, peaked around 0800 h, and ceased before 1300 h. Unlike the laboratory strain, wild-caught females deposited a substantial portion (more than 25%) of their eggs after sunrise.

Experiment 2 – Caged individuals oviposit in one continuous bout

Ovipositional periodicity of gravid and hypergravid females held individually was similar, although more gravid females contributed to the second ovipositional pulse than did hypergravids. A paired t-test of the total mean number of eggs oviposited per *An. gambiae* female per h (1700-1900 and 2100-2200 h) revealed no significant difference between gravid and hypergravid states ($p = 0.61$). However, correlation analysis revealed a significant positive correlation between the length of the preoviposition interval and the total number of eggs deposited ($p = 0.03$).

The time at which individual females initiated oviposition was highly variable (Figure 2.4). The mean length of the preoviposition interval was 3.5 h with a variance of 9.5, and the mean length of the oviposition interval was 2.5 h with a variance of 1.0. According to a modified Levene's test, the length of the preoviposition interval was more variable than the length of the oviposition interval ($p < 0.0001$). However, there was no correlation between

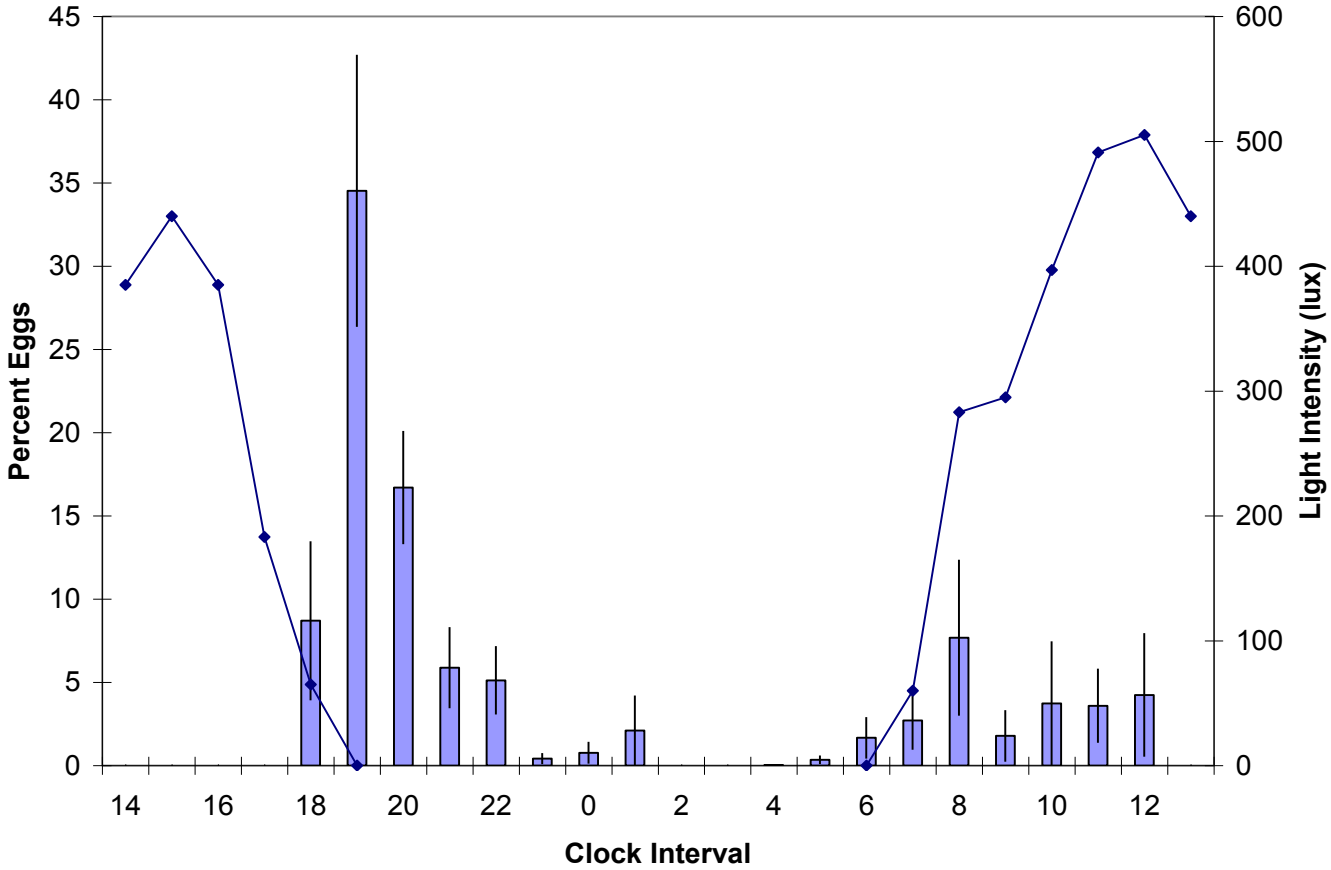

Figure 2.3. Ovipositional periodicity of house-collected *Anopheles gambiae* groups. Mean percent of eggs oviposited per hr by caged house-collected groups (100 females per replicate; total eggs = 11,007). Measurements of outdoor light intensity, represented in the graph as a dark blue line, were taken 5/11/2004 in Kisumu, Kenya.

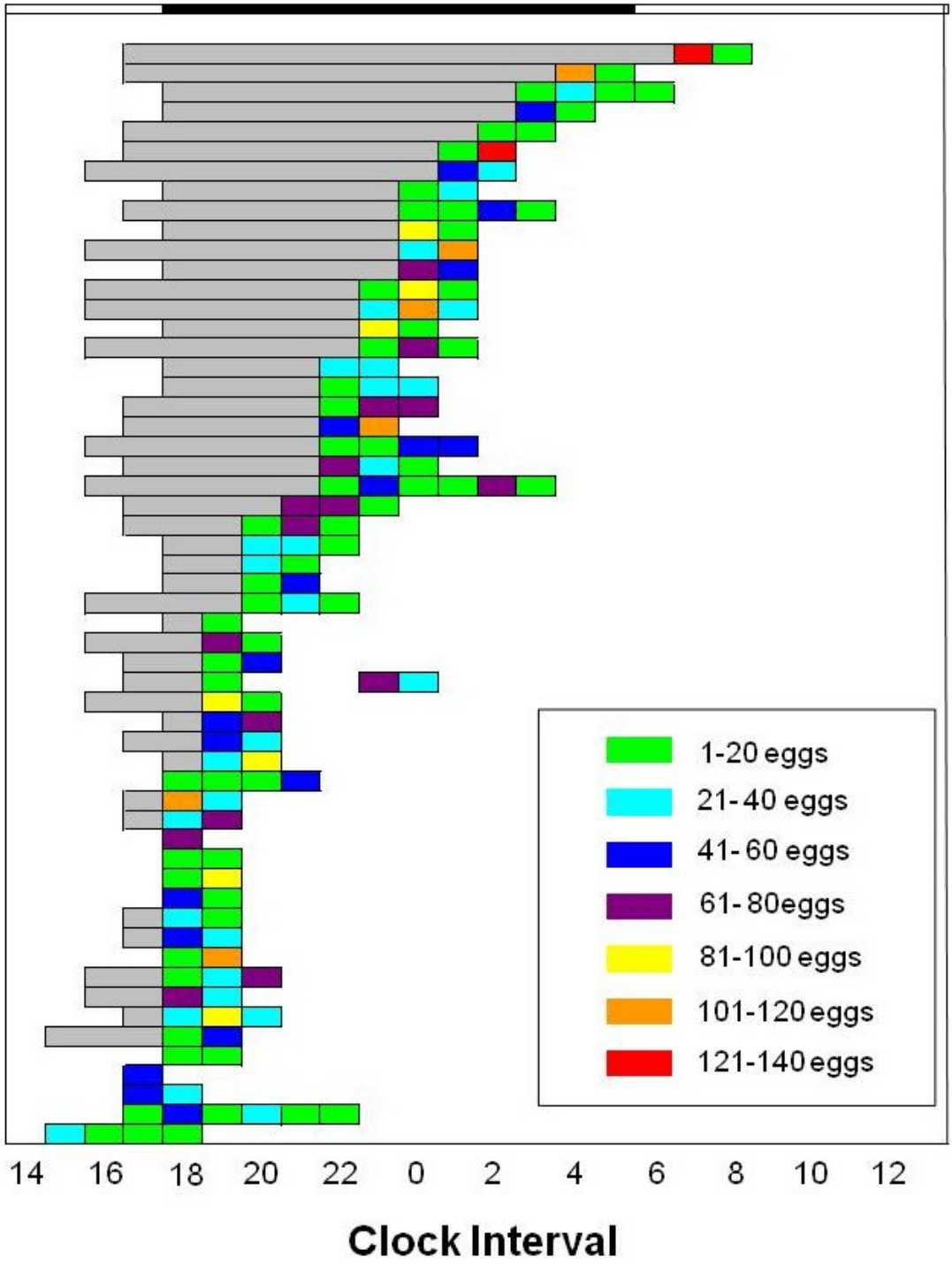

Figure 2.4. Ovipositional patterns of individual *Anopheles gambiae* over 24 hrs. Each horizontal cluster of rectangles represents a single individual. Shading classifies the number of eggs deposited per individual per hr (n = 56) during the oviposition interval.

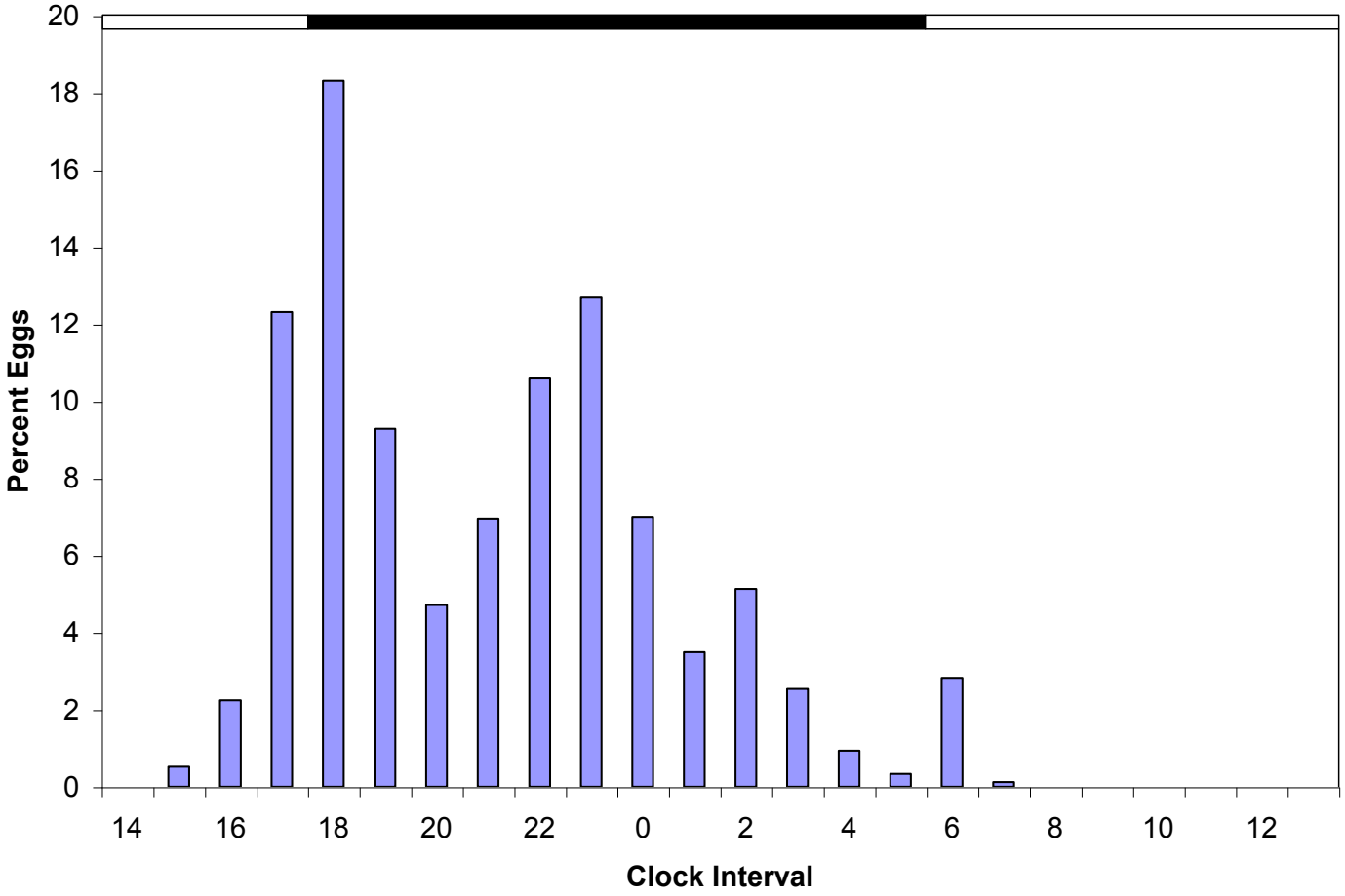

Figure 2.5. Accumulated ovipositional patterns of individual *Anopheles gambiae* over 24 hrs. Percent of eggs oviposited per hr by caged individual *An. gambiae* (total eggs = 4,815).

the length of the preoviposition interval and the length of the oviposition interval ($p = 0.51$). Compiled individual oviposition was similar to patterns of groups (Figure 2.5); egg deposition occurred throughout scotophase and even during certain hours of photophase. Seven percent of individuals commenced egg deposition before lights off and one individual initiated oviposition after lights on. Interestingly, Figure 2.4 documents that most females oviposited without detectable interruption and those females spread their eggs continuously over a few consecutive clock intervals. Only one individual out of 56 exhibited two ovipositional pulses; she commenced at 1900 h, ovipositing 12 eggs, and then paused until 2300 h before depositing another 105 eggs.

Experiment 3 – An. gambiae *are capable of mid afternoon oviposition*

Seven out of the 12 cages from which an ovipositional resource had been withheld until mid afternoon produced eggs (Table 2.1). All of the 12 cages provided with an evening ovipositional resource produced eggs. Cages provided only with a mid afternoon ovipositional resource produced 641 total eggs, which is equivalent to 6% of the eggs produced by cages provided an evening ovipositional resource. Eggs from cages provided only a mid afternoon ovipositional resource were spread over the entire 4 h period. Sixty-seven percent were deposited in the first 2 h, and approximately 24% were deposited between 1400 and 1500 h. The remaining 9% were deposited in the last h. Two hundred and three eggs were oviposited between 1200 and 1600 h in cages previously exposed to an ovipositional resource the evening prior.

Discussion

An. gambiae deposits eggs in two ovipositional pulses per 24 h. Both laboratory-strain individuals and house-collected groups of *An. gambiae* produced a large pulse of eggs

Table 2.1. Mid afternoon egg output by *Anopheles gambiae* as influenced by previous access to an ovipositional resource.

Treatment		**Total eggs per cage per period**	
With ovipositional resource the previous evening		1700 - 1200 h	1200 - 1600 h
Cage No.	Individuals / cage		
1	14	252	0
2	18	363	0
3	17	77	0
4	16	512	1
5	20	790	10
6	20	818	38
7	20	567	154
8	20	872	0
9	19	1888	0
10	20	1855	0
11	20	1251	0
12	20	1793	0
Total	224	11038	203
Without ovipositional resource the previous evening			
Cage No.	Individuals / cage		
1	14	-	3
2	18	-	9
3	15	-	0
4	18	-	161
5	20	-	0
6	20	-	128
7	20	-	0
8	20	-	51
9	18	-	6
10	20	-	184
11	18	-	99
12	20	-	0
Total	221	-	641

commencing at scotophase and peaking 1-2 h later. These results agree with those of Haddow and Ssenkubuge (1962) and Sumba *et al.* (2004). In our work with both the laboratory strain and house-collected strain, we also observed a second smaller ovipositional pulse a few h after the first pulse. The second pulse by laboratory strain groups occurred between 0 and 1100 h, while this second pulse occurred between 0500 and 1300 h for the house-collected strain. For individuals, the onset of the second pulse occurred earlier than its occurrence in the group tests (Figure 2.5). Most eggs were deposited between 2300 and 0 h.

Between the first and second ovipositional pulses in all groups, egg deposition sharply declined. While both laboratory and house-collected strains decreased ovipositional activity at 0 h, laboratory strain egg deposition resumed at 0100 h, whereas oviposition by house-collected females remained sparse until 0600 h. We speculate that the significant midnight decline in egg deposition may be the result of an endogenous rhythm. The length of the quiescent period between pulses may be a direct result of exposure to environmental conditions, such as early morning low temperatures probably experienced by house-collected, but not laboratory strain females during these tests.

Jones and Gubbins (1978) reported that peak flight by *An. gambiae* occurs immediately after lights off and that a second smaller peak in activity occurs between 6 and 10 h later. This suggests that flight activity is regulated by a circadian rhythm that could secondarily influence ovipositional patterns (Jones and Gubbins 1978, Jones *et al.* 1972). Increasing flight activity during the onset of scotophase would increase the probability that a female encounters a suitable ovipositional resource. These peak flight times described by Jones *et al.* (1972) and Jones and Gubbins (1978) may contribute to the dusk and early morning peaks in oviposition that we have recorded.

Our research established that individual females rarely split their eggs over two distinct time periods but rather lay eggs steadily after oviposition begins. We conclude that

the two pulses in oviposition by groups are not the result of individual females spreading their eggs over two distinct time periods. Instead, some individual females delay the onset of oviposition to create the second peak. There was much greater variability in the preoviposition interval (i.e. the time interval prior to when a female initiated oviposition), than there was in the amount of time devoted to oviposition. In the case of the single female who split her eggs between two ovipositional periods, an interruption caused by the exchange of the paper toweling could explain this single aberration.

While the rates of egg deposition by gravid and hypergravid females were not found to be different, a statistically significant positive correlation existed between the length of the preoviposition interval and the total number of eggs deposited per female. Individuals with longer preoviposition intervals tended to deposit slightly more eggs. However, this correlation likely has little biological significance due to the considerable scatter in the data. This is demonstrated by the width of the 95% confidence intervals (CI) surrounding the mean total numbers of eggs per preoviposition interval. The width of the 95% CI surrounding mean egg deposition for individuals with a 14 h preovipositional interval was 64 (actual 95% CI: [90, 154]) eggs, *vs.* 20 (actual 95% CI:[80, 100]) eggs when the preoviposition interval was 4 h.

An. gambiae has the capacity for afternoon oviposition in full light. Females denied an ovipositional resource for 18 h oviposited between 1200 and 1600 h, when the ovipositional resource was introduced. In some cases, eggs were found on the ovipositional resources between 1200 and 1600 h even when the mosquitoes had a resource beginning at 1700 h on the previous night.

Visual contrast of the ovipositional substrate is an important stimulus for oviposition and egg placement. Huang *et al.* (2007) reported that a black ovipositional dish on a white or grey floor received many more eggs than any other white-black or grey-black combination of

ovipositional substrate and background. Clay soil in Kisumu, Kenya, appears black when wet and grey when dry, and discrimination between grey and black coloration improves at light levels of 2.1 x 10^{-3} W m^{-2}, which is equivalent to late dusk or early dawn (Huang *et al.* 2007). When ovipositional resources are sparse, it may benefit *An. gambiae* to forage for ovipositional sites before full darkness and at or after dawn, when visual contrast would be more detectable.

Our overall results establish that oviposition by *An. gambiae* is not restricted only to one specific time of day, and oviposition is not fully inhibited by high light levels. Gravid females can initiate oviposition as soon as an ovipositional resource becomes available. Thus, ovitraps, a tool to monitor *An. gambiae* population growth and help predict malaria epidemics, should remain available throughout the full 24 h diel to be maximally effective. Further study of abiotic factors such as daily temperature and relative humidity fluctuations and their contribution to patterns in flight activity in the field may be of interest. High temperature and low RH may limit mid-afternoon oviposition in the field. During the daytime in the tropics, air and soil temperatures typically exceed the optimum temperature for oviposition - > 25 ºC (Huang *et al.* unpublished data).

In conclusion, *An. gambiae* populations are ovipositionally flexible. Rather than confining oviposition to a specific brief period during 24 h, as is true for many insects, *An. gambiae* can oviposit at any time after their eggs have fully developed and they have access to an ovipositional resource. But, they most commonly begin oviposition and deposit the majority of eggs shortly after dusk. Once oviposition commences, individual females deposit their eggs over a continuous 2 to 3 h period without interruption.

CHAPTER 3:

EGG PLACEMENT BY *ANOPHELES GAMBIAE* (DIPTERA: CULICIDAE) AS INFLUENCED BY RESTING SURFACE SLOPE AND ENCLOSURE

Abstract

In these experiments, laboratory-reared *Anopheles gambiae s.s.* were as likely to rain eggs down from a vertical perch, as to oviposit while sitting horizontally on a substrate of moist mud or open water. Tall cylinders with moist, dark walls provided an enclosed vertical resting surface from which *An. gambiae* deposited eggs. Similar numbers of eggs were deposited in these cylinders as on dark and moist horizontally-positioned ovipositional substrates. Likewise, *An. gambiae* oviposited equally from a vertical perch among emergent surrogate reeds vs. sitting horizontally on mud. Open ovipositional resources with exposed resting surfaces presenting 45°, 90°, and 135° angles relative to the cage floor received fewer eggs than a horizontally positioned, moist and dark ovipositional dish, even though more *An. gambiae* females settled on these angled resting surfaces post-oviposition. We conclude that vertical resting sites are adequate perches from which oviposition can occur if they provide high humidity and the paramount dark and wet ovipositional cues for *An. gambiae* oviposition.

Introduction

Many insects accommodate the physical structure and dimensionality of their surrounding environment during ovipositional site selection (Harris and Miller 1982, 1984, Prokopy and Owen 1983, Roessingh and Städler 1990). Mosquito breeding habitats commonly occur at the interface of aquatic and terrestrial environments, and may have 3-dimensional structure. Visual/physical cues arising from the structure of the surrounding

environment may play an important role in ovipositional site selection for some mosquito species (Overgaard 2007).

By definition, egg deposition by *Anopheles gambiae s.s.* Giles determines the distribution of larvae of this important malaria vector species in nature, which in turn influences resultant production of the adults (Mutuku *et al.* 2006b). *An. gambiae* was once thought to deposit most of their eggs in small sunlit pools of rainwater devoid of vegetation, in close proximity to human dwellings (Holstein 1954). However, it is clear that the ovipositional plasticity of *An. gambiae* extends egg deposition beyond these habitats (Muirhead Thomson 1951, Holstein 1954, Gillies and De Meillon 1968, Huang *et al.* 2006; Miller *et al.* 2007). Indeed, *An. gambiae* larvae have been discovered in such habitats as inundated agricultural fields (Minakawa *et al*. 1999, Munga *et al.* 2005), swamp margins (Minakawa *et al.* 2004), drainage ditches (Mutuku *et al.* 2006), and tree holes (Omlin *et al.* 2007). In fact, a cohort of offspring from a single *An. gambiae* female may even be distributed among multiple mosquito breeding habitats (Chen *et al.* 2006), suggesting that ovipositing females use multiple sites during an ovipositional cycle.

In the laboratory, females readily oviposit within grassy vegetation (Huang *et al.* 2006a), on sand that is dry to the touch as perceived by a human (Huang *et al.* 2005), and on agarose media (Huang *et al.* 2006b). The most important determinants of *An. gambiae* oviposition described to date are darkness and wetness (McCrae 1984, Huang *et al*. 2005, Huang *et al.* 2007). Dark substrate juxtaposed to a lighter background provides visual contrast and promotes *An. gambiae* oviposition (McCrae 1984, Huang *et al*. 2007), particularly during peak ovipositional times of dusk and dawn (Sumba *et al*. 2004, Fritz *et al*. 2008) when ambient light levels are low. Indeed, dark mud appears to receive the preponderance of *An. gambiae* eggs (Miller *et al.* 2007). Larvae eclosing there can crawl into puddles or be carried there during rains.

Ovipositional posture has rarely been investigated in *An. gambiae*, but appears to be flexible. McCrae (1984) reported that coastal Kenyan *An. gambiae* deprived of a muddy border around a pool of water oviposited while in flight. However, a full third of his test population oviposited from a settled posture onto ovipositional resources. More recent studies (Bray 2003, Huang *et al*. 2006, Miller unpublished) suggest that oviposition during flight is less common than previously reported, even in wild-caught populations. Upon detection of the ovipositional site, laboratory-reared anophelines, including *An. gambiae*, land and oviposit on the water surface (Bates 1940, McCrae 1984) or more commonly on mud (Miller *et al*. 2007). Other anophelines perch vertically on emergent vegetation and drop eggs onto the water below (Giglioli 1965, Clements 2000). Based upon previous reports of their ovipositional flexibility, we postulated that *An. gambiae* may also exhibit this behavior. Here we extended the analysis of *An. gambiae* ovipositional site preference to 3 dimensions, and attempt to characterize *An. gambiae* egg deposition patterns as influenced by the 3-dimensional objects to which they are exposed. If such behavior were to be found, such data would suggest that some larval habitats of this important malaria vector have been overlooked.

Methods

Mosquitoes and bioassay conditions

Kisumu strain *An. gambiae*, reared according to Huang *et al*. (2005), was used for high and low humidity ovipositional tests conducted in a previously described mosquito rearing facility at Michigan State University (MSU) (Huang *et al*. 2005). Some low humidity ovipositional tests for Experiments 1 and 3 were conducted at the Entomology laboratory of the Kenyan Medical Research Institute (KEMRI), in Kisian, Kenya, using the KEMRI Kisumu strain *An. gambiae* colony.

Forty 5 d old females were blood-fed via artificial membrane feeder (Huang *et al.* 2005) 3 d prior to use in ovipositional tests. Once gravid, females were inserted into 60 × 60 × 60 cm white BugDorm-2 dome insect rearing cages (MegaView Science Education Services Co., Taiwan) via mouth aspirator. All tests were performed under a 12L:12D h photoperiod. At KEMRI, low light was provided during scotophase by outdoor security lights shining through the window, while a small tungsten night-light (4 lux) provided low light in the colony room at MSU. For all tests, ambient temperature was ca. 25ºC. The RH for low humidity tests was 35% (±10). A Kenmore Whole House Humidifier (Model 758.154120; Sears, Roebuck and Co. Hoffman Estates, IL), as well as damp white kitchen towels draped over the cages walls increased the RH for high humidity tests to 80% (±10). Mosquitoes were provided access to a 10% honey solution *ad libitum* throughout tests.

Experiment 1- Exposed resting surface slope preference

In this choice test, ovipositional resources were composed of a horizontal and a vertical component (Figure 3.1). Three of four ovipositional resources contained vertical components sloped at varying angles relative to the floor. Each vertical component was made using a 13 × 9 × 2 cm plastic dish (The Glad Products Co., Oakland, CA, U.S.A.). Three layers of well-water-moistened Spontex® sponge cloth (Supa Brite, Nairobi, Kenya) were cut to fit inside each rectangular dish, then stapled in place. These were covered with black paper printed from an HP LaserJet 4100 PCL 5 (Hewlett-Packard Co., Palo Alto, CA, U.S.A.), and then moistened to stimulate oviposition (Huang *et al*. 2005). Rectangular dishes were hot-glued to the horizontal component of the ovipositional resource, the bottoms of dry 100mm diam × 35mm clear plastic Petri dishes, at 45º, 90º, and 135º angles to the floor. A fourth ovipositional resource consisted of a 2cm deep rectangular dish, not secured to a Petri dish base, placed horizontally on the floor. Additionally, the Petri dish base served as a

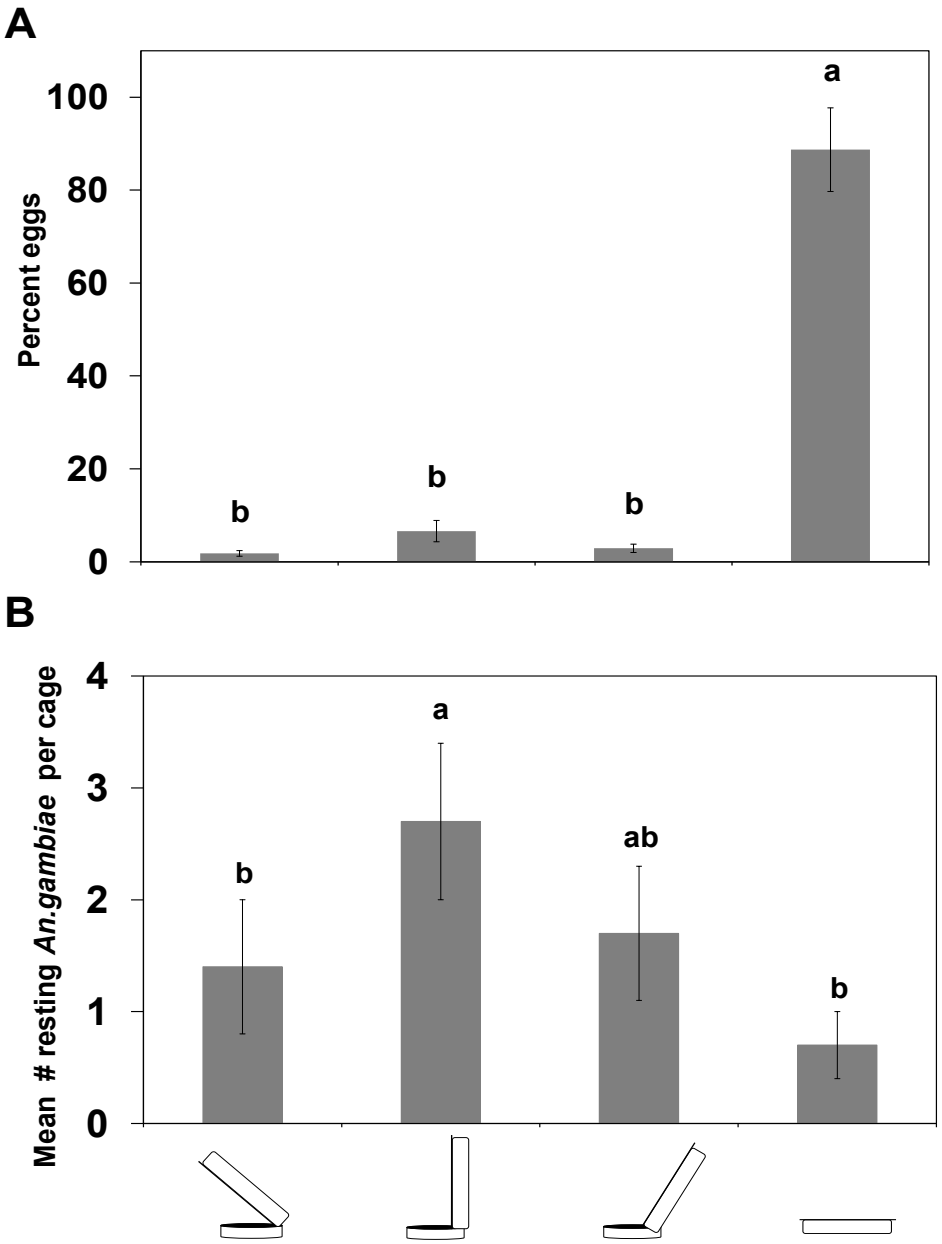

Figure 3.1. A. Mean percentage of *Anopheles gambia*e eggs (± 1 SEM) collected from ovipositional resources with exposed resting surfaces at varying inclines with respect to the cage floor (total eggs = 17,134; mean no. eggs/female = 37). B. Mean number of resting mosquitoes (± 1 SEM) counted on ovipositional resources with exposed resting surfaces at varying inclines with respect to the cage floor. Bars within a graph sharing the same letter are not significantly different ($p>0.05$).

moisture reservoir; water was added to the Petri dish bottoms at the start of each trial to replace evaporating moisture from paper and sponges of the vertical component. To discourage egg deposition in the water-filled Petri dish bases, we covered the entire base with one layer of aluminum foil, and then a layer of dry, black paper.

The cage floor was covered with dry white paper, and divided into 4 quadrants of equal size; the center of each received a different ovipositional resource. Experiment 1 was replicated with 6 different groups of 40 gravid *An. gambiae* at both high and low humidity, and ovipositional resources were rotated to a new position in the cage for the testing of each new group. Ovipositional resources were presented to females 1 h prior to the onset of scotophase, and were removed *ca.* 20 h later. For each cage, oviposition was monitored over two consecutive nights; loaded ovipositional resources were replaced with fresh ones between consecutive nights. Both the horizontal and vertical components of each ovipositional resource were examined under a dissecting microscope daily for the presence eggs. Egg totals from each cage over the two nights were summed for respective treatments.

Experiment 2 – Resting surface counts

The post-ovipositional sitting preferences of mosquitoes from six cages used in Experiment 1 were quantified. After two nights of testing, but before ovipositional resources were collected, the total numbers of mosquitoes resting on ovipositional resources in these cages were counted. Mosquitoes were dislodged from their resting places by waving a human hand near each ovipositional resource, the walls, and the cage floor. After 15 min, resting counts resumed, and the process was repeated. Three resting counts were taken for each cage of mosquitoes, and mean values calculated.

Experiment 3 - Enclosed resting surface slope preference

Two vertically positioned cylinders of differing heights were designed to provide an enclosed resting surface around the perimeter of an ovipositional resource. Dry, black paper in a Petri dish bottom, then covered with Parafilm deterred females from resting on the floor of the ovipositional resource while depositing eggs. The walls of the cylinders were made from moistened Spontex® sponge cloth cut into 20 × 2.7 cm and 20 × 10 cm pieces, then rolled into tubes. These sponge tubes of equal diameter, but differing in height, fit inside the rim of 100mm diam × 35mm high clear plastic Petri dish bases. The outside surfaces of the cylinders were lined with aluminum foil, then white paper, while the insides were lined with black paper, to create a dark, moist enclosed vertical surface. A Petri dish bottom was over-layed with three moist circular pieces of sponge cloth and covered with a circular piece of black paper, so as to provide a horizontal surface on which ovipositing females could rest.

The three cylinder treatments were spaced equidistantly from each other on a white paper-covered floor. In tests conducted under both high and low humidity, ovipositional resources were rotated to a new position within the cage for each of 8 replicate groups of *An. gambiae.* Ovipositional resources were presented to gravid females 1 h prior to the onset of scotophase, and were removed from cages *ca.* 20 h later. Tests were conducted over two consecutive nights, and each night, fresh ovipositional resources replaced loaded ones. The sum of the eggs found on each ovipositional resource over two consecutive nights was recorded.

Experiment 4 – Presence/absence of surrogate vegetation

Two rectangular opaque plastic dishes measuring 12 × 10 × 3 cm were used as ovipositional resources. In the center of one dish, 1.4 × 17 mm wire nails were hot-glued vertically, but with the head down within a 10 × 2.5 cm strip in 3 staggered rows. The

bottom of the dish was covered with a layer of Michigan soil, followed by a layer of brown paper towel. Glass tubes (12 cm high and 0.3 cm diam) rounded and sealed at one end, and open at the other were painted green and coated with wax (Harris 1987) to make removable surrogate reeds. These surrogate reeds slipped over the nails during oviposition tests, but could be removed and inspected for eggs during data collection. In the bottom of the other rectangular dish, the soil was layered so as to create a shallowly sloped surface leading to a 10 × 2.5cm strip of flat soil substrate. A layer of brown paper towel covered the soil for ease of egg collection. Both rectangular dishes were placed equidistantly from the walls of a 37 × 37 × 29 cm clear Plexiglas cage, and then filled to the brim with filtered well water immediately prior to initiation of oviposition tests. Five gravid *An. gambiae* were inserted into a cage via mouth aspirator *ca.* 15 min prior to the onset of scotophase. Tests ran for 20 h, at which time dishes were carefully removed from the cages so as not to spill any water. Water from each ovipositional resource was collected with a disposable plastic Pasteur pipette and filtered through white paper. Eggs from filter paper, as well as any on the brown paper towel were counted and recorded. Surrogate reeds were also removed from the ovipositional resource, and each reed was washed with water from a squirt bottle into a plastic cup. This water was also poured through the filter funnel to ensure that all eggs sticking to the surrogate reeds were collected. Tests were replicated with eight new groups of mosquitoes.

Visual observations of mosquito ovipositional behavior were made in four of the replicates. An observer monitored activity in the arena for two h after insertion of the mosquitoes. An Energizer® 6 LED Headlight (Eveready Battery Company, Inc. St. Louis, MO, USA) was set to red night vision and worn by the observer to improve visibility. Data were collected on 1) where oviposition began, 2) ovipositional posture assumed, and 3)

whether ovipositing mosquitoes switched ovipositional resources for all individuals beginning oviposition 2 h post-insertion.

Data collection and analysis

All analyses were performed using R version 2.7.2 (R Development Core Team, Vienna, Austria). For experiments 1 and 3, a likelihood ratio test was used to determine whether ambient humidity influenced egg deposition. Data from Experiments 1 and 3 (Table 3.1) were arc sine square root transformed, and analyzed using ANOVA. Means were separated by Tukey's honestly significant difference (HSD) test. Data from Experiment 2 were fitted to the Poisson distribution using Akaike's Information Criterion (AIC), and a likelihood ratio test determined the effects of surface angle on *An. gambiae* resting behavior. Bootstrapped 95% confidence intervals (N=1000) were compared across treatments for separation of means. A paired t-test was used to analyze egg deposition from Experiment 4.

Results

In Experiment 1, *An. gambiae* deposited most of their eggs in association with dark, wet horizontal surface of the rectangular dish, and few eggs when resting on any exposed vertical component of an ovipositional resource ($df = 3$, $F = 217.46$, $p < 0.001$; Figure 3.1a). Humidity did not influence egg deposition ($df = 1$, $F = 0.35$, $p = 0.55$). In Experiment 2, more females rested on the vertical components of ovipositional resources presenting angles of 90° and 135° relative to the cage floor ($df = 1$, $\chi^2 = 8.04$, $p = 0.005$; Figure 3.1b). Room RH slightly influenced the site of greatest egg deposition in Experiment 3, resulting in a significant interaction between RH and ovipositional resource ($p = 0.01$; Table 3.1). At low ambient RH, the greatest proportion of eggs was deposited in the tall cylindrical ovipositional resource ($p = 0.0001$; Figure 3.2). However, equal numbers of eggs were deposited in the

Table 3.1. Analysis of variance for Experiment 3. An asterisk denotes a significant p-value.

Source	df	SS	MS	F value	Pr (>F)
Treatment	2	3.05	1.52	28.94	1.98 e-08 *
Humidity	1	0.02	0.02	0.32	0.57
Treatment × Humidity	2	0.54	0.27	5.15	0.01 *
Residuals	39	2.05	0.05		

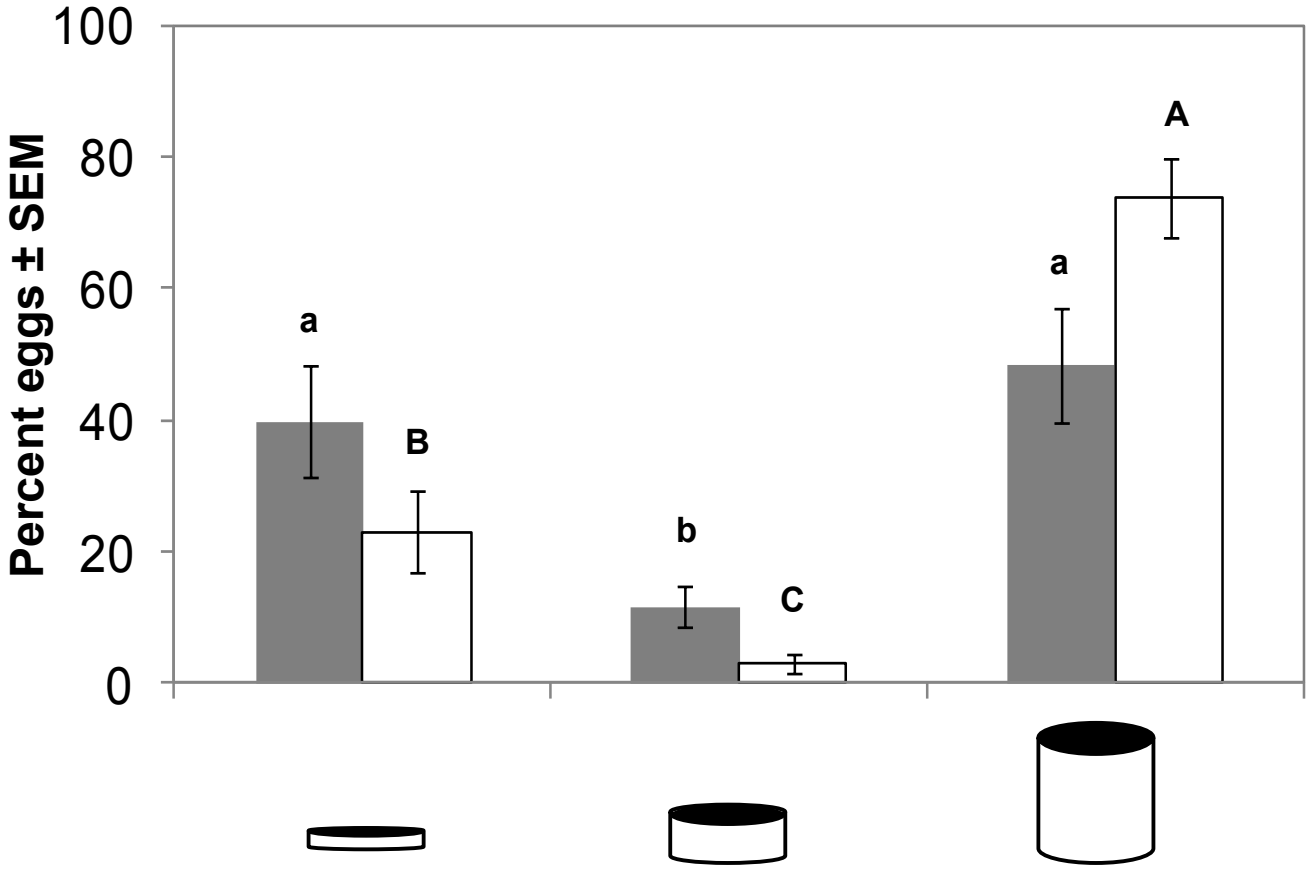

Figure 3.2. Mean percentage of *An. gambiae* eggs (± 1 SEM) deposited on ovipositional resources enclosed by no walls, 2.7cm or 10cm high walls (total eggs = 16,813; mean no. eggs/female = 47). Dark and light bars indicate high and low RH respectively. Mean separation was applied within each RH, represented by upper *vs.* lower case letters. Bars within an RH treatment sharing the same letter are not significantly different ($p>0.05$).

Figure 3.3. Map of egg deposition within the tall vertical cylinder adapted from photographed results of the first replicate of Experiment 3. Black dots represent individual eggs, while outer and inner black circles represent the Petri dish and sponge walls respectively.

horizontal and tall cylindrical ovipositional resources when ambient RH was high ($p = 0.65$). Most eggs deposited in the tall cylindrical treatment were found within 1 cm from the cylinder wall (Figure 3.3). At both high and low RH, the short vertical cylinder received the fewest eggs.

In Experiment 4, egg counts did not differ between the ovipositional resource containing emergent surrogate reeds *vs.* the flat moist surface ($df = 7$, $t = -0.19$, $p = 0.86$; mean no. eggs/female = 60). The mean number of eggs per ovipositional resource was 142 and 155, respectively. Three individuals were observed ovipositing in two of the replicates that were observed. All began oviposition within the surrogate reeds while perched on a stem, and dropped eggs onto the surface of the water below. One individual hopped from stem to stem between ovipositional bouts, but none switched to the soil treatment. No individuals were observed dropping eggs while hovering over either treatment.

Discussion

An. gambiae is ovipositionally flexible, and uses varied ovipositional sites. Previously, *An. gambiae* was reported to oviposit in flight while hovering over an ovipositional resource (McCrae 1984). Yet egg deposition from flight has neither been quantified, nor has it been confirmed that hovering females always drop eggs. Subsequent studies suggest this behavior is rare in the laboratory (Bray 2003). There, females oviposit from a sitting position on moist mud (Huang *et al*. 2006a, Miller *et al*. 2007), and can hover over an ovipositional resource without depositing eggs (Miller *et al*. unpublished). The *An. gambiae* used in the present study were tested for their propensity to oviposit while in flight. In a Plexiglas cage measuring 128 × 63 × 50 cm, females deposited eggs while sitting on a 15

cm diam Petri dish containing moist mud twice as often as they deposited eggs while hovering (Huang *et al.* unpublished). Perhaps the discrepancy between these results and those reported by McCrae (1984) can be explained by the use of different *An. gambiae* populations; McCrae used *An. gambiae s.l.*, while *An. gambiae s.s.* was used in the present study. A more likely explanation is that the Petri dishes used by McCrae did not provide a suitable resting surface onto which *An. gambiae* could settle during an ovipositional bout.

Furthermore, egg deposition patterns within the tall vertical cylinder (Figure 3.3) revealed the location from which *An. gambiae* oviposited. If eggs were deposited in flight, the distribution of eggs within the tall vertical cylinder should have been random; it would have been equally or more likely that eggs occurred in the center of the cylinder floor than along the cylinder wall. Instead, greater than 95% of the eggs deposited within the tall vertical cylinder were found within 1 cm from the wall (Figure 3.3). These results demonstrate that *An. gambiae* females do oviposit from a settled posture, even while sitting on a vertical resting surface over an ovipositional resource.

In Experiment 1, 8-fold more eggs were deposited on the horizontal ovipositional resource than on any other resource. Yet in Experiment 2, this same resource was least likely to be accepted by *An. gambiae* as a resting site. Furthermore, *An. gambiae* was equally or more likely to oviposit from a vertical perch in an enclosure, *vs*. while sitting on a horizontal resting surface in Experiment 3. Our interpretation of the broader distribution of eggs in Experiment 3 relative to Experiment 1 is that the tall cylinder received eggs that would have been deposited in the horizontal treatment if the tall cylinder had not been present. These data, in conjunction with *An. gambiae* resting site preferences found in Experiment 2 suggest that egg deposition can be influenced by resting site. *An. gambiae* is more likely to accept a vertical slope *vs*. a horizontal surface for resting. If such a resting site contains the critical

ovipositional cues of darkness and wetness, females may simply deposit their eggs from their vertical resting position. We speculate that higher humidity levels, resulting from a greater wet surface area, and darker conditions within the tall cylinder enhanced *An. gambiae* willingness to oviposit while resting inside the cylinder.

In the field, vegetation can present vertical resting sites near dark and moist ovipositional sites. Laboratory-reared females used in Experiment 4 equally accepted surrogate emergent vegetation *vs.* flat, bare soil, thus confirming the results of Huang *et al*. (2006a) who used natural vegetation. In the current study, *An. gambiae* was more likely to oviposit on vegetation over bare soil than the *An. gambiae* used by Huang *et al*. (2006a). Such differences could be attributed to: 1) the larger stem diameter of our surrogate vegetation providing a more stable perch from which *An. gambiae* females could oviposit, or 2) the sparse surrogate vegetation density providing greater access to resting sites for gravid females.

We speculate that the willingness to oviposit while resting on a vertical surface may be correlated with probability of ovipositing in vegetation. Like *An. melas* and *An. funestus*, feral *An. gambiae* may also perch on vegetation and release eggs onto water and damp soil below. Although most larval production occurs in small sunlit pools of open water, even putatively unproductive larval habitats can enhance malaria transmission (Le Menach *et al.* 2005). Understanding the potential suitability of different aquatic habitats for *An. gambiae* oviposition would improve vector control efforts. Our results suggest that larval sampling and control efforts for this vector of important human diseases should be broadened to include pools containing, or surrounded by emergent vegetation, or other vertical resting surfaces.

PART II:

STUDIES ON FEASIBILITY OF AVERMECTIN-TREATED CATTLE AS A VECTOR CONTROL MEASURE IN SUB-SAHARAN AFRICA

CHAPTER 4:

AVERMECTINS AND MOSQUITO CONTROL

Chemical and pharmacological properties of the avermectins

The avermectins and milbemycins, otherwise known as the macrocyclic lactones, belong to a group of related chemicals derived from *Streptomyces* microorganisms (Martin *et al.* 2002). This group is named for the base structure of its compounds: they are 16-membered lactone rings, onto which are appended different functional groups (Figure 4.1). Avermectins and milbemycins are known for their broad-spectrum endo- and ectoparasitic activity. These lipophilic compounds dissolve easily in most organic solvents and are remarkably safe for use in animals and humans (McKellar and Benchaoui 1996). In veterinary and human medicine, ivermectin and similar compounds are used to manage arthropod pests (Wilson 1993), e.g. bovine ectoparasites, such as *Chorioptes bovis*, *Sarcoptes scabiei* var. *bovis, Haematobia irritans*, and *Haematopinus eurysternus* as well as to control the tick, *Boophilus microplus* (Benz *et al.* 1989). Ivermectin is also used to rid cattle of gastrointestinal nematodes (Benz *et al.* 1989), which are generally thought to have severe world-wide economic consequences for the cattle industry (Gibbs and Herd 1986). In 1982, ivermectin was first introduced for the treatment of *Onchocerca volvolus* in humans (Aziz *et al.* 1982), and is now extensively used for Onchocerciasis control in endemic regions. Ivermectin is also used for the treatment of humans affected by filariasis, scabies, strongyloidiasis, and other gastrointestinal nematodes (Dourmishev *et al.* 2005).

For the treatment of cattle, ivermectin is either applied topically, by subcutaneous injection, intravenously, or in a bolus. In humans, ivermectin is administered as an oral tablet. Bioavailability of the macrocyclic lactones, and thus the efficacy and persistence

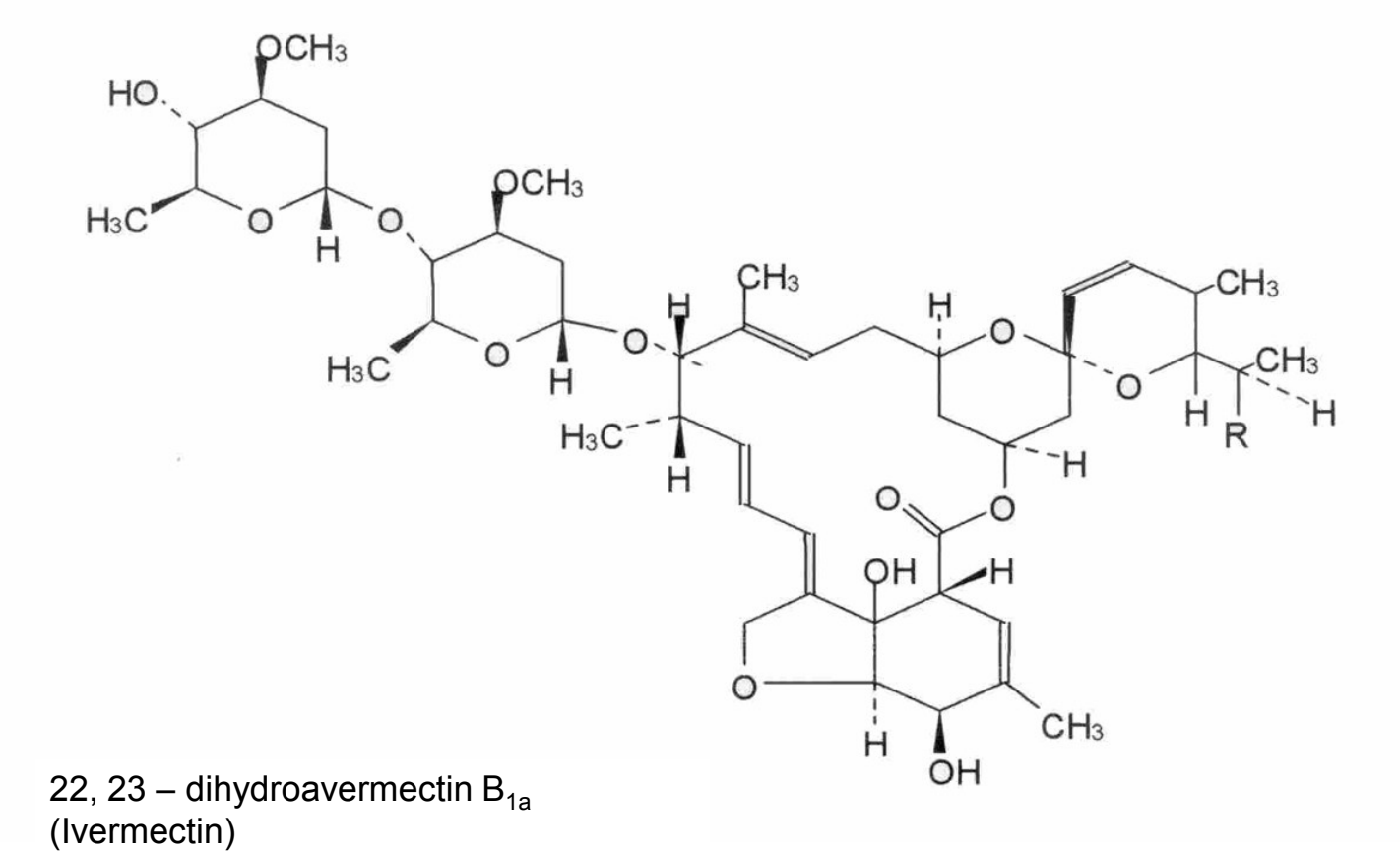

Figure 4.1. Chemical structures of ivermectin (Adapted from Edwards 2003).

of the drug, varies depending on the formulation used for treatment (Edwards *et al.* 1988, Fink and Porras 1989, Escudero *et al.* 1999). For example, the biological half-life of ivermectin is 2.8 d in cattle treated intravenously, but 8.3 d in cattle treated via subcutaneous injection (Fink and Porras 1989). Regardless of treatment formulation, the main route of excretion is in the feces (Chiu and Lu 1989). Some mosquitoes die after taking a bloodmeal containing ivermectin (Tesh and Guzman 1990, Gardner *et al.* 1993, Bockarie *et al.* 1999). However, the lethal and sublethal effects on *An.gambiae s.l.* after blood feeding directly on ivermectin-treated cattle or humans have never been investigated.

Alternatives to ivermectin

Moxidectin, doramectin, and eprinomectin are all used for the treatment of bovine endo- and ectoparasites. Moxidectin is thought to more effectively control nematodes than arthropods (Mckellar and Benchaoui 1996). It differs from ivermectin by three functional groups: 1) the C-13 disaccharide found in ivermectin is absent in moxidectin, 2) a methoxime moiety is found at C-23 in moxidectin, but absent in ivermectin, and 3) an olefinic side chain is found at C-25 in moxidectin, but is absent from ivermectin (Rock *et al.* 2002). Moxidectin is more lipophilic than ivermectin. In cattle, the half-lives of moxidectin and ivermectin in fatty tissue are 12-14 d (Rock *et al.* 2002) and 7-8 d (Chiu and Lu 1989) respectively. This property may give moxidectin a longer mean plasma residence time than ivermectin (Lanusse *et al.* 1997). However, cattle treated with equivalent dosages of these two drugs do not have significantly different peak plasma concentration values (Lanusse *et al.* 1997).

Doramectin was derived via mutational biosynthesis of avermectin A1, whereas ivermectin is a derivative of avermectin B1 (Goudie *et al.* 1993). The presence of a cyclohexyl group on C-25 can be used to distinguish doramectin from ivermectin (Conder and Baker 2002). It is highly efficacious against nematode parasites (Jones *et al.* 1993), and

in some cases outperforms ivermectin (Goudie *et al*. 1993). After cattle are administered subcutaneous injections of either doramectin or ivermectin at 200µl/kg, examination of their blood plasma reveals that doramectin peaks higher and persists for longer than does ivermectin (Lanusse *et al.* 1997, Toutain *et al.* 1997).

Eprinomectin was discovered in 1997, and is only commercially available as a topical formulation. It was purposefully derived to create a product that was highly effective against livestock gastro-intestinal parasites, yet had low milk partitioning (Shoop and Soll 2002). Like ivermectin, it is derived from avermectin B1, but is distinguished by the presence of an amino group at C-4 (Shoop *et al.* 1996). Cattle and deer are treated topically with Eprinex (containing eprinomectin) at 500 µg/kg, while other avermectins are typically only applied at 200 µg/kg. Interestingly, eprinomectin is three-fold more effective at reducing endoparasites in cattle than ivermectin, even when the drugs are applied at the same rate. This may be, in part, due to the greater bioavailability of eprinomectin; blood plasma availability of eprinomectin is twice that of ivermectin in cattle (Alvinerie *et al.* 1999).

The modes of action (Arena *et al.* 1995) and routes of excretion (Mehlhorn 2008) are similar among the avermectins. Unlike ivermectin, moxidectin and eprinomectin do not have meat or milk withdrawal times, and all three ivermectin alternatives are approved for use in pregnant animals. Hosts o f *An. gambiae s.l.* that cannot be treated with ivermectin, such as pregnant or lactating cattle, could safely be treated with moxidectin, doramectin, or eprinomectin.

Mode of action of the avermectins

It was previously suggested that avermectins and milbemycins are activators of both GABA-gated and glutamate-gated chloride channels. Glutamate is a stimulatory transmitter found at nerve-muscle junctions (only in invertebrates) (Fox and Lloyd 1999), whereas γ-

aminobutyric acid (GABA) is an inhibitory postsynaptic transmitter of the central nervous system (Nation 2002). Fritz *et al.* (1979) were the first to report that the inhibitory postsynaptic potentials in lobster stretcher muscles perfused with avermectin B_{1a} (AVM) were irreversibly blocked. The inhibitory neurotransmitter at the lobster stretcher muscle neuromuscular junction was believed to be GABA (Fritz *et al.* 1979). Later, Kass *et al.* (1980) reported that AVM inhibits transmission between interneurons and excitatory motoneurons in the ventral nerve chord of nematodes; they suggested that AVM acted as a GABA agonist in these animals. Feng *et al.* recently showed that AVM does interact with nematode GABA receptors (2002), though others report that AVM inhibits rather than activates these receptors (Martin and Pennington 1988). The high concentration of AVM needed to activate GABA receptors (Wolstenholme and Rogers 2005) in nematodes, and the inactivity of AVM on mammalian peripheral GABA receptors also indicate that the GABAnergic receptors (Martin *et al.* 2002) are not the main target of the avermectins. The macrocyclic lactones act mainly on glutamate-gated chloride channels, which are important in signal transmission to locomotory and pharyngeal muscles in nematodes (Martin *et al.* 2002, Wolstenholme and Rogers 2005). They cause paralysis and death in target organisms, and can also cause sterility in some arthropods by blocking oviposition (Hollingworth 2006).

Safety of ivermectin and moxidectin in vertebrates

The macrocyclic lactones are widely used in human and veterinary medicine for the treatment of endo- and ecto-parasites. They powerfully affect glutamate-gated chloride channels, yet this group of ligand-gated Cl channels is not found in vertebrates (Martin *et al.* 2002). Cattle can tolerate dosages of ivermectin and moxidectin well above the recommended therapeutic doses. The acute toxicity of ivermectin applied orally and subcutaneously is 10 mg/kg. Single concentrated doses of 6.0 mg/kg can be administered to

cattle without ill effects (Pulliam and Preston, 1989). Injectable moxidectin has been used safely in cattle at dosages to 1.0 mg/kg (Yazwinski *et al.* 2006). In humans, therapeutic dosages of ivermectin are well tolerated up to 200 μg/kg (Greene *et al.* 1989). To date, no other avermectin has been approved for human use.

Current vector control methods

In sub-Saharan Africa, current vector control strategies include: 1) indoor spraying of insecticides targeting adults, 2) personal protection measures such as ITNs and insecticide-treated curtains, 3) larvicidal compounds such as *Bacillus thuringiensis var. israeliensis* and 4) environmental manipulation for prevention of successful breeding (Touré 2001). Yet ITNs and IRS continue to be the most inexpensive and effective tools used for community-wide vector control and prevention of malaria epidemics (Rozendaal 1997). ITNs successfully limit survivorship of mosquitoes that contact the net, and can reduce malaria transmission by 90 percent in villages where nets are deployed to cover all sleeping spaces (Gimnig *et al.* 2003). ITN use can reduce childhood mortality by 17% or more in Sub-Saharan Africa (Lengeler 2004). Mass distribution programs in rural Africa have the potential to significantly increase ITN coverage to even the poorest families. Between 2004 and 2006, ITN use by rural Kenyan children increased from 7 to 67% due to expansion of a subsidized clinical distribution system (Noor *et al.* 2007). Even unprotected families receive some of the benefit of high mosquito mortality from ITN-protected neighbors; increased malaria protection is experienced in villages where ITN coverage is greater than 50% (Hawley *et al.* 2003). In Western Kenya, *An. gambiae* is being extirpated in areas where ITN coverage reaches over 90% (Bayoh *et al.* 2010). However, malaria transmission still occurs in areas where ITN coverage is high (Walker, personal communication). Although *An. gambiae* populations are declining, the entomologic inoculation rate remains high enough for malaria

infection to recur at least once per person per year (Bayoh, personal communication). Furthermore, ITNs apply selection pressure and push *An. arabiensis* populations toward exophily, which may lead to behavioral resistance to indoor control measures in these vector populations. Tirados *et al.* (2006) found that only 16% of *An. arabiensis* females entering a house at night rested there the following morning, suggesting that females feed indoors but leave immediately after feeding. While indoor vector control measures successfully control endophilic vector populations, a vector control measure targeting exophilic populations is needed.

In the 1950s and '60s, over-reliance on a single vector control measure, DDT, led to rapid evolution of insecticide resistance (Nabarro and Tayler 1998). Heavy reliance on pyrethroids used in ITN treatment, and lack of affordable alternatives to ITNs, could potentially lead to pyrethroid resistance. Currently, *An. gambiae s.l.* populations resistant to both pyrethroids, and DDT used in IRS have been documented in several African countries (Chandre *et al.* 1999). To improve malaria control, a multi-tactic approach that supplements ITNs and IRS is urgently needed. The following investigations aimed to:

1) Demonstrate that vector populations were feeding upon, and likely persisting due to cattle hosts present in areas of high ITN coverage,
2) Examine the cost of blood feeding upon ivermectin- and moxidectin-treated cattle to *An. gambiae s.l.*
3) Compare the toxicities of all four cattle dewormers on *An. arabiensis* in a controlled laboratory setting.

CHAPTER 5:

TOXICITY OF BLOODMEALS FROM IVERMECTIN-TREATED CATTLE TO *ANOPHELES GAMBIAE S.L.*

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Abstract

Two cattle dewormers, ivermectin and moxidectin, were tested for lethal and sublethal effects on the malaria vectors *Anopheles gambiae s.s.* and *An. arabiensis.* In the laboratory, direct addition of ivermectin to bovine blood reduced mosquito survivorship and fecundity. The LC_{50} of ivermectin for *An. gambiae s.l.* laboratory populations was 19.8 ± 2.8 ppb. In the field, commercially available formulations containing ivermectin and moxidectin were injected into cattle at three times the recommended rate. Ninety percent of *An. gambiae s.s*. that fed on ivermectin-treated cattle within 2 wk of treatment failed to survive >10 d post blood meal. No eggs were deposited by *An. gambiae s.s.* fed on ivermectin-treated cattle within 10 d of treatment. Survivorship and egg production of mosquitoes feeding on moxidectin-treated cattle were no different from those feeding on untreated cattle. These results suggest that treatment of cattle with ivermectin could be used as part of an integrated control program to reduce zoophilic vector populations that contribute to malaria transmission.

Introduction

Malaria continues to burden human populations throughout the tropics, annually killing over 1 million African children. Two mosquitoes of the *Anopheles gambiae* complex, *An. gambiae* sensu stricto and *An. arabiensis*, are widely distributed throughout Sub-Saharan

Africa, and along with *An. funestus*, are the principle vectors of malaria. Although morphologically indistinguishable, the species in the *An. gambiae s.l.* complex differ in blood host utilization, resulting in variable vectorial capacity. *An. gambiae* is predominantly anthropophagic, and *An. arabiensis* feeds primarily on cattle, yet both species will opportunistically feed on either host (Highton *et al*. 1979, Githeko *et al*. 1996, Tirados *et al*. 2006).

Where densely deployed, pyrethroid-treated bed nets reduce *An. gambiae s.l.* populations. However, it is unlikely that malaria will be suppressed to tolerable levels unless the cattle-mosquito connection is simultaneously broken. This is attributable to *An. arabiensis*; despite feeding primarily upon cattle throughout its broad distribution, this mosquito is still an efficient vector of malaria (Trape *et al*. 1992, Mendis *et al*. 2000). Concerns over insecticide (Chandre *et al*. 1999, Müller *et al*. 2008, Munhenga *et al*. 2008**,** Awolola *et al*. in press) and behavioral resistance management (Molineaux and Grammiccia 1980), also call for the implementation of novel vector control measures that can augment bed nets and indoor residual sprays.

Ivermectin and similar macrocyclic lactones are used in veterinary and human medicine against a range of helminths and arthropod pests (Wilson 1993). It is ingested by humans for the treatment of ectoparasites *Sarcoptes scabies* and *Pediculus humanus,* as well as endoparasites such as *Strongyloides stercoralis*, *Wuchereria bancrofti* and *Onchocerca volvulus.* Ivermectin is administered to cattle via subcutaneous injection, topically as a pour-on, or as a bolus to treat the ectoparasites *Chorioptes bovis*, *Sarcoptes scabiei* var. *bovis, Haematobia irritans*, *Haematopinus eurysternus*, and *Boophilus microplus* (Benz *et al*. 1989), as well as gastrointestinal nematodes.

Some mosquitoes die after taking a blood meal containing ivermectin. All *An. stephensi* Liston fed on the blood of mice treated with 2.8 mg/kg ivermectin died

(Pampiglione *et al*. 1985). Tesh and Guzman (1990) reported that blood-ivermectin levels greater than 10 µg/ml were lethal to *Ae. aegypti, Ae. albopictus*, and *Cx. quinquefasciatus*. Even at sublethal doses, surviving mosquitoes suffered reduced egg production and hatchability (Tesh and Guzman 1990). Seventy percent of blood-fed *An. punctulatus* collected from human dwellings 4 d after ivermectin treatment of human inhabitants, died 24 h post-bloodmeal (Bockarie *et al*. 1999). More than 90% of *An. farauti* fed on the arm of an ivermectin-treated human volunteer died when the feeding occurred within 10 d after ivermectin ingestion (Foley *et al*. 2000). To our knowledge, the lethal and sublethal effects of ivermectin-treated blood on *An. gambiae s.l*. have never been investigated. Therefore, in this study we quantified the effects of anthelmintic-treated cattle on the survivorship and fecundity of the important malaria vector *An. gambiae s.l.*

Methods

Mosquitoes

We used Kisumu strain *An. gambiae* laboratory colonies: one was reared at Michigan State University (MSU) according to the methodology of Huang *et al.* (2005), and the other at the Centre for Global Health Research, Kenya Medical Research Institute (KEMRI), Kisumu, Kenya. One experiment included *An. arabiensis* of the Dongola strain. This colony was provided to MSU in January of 2008 by the Centers for Disease Control and Prevention, Malaria Research and Reference Reagent Resource Center (MR4; American Type Culture Collection, Manassas, VA). These laboratory-reared *An. arabiensis* were handled identically to *An. gambiae* at Michigan State University. Eclosed mosquitoes were placed in 30cm^3 BugDorm-1 (MegaView Science Co. Ltd., Taiwan) rearing cages and were always held at 28±1^{0}C and 80±10% RH under a 12:12 LD photoperiod.

Delivery of ivermectin-treated blood via artificial membrane feeder

Ivermectin (> 90% B1a; Sigma-Aldrich Corp., St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of 10 ppm. A 10:1 serial dilution of stock solution in DMSO was used to make 6 treatments (1000, 100, 10, 1, 0.1, and 0.01 ppb). Heparinized bovine blood (Lampire Biological Laboratories, Inc., Pipersville, PA) was added to 1.5 ml of each treatment solution to make 6 treated blood solutions of 15 ml each. The negative control was 1.5 ml of DMSO in 13.5 ml of bovine blood. Treatments of 2, 4, 6 and 8 ppb ivermectin in bovine blood were offered to both *An. gambiae* and *An. arabiensis,* so as to refine LC_{50} estimates. Each treatment was presented to 20 mosquitoes transferred via mouth aspirator to cages made from circular, clear plastic food containers measuring 10 cm in diameter. The center was removed from the lid of each container and replaced with netting material through which mosquitoes could blood feed. The bottom of each container was cut off and replaced with a new, removable cage floor filled with moist sand, and covered in brown paper towel (Georgia Pacific, Atlanta, GA) for oviposition. Treated blood was offered to cages of mosquitoes for 30 min via artificial membrane feeder (Huang *et. al* 2005). Then, all unfed females were removed. Mosquitoes were monitored daily for mortality and oviposition for 9 d post blood-meal. Dead mosquitoes were counted and removed, and ovipositional substrates were inspected and eggs counted daily. Ten percent honey solution was provided to all mosquitoes *ad libitum* during the post-treatment period; six 1.5 cm diam cotton balls were soaked in the solution, then placed on top of each cage.

Approximately 100 eggs from each loaded ovipositional substrate were separated according to treatment, and submerged in clear plastic 100 x 15 mm petri dishes containing filtered tap H_2O. Petri dishes were held on the laboratory bench top at ca. 22^0C under 12:12 LD photoperiod. First instars were counted and removed daily with a Pasteur pipette. Larval counts ceased when no new first instars were found for 3 consecutive d.

Mosquito feeding bioassay on ivermectin- and moxidectin-treated cattle

Nine zebu bulls between one and three years old were purchased in Kisumu, Kenya. These bulls were incorporated into a local herd. They grazed daily and were housed as in traditional Luo husbandry: tied from sun-down until 10 am within the open air center of a housing compound. These bulls were weighed on a WB5000 portable scale (Salter Brecknell, Fairmont, MN). They were partitioned into 3 groups of 3 bulls each, so as to block by weight. Groups were randomly assigned to one of three treatments: injectable Ivomec (ivermectin; Merial, Duluth, GA) at 3 mL/50 kg (600 μg/kg AI), injectable Cydectin (moxidectin; Fort Dodge Animal Health, Fort Dodge, IA) at 3 mL/50 kg (600 μg/kg AI), and a 0.9% sterile saline injection at 3 mL/50 kg. Prior to treatment, cages of *An. gambiae* were fed on each animal to establish a baseline for mortality not due to chemical. Cages consisted of a clear plastic cone with a cotton-plugged port at the narrow end for insertion of 30 mosquitoes, and covered at the wide end with white netting.

Prior to blood feeding, a *ca*. 6 cm diam circular patch was shaved (or reshaved) from the rib cage immediately behind the foreleg of each animal. A loaded cage was snuggly affixed by means of a size 32 brassiere. Cattle were loosely tethered to a tree trunk during the 30 min blood-feeding, and up to 3 bulls at one time were donating blood under the care of one or two human attendants. Starting 1 day post treatment, cohorts of 3-d-old mosquitoes were fed on the treated and untreated cattle every three days for up to 23 d post-treatment.

After transport to the KEMRI insectary, cattle-fed mosquitoes within one of the three chemical treatment groups were segregated into two sub-groups according to blood meal size. Females with distended abdomens in which blood was clearly visible in greater than 50% of the abdominal length were classified as having taken a large blood meal. Females lacking distended abdomens, with blood visible in less than 50% of the abdominal length were classified as having taken a small blood meal. Cages were monitored daily for mortality and

oviposition until all females in each cage died. Each day, dead females were counted and removed, and eggs deposited were counted under a dissection microscope.

Statistical Analysis

When ivermectin-treated blood was delivered via artificial membrane feeder, the LC_{50} and LC_{95} estimates for *An. gambiae* and *An. arabiensis* were identical. Mortality data for the two species were therefore pooled, and a single LC_{50} and LC_{95} was calculated using PROBIT analysis. Residual variation in egg production was modeled using a gaussian distribution. Parameter estimates were calculated using maximum likelihood, and treatment effect, in groups where eggs were produced, was determined by an F-test. Egg viability residuals were modeled using a normal distribution, and an F-test tested for differences in viability due to treatment.

When mosquitoes fed upon treated cattle, survivorship data were compared across treatment groups using the Cochran-Mantel-Haenszel χ^2 analysis (Parmar and Machin 1995). Kaplan-Meier survival analysis (Parmar and Machin 1995) was used to compare mosquito survivorship curves across pre- and post-treatment intervals within the ivermectin treatment group. Bootstrapping was used to calculate the 95% CIs surrounding the median survivorship estimates at each post-treatment interval in Figure 5.1. Where mortality was substantial, survivorship as influenced by blood meal size was compared across post-treatment intervals using a log-rank test. Maximum likelihood was used to estimate egg deposition at each post-treatment interval across treatments. An F-test tested for the effect of treatment on egg deposition at each post-treatment interval. All data were analyzed using R statistical software version 2.7.2 (R Development Core Team 2008).

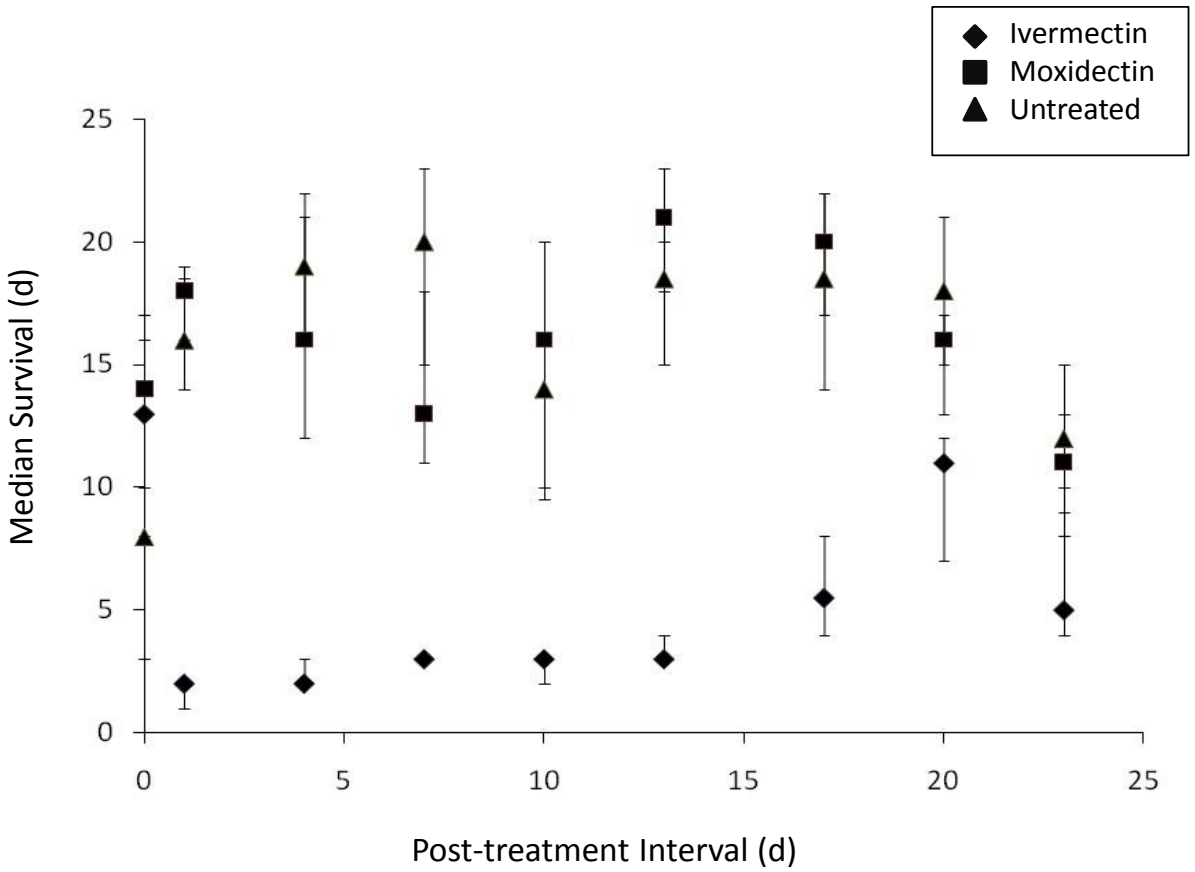

Figure 5.1. Median survival time (d) of *Anopheles gambiae s.s.* with bootstrapped confidence intervals (N = 1000). *An. gambiae s.s.* sample sizes were n= 643, n= 615, and n = 666 for ivermectin, moxidectin and untreated groups respectively.

1

Results

An. gambiae s.l. *LC_{50} and LC_{95} estimates from lab feedings of ivermectin-treated blood*

Laboratory tests of ivermectin-treated bovine blood established that the LC_{50} and LC_{95} for *An. gambiae s.l.* at 19.8 ± 2.8 ppb, and 77.7 ± 8.1 ppb respectively. No eggs were produced by either species in the three highest ivermectin-in-blood treatments. The numbers of eggs produced per blood fed female in the three lowest treatment groups and the untreated group were indistinguishable ($p = 0.85$). Nearly all deposited eggs were viable; hatchability reached > 86% across treatments (Table 5.1).

Survivorship and fecundity of An. gambiae *fed on treated bulls*

The blood of ivermectin-treated cattle reduced survivorship and limited the fecundity of laboratory-reared *An. gambiae*, while moxidectin did not ($p < 0.001$; Figure 5.1). The median survivorship of *An. gambiae* feeding on the blood of ivermectin-treated cattle 1 d post-treatment was only 2 d. Thereafter, survivorship of *An. gambiae* ingesting ivermectin increased slowly over post –treatment intervals (Figure 5.1). Survivorship was significantly lower after feeding on ivermectin-treated cattle than on untreated cattle for up to 20 d post treatment (Figure 5.1). Furthermore, the 10 d survival probability was <10% for *An. gambiae* fed on ivermectin-treated cattle within two wk after cattle treatment (Figure 5.2).
Relative to partial feeding, taking a full meal of ivermectin-containing blood significantly reduced mosquito survivorship for > 2 weeks after cattle treatment (Table 5.2). For 1-10 d post-treatment, neither the fully-fed nor the longer-lived partially-fed mosquitoes produced eggs (Figure 5.3). Oviposition was reduced in mosquitoes feeding on ivermectin-treated cattle ($p = 0.07$; Figure 5.3), though egg deposition 20 d post-treatment and thereafter of mosquitoes that fed on ivermectin-treated cattle no longer differed from the negative control.

Table 5.1. Viability of eggs laid by *Anopheles gambiae s.s*. and *An. arabiensis* fed treated *vs*. untreated blood meals. No difference in viability was observed (p = 0.78).

Treatment (ppb)	Species	Eggs Hatched	Total Eggs	Percent Hatch
1.00	*An. gambiae*	496	565	86.3
	An.arabiensis	528	621	
0.10	*An. gambiae*	574	593	89.2
	An.arabiensis	377	457	
0.01	*An. gambiae*	621	722	87.1
	An.arabiensis	268	300	
DMSO only	*An. gambiae*	458	481	93.1
	An.arabiensis	297	332	

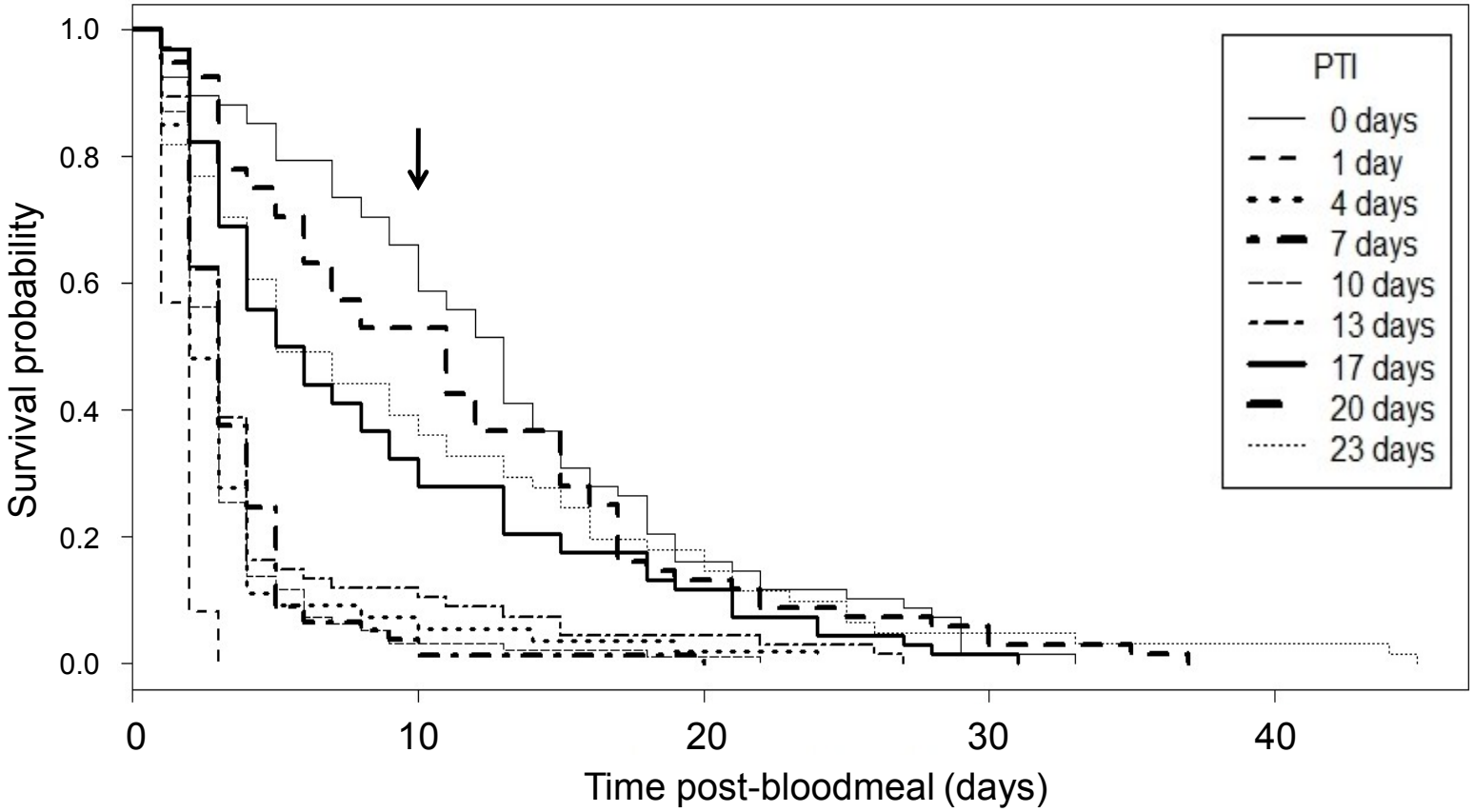

Figure 5.2. Kaplan-Meier survivorship curves for *Anopheles gambiae s.s.* that blood-fed upon ivermectin-treated cattle. Each curve corresponds to a unique post-treatment interval. The arrow indicates the required development time for *Plasmodium falciparum* in the body of a mosquito at an external temperature of 27° C (Patz and Olson, 2006).

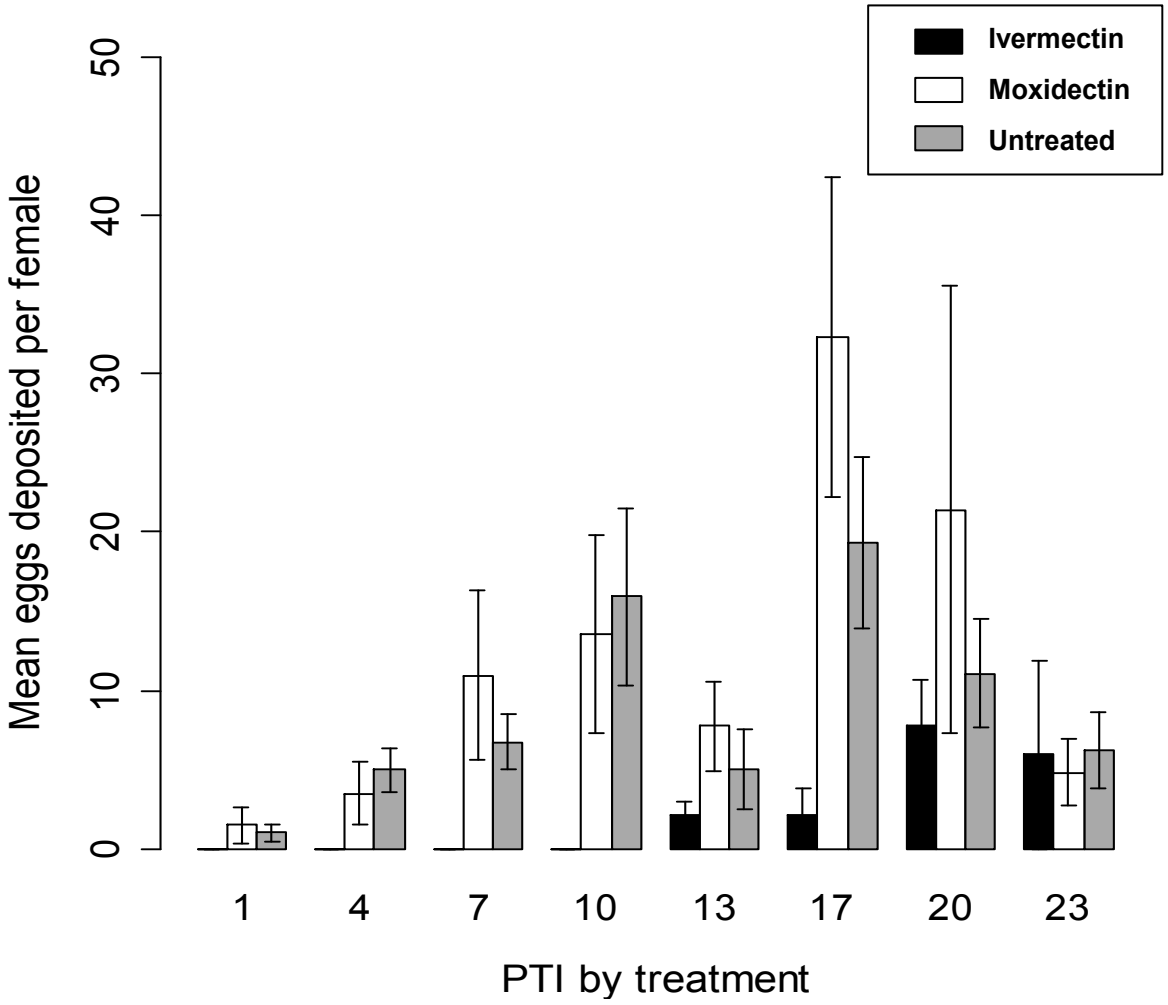

Figure 5.3. Mean (±95% CIs) number of eggs deposited per female per treatment group over 8 different post-treatment intervals (d).

Table 5.2. Log-rank test for differences in survivorship in *Anopheles gambiae s.s.* taking different blood meal sizes within the ivermectin treatment group. P-values are unadjusted for multiple comparisons.

Post-treatment Interval (d)	Blood meal size	N	Observed	Expected	χ^2	df	p
1	Full	59	59.0	64.0	4.3	1	0.0373
	Partial	27	27.0	22.0			
2	Full	23	23.0	17.8	4	1	0.0459
	Partial	31	31.0	36.2			
7	Full	38	38.0	27.8	10.4	1	0.00127
	Partial	39	39.0	49.2			
10	Full	68	68.0	69.3	0.2	1	0.698
	Partial	26	26.0	24.7			
13	Full	38	38.0	31.1	4.3	1	0.0377
	Partial	29	29.0	35.9			
17	Full	51	51.0	48.3	0.6	1	0.436
	Partial	17	17.0	19.7			
20	Full	57	57.0	59.8	1.2	1	0.267
	Partial	11	11.0	8.2			
23	Full	42	42.0	45.2	1	1	0.306
	Partial	19	19.0	15.8			

Discussion

Ivermectin persisted in the blood of Zebu cattle, and reduced survivorship and fecundity of laboratory-reared *An. gambiae* populations for nearly 3 wk post treatment. If applied at the onset of the rainy season, ivermectin-treated cattle could delay the *An. gambiae s.l.* population explosion, possibly precluding malaria epidemics. During those 3 wk, up to 90% of *An. gambiae s.l.* feeding on cattle would die prior to transmitting parasites (Figure 5.2).

Based upon our LC_{50} estimates of laboratory strain *An. gambiae s.l.,* we speculate that ivermectin-treated cattle would similarly reduce survivorship in *An. arabiensis* populations. In a laboratory setting, it takes several days for a lethal blood meal to kill laboratory-reared *An. gambiae*. During this time, these females were 1) unable to digest their blood meals normally, 2) lethargic, and 3) unable to fly. These conditions would likely hasten mortality in the field, where hazards of desiccation, extreme temperatures, and predation are expected (Foley *et al*. 2000).

Our results were similar to those of Bockarie *et al*. (1999); after 28 d elapsed between treatment and blood feeding, humans treated with ivermectin at a rate of 400 µg/kg were no longer lethal to *An. punctulatus*. Foley *et al*. (2000) document a convincing reduction in survivorship of *An. farauti* laboratory populations for 14 d after human ingestion of 250 µg/kg ivermectin.

Insecticide-treated nets (ITNs) are reducing vector populations, as well as malaria transmission. In Western Kenyan villages, where ITNs are deployed to cover all sleeping spaces, malaria transmission has been reduced by up to 90% (Gimnig *et al*. 2003). Furthermore, *An. gambiae* is being extirpated in villages where ITN coverage reaches 90% or more (Walker *et al*. unpublished). In these same villages, however, both *An. arabiensis* and malaria transmission persist (Walker *et al*. unpublished). Mass treatment of humans with

ivermectin could reduce malaria transmission and preclude epidemics (Bockarie *et al*. 1999). It has also been suggested that mass treatment of livestock may reduce malaria transmission by zoophilic vectors, such as *An. farauti* (Iakubovich *et al*. 1989, Foley *et al*. 2000).

The use of anthelmintic drugs to supplement ITNs represents a novel, integrated approach to malaria vector control. Previously, DDT and pyrethroids have been used in indoor residual spray programs and ITNs respectively for suppression of vector populations (Beier *et al*. 2008). However, cross-resistance between these classes of insecticide has been reported (Chandre *et al*. 1999). Furthermore, there is no widely-used malaria management strategy that simultaneously targets both zoophagic and anthropophagic vector populations. Additionally, no convincing cross-resistance of avermectins with pyrethroids has been reported to date (Cochran 1990, Rugg *et al*. 2007), giving this combination of vector control measures unique insecticidal resistance management potential. Treatment of cattle with ivermectin fulfills the need for new insecticidal compounds that can safely be used in proximity to humans, while targeting overlooked zoophagic vector populations.

CHAPTER 6:

LETHAL AND SUBLETHAL EFFECTS OF AVERMECTIN/MILBEMYCIN PARASITICIDES ON THE AFRICAN MALARIA VECTOR, *ANOPHELES ARABIENSIS* (DIPTERA: CULICIDAE)

Abstract

Four cattle parasiticides of the avermectin/milbemycin class were examined for their lethal and sublethal effects on the zoophilic, African malaria vector *Anopheles arabiensis*. Ivermectin, moxidectin, doramectin, and eprinomectin were mixed with bovine blood and provided to laboratory-reared *An. arabiensis* in a membrane feeder. Ivermectin and eprinomectin were lethal to *An. arabiensis* at low concentrations (LC_{50}s of 7.9 ppb and 8.5 ppb, respectively). While the lethality of doramectin (LC_{50} of 23.9 ppb), was less than that of ivermectin and eprinomectin, it markedly reduced egg development. The concentration of moxidectin required to reduce survivorship and egg production in *An. arabiensis* was >100 fold greater than for ivermectin or eprinomectin. Moxidectin was weak in its actions against *An. arabiensis* relative to the other three chemicals. These results suggest that cattle treated with ivermectin or eprinomectin in the prescribed range of low dosages as parasiticides have blood toxic to zoophilic malaria vectors. Regionally-coordinated, seasonal treatment of cattle could suppress *An. arabiensis* populations, thereby reducing malaria transmission. Doramectin (although less toxic) would have population level effects on egg production if used in this manner.

Introduction

Avermectins are glutamate-gated chloride channel activators that cause reduced motor activity and paralysis in both insects and nematodes (Martin *et al*. 2002). Ivermectin is a

member of the avermectin class, capable of reducing endo- and ectoparasite burden in vertebrates. In humans, ivermectin is ingested or applied topically to treat endo- and ectoparasitic infestations of the following: *Sarcoptes scabies*, *Pediculus humanus*, *Strongyloides stercoralis*, *Filaria bancrofti* and *Onchocerca volvulus (*Dourmishev *et al.* 2005*)*. In cattle, the species spectrum of ivermectin activity ranges from parasitic nematodes, mites, ticks, lice, and fly larvae (Benz *et al.* 1989) to certain species of mosquito (Iakubovich *et al.* 1989, Tesh and Guzman 1990, Bockarie *et al*. 1999, Foley *et al.* 2000, Fritz *et al.* 2009, Chaccour *et al.* 2010). Circulating ivermectin in the blood of treated hosts reduces survivorship of the mosquitoes that fed on them in both laboratory and controlled field settings (Bockarie *et al.* 1999, Fritz *et al.* 2009, Chaccour *et al.* 2010). For example, treatment of cattle at 600 μg/kg AI resulted in sufficiently high ivermectin blood plasma titers to produce >90% mortality in the *Anopheles* mosquitoes feeding upon them within 2 wk post-treatment (Fritz *et al.* 2009).

The most widespread vector control methods currently in use are insecticide-treated bed nets and indoor residual spraying, both of which reduce mosquito survivorship and successful human blood meal acquisition (Molineaux and Gramiccia 1980, Killeen and Smith 2007, Siegert *et al.* 2009). These control measures are relatively less effective at reducing blood meal acquisition (Killeen and Smith 2007), and vector abundance (Bayoh *et al*. 2010) for zoophilic vectors like *An. arabiensis*, compared with anthropophilic vectors like *An. gambiae*. In some cases, cattle serve as adequate hosts when humans are unavailable (Lefevre *et al*. 2009), allowing vector populations to persist. In malaria endemic areas, timed mass treatments of livestock with ivermectin could reduce zoophilic vector populations at the onset of the rainy season, precluding epidemics (Foley *et al.* 2000, Fritz *et al.* 2009). In addition, benefits for cattle shareholders would extend beyond malaria control, by reducing parasite burden of the cattle (Ciordia *et al.* 1984, Roncalli and Benitez Usher 1988, Dimander

et al. 2003), and thus increase the health and productivity of cattle herds (Ciordia *et al.* 1984, Dimander *et al.* 2003). The utility of ivermectin, however, is limited for malaria control because it currently is not labelled for use in lactating animals, which can comprise *ca.* 20% of a cattle population in traditional agro-pastoral regions of Africa (Voh and Otchere, 1988). It also requires post-treatment milk and meat withdrawal periods for treated animals, limiting the application of this parasiticide for vector control even further.

The parasiticides moxidectin, doramectin, and eprinomectin are closely related to ivermectin and part of the avermectin/milbemycin family. The mean residence time for moxidectin and doramectin is longer than for ivermectin in the blood of cattle (Lanusse *et al.* 1997), and commercially available pour-on formulations of moxidectin and eprinomectin do not require meat or milk withdrawal intervals following treatment. The potential use of these compounds for malaria vector control has not been thoroughly investigated, however. Moxidectin-treated cattle do not reduce *An. gambiae s.s*. mosquito survivorship as effectively as did cattle treated with ivermectin at the same rate (Fritz *et al.* 2009). Still, the toxic dose of moxidectin required for *An. gambiae* complex mosquitoes has not been determined. The aim of this study was to determine the concentrations of moxidectin, doramectin, and eprinomectin that reduced survivorship and fecundity of the opportunistic malaria mosquito, *An. arabiensis*.

Methods

Mosquitoes

In 2009, *An. arabiensis* of the Dongola strain were acquired from the Malaria Research and Reference Reagent Resource Center (MR4), and reared according to Huang *et al.* (2005). Thirty min prior to blood feeding, groups of twenty 3-5 d old females were aspirated into small, circular plastic cages (10 cm diam.). White mesh covering the top of the

cage permitted blood feeding via artificial membrane feeder, while brown paper towel over moist mud served as an ovipositional resource covering the cage floor. Throughout the study, cages of adults were always provided cotton moistened with 10% honey solution and held at 25 ^{0}C and 80±10% RH under a 12L: 12D cycle.

Preparation of treated blood

Ivermectin [22,23-Dihydroavermectin B_1], doramectin [25-cyclohexyl-5-O-demethyl-25-de(1-methylpropyl) avermectin A_{1a}; extracted as below], eprinomectin [4"-(epi-acetylamino)-4" deoxyavermection B_1], and moxidectin [(2aE,4E 5'R,6R 6'S,8E,11R,13S,15S,17aR,20R, 20aR,20bS)-6'-[(E)-1,2-dimethyl-1butenyl]-5'6,6',7,10,11,14,15,17a,20,20a,20b-dodecahydro-20,20b-dihydroxy-5'6,8,19,tetra-methylspiro[11,15-methano-2H,13H,17H-furo[4,3,2-pq][2,6]benzodioxacyclooctadecin-13,2'-[2H]pyrano]-4',17(3'H)-dione,4'-(E)-(O-methyloxime)] were dissolved in dimethyl sulfoxide (DMSO), then added to defibrinated bovine blood (Hemostat Laboratories, Dixon, CA, USA) in a 15 mL plastic conical centrifuge tube and inverted 6 times. Initial tests of each drug consisted of a 10-fold serial dilution of drug in DMSO, so as to achieve these final concentrations of active ingredient (AI) in blood: 1000, 100, 10, and 1 ppb. Control blood contained equivalent concentrations of DMSO. For each drug, cages of mosquitoes were randomly assigned to a single blood concentration, and fed only once throughout the course of the study. The results of the initial test determined the blood concentrations of each AI used in subsequent analyses of survivorship and fecundity (Table 6.1). Mosquitoes were blood-fed via artificial membrane feeder, using parafilm as a membrane for tests of the drugs eprinomectin and moxidectin. Pork sausage casing (Great Lakes Butcher Supply, Howell, MI, USA) was substituted for parafilm during ivermectin and doramectin tests, because it enhanced mosquito acceptance of the artificial feeder. After blood-feeding was complete,

Table 6.1. Blood concentrations used to determine the effects of each AI on *An. arabiensis* survivorship and fecundity.

Drug	Concentration AI in blood (ppb)
Eprinomectin	100, 10, 7, 4, 1, 0.1
Ivermectin	100, 10, 7, 4, 1, 0.1
Doramectin	100, 70, 40, 10, 7, 4
Moxidectin	50000, 10000, 5000, 1000, 500, 100

unfed mosquitoes were removed from each cage using a mouth aspirator, and the total number of blood-fed mosquitoes was recorded. Cages of mosquitoes were held for 9 d post blood-feeding.

Doramectin extraction and HPLC

Pure doramectin was unavailable, so it was extracted from a commercial injectable formulation for cattle (Dectomax, Pfizer Inc., New York, NY, USA). Five mL of ethanol was added to 5 mL Dectomax, and the mixture was allowed to separate after shaking in a separatory funnel (15 min). The top layer was reserved, while 5 mL EtOH was again added to the bottom layer. This process was repeated 3 times, for a total collection of 15 mL EtOH and doramectin. Thin-layer chromatography confirmed the presence of doramectin and absence of other solutes in the extract. Five µl of the doramectin extract was blotted onto a precoated silica-gel 60 F_{254} aluminum backed sheets. These were developed at room temperature using a mixture of hexane-acetone-decane-methanol (59:30:10:1, v/v). Sheets were viewed in a dark room under a 340-380 nm UV lamp. After doramectin was confirmed present, the extract was dried under a stream of nitrogen for 12 h in a dark hood. The final product was dissolved in DMSO to make a stock solution of expected concentration 10 ppm. The entire extraction process was replicated 3 times to produce 3 different doramectin extracts, each used twice during the study. HPLC was used to quantify total doramectin in two of the extracts.

Samples of each extract were diluted with DMSO to a working level for the HPLC/MSD. The LC system was a Waters 2695 HPLC and analytical column Waters XBridge (3.0 x 50mm) packed with 3.5µm C18 stationary phase. Samples (10µl) were eluted using an isocratic mobile phase consisting of 50% acetonitrile, 50% water and 0.1% triethylamine at a flow rate of 0.25ml min-1. Mass spectrometry (MS) was run at basic pH

conditions in order to promote the formation of analyte anions. Detection by MS in negative ion mode required an atmospheric pressure chemical ionization interface (APCI), and was performed on a Waters MicroMass ZQ mass spectrometer. Ions 591.5 and 815.6 were monitored for detection of doramectin; ion 591.5 was used for quantitation and 815.6 for confirmation.

Data collection and statistical analysis

Beginning 1 d post-blood-meal, cages were examined for the presence of mosquito carcasses and egg production every other day. Dead females were counted and removed from the cage floor; eggs were counted under a dissection microscope. After 9 d of observation, mosquitoes from each cage were frozen at -20°C, and then dissected for the presence of eggs. Eggs present of Christopher's stages IV and V were counted under a dissection microscope.

Logistic regression was used to model survivorship and egg deposition data for each drug. For all drugs, survivorship residuals were specified as binomially distributed, and Abbott's correction was applied to the mean proportion of individuals deceased at each dose. A log transformation was applied to dosage within each drug treatment, and maximum likelihood estimation (MLE) was used to estimate parameters for mortality models. LC_{50} and LC_{95} estimates from these models were calculated using the MASS library dose.p program for R statistical software (v. 2.7.2; Anon. 2010).

Residual variation in egg production was modeled using a Poisson distribution for each drug. The effect of drug dosage on egg development was explored using a mixed effects model; drug dosage was specified as fixed, while date of feeding was specified as random. A likelihood ratio test (LRT) determined the effect of drug dosage on egg production. To determine whether drug and drug dosage influenced oviposition relative to

total egg development, all egg deposition below each LC_{50} was expressed as a proportion over total eggs developed (oviposited + dissected). Within each treatment group, the proportion of eggs deposited by females fed drug dosages below the LC_{50} was compared with the proportion of eggs deposited by females fed blood treated only with DMSO. Data were fit to a generalized linear model with a quasibinomial distribution, and drug contributions to the proportion of eggs oviposited relative to the total eggs developed (oviposited + dissected) were assessed with a t-test.

Results

A summary of the lethality of the parasiticides on *An. arabiensis* can be found in Table 6.2. Mortality elicited by treated blood was highly variable among the four compounds. Eprinomectin and ivermectin were highly lethal to *An. arabiensis*; LC_{50} values were 8.48 ppb and 7.85ppb, respectively. While the concentration required to kill 95% of blood feeding *An. arabiensis* tended to be lower for eprinomectin than for ivermectin, the difference was not statistically significant based upon their overlapping 95% confidence intervals. Average recovery of doramectin was 80% as determined by HPLC. Thus, expected doramectin dosages (Table 6.1) were multiplied by a correction factor of 0.8 prior to statistical analysis. Doramectin and moxidectin were comparatively less lethal, requiring, respectively, 3 and 148 times the blood concentration to kill *An. arabiensis* than either eprinomectin or ivermectin. As expected, increasing the amount of drug in the proffered blood increased mosquito mortality across all four dewormers (Figures 6.1and 6.2).

Three of the four drugs also exerted sub-lethal effects. Surviving individuals having fed upon ivermectin-, doramectin-, and moxidectin-treated blood produced fewer eggs than

Table 6.2. Abbott's corrected LC_{50} and LC_{95} values for *An. arabiensis* fed upon eprinomectin-, ivermectin-, doramectin- and moxidectin-treated blood, given in parts per billion of drug in the mosquito blood meal. The α and β values represent the intercept and slope of the logistic regression analyses, respectively.

Drug	N	df	α	β	LC_{50} (95% CI)	LC_{95} (95% CI)
Eprinomectin	451	5	-3.66	1.71	8.5 (7.2, 10.0)	47.4 (28.2, 79.8)
Ivermectin	518	5	-2.17	1.06	7.9 (6.2, 9.9)	128.1 (62.1, 264.4)
Doramectin	585	5	-3.18	1.00	23.9 (19.1, 30.0)	453.0 (236.2, 868.7)
Moxidectin	621	5	-0.25	1.51	1181.0 (999.4, 1395.7)	8305.3 (5758.7, 11978.1)

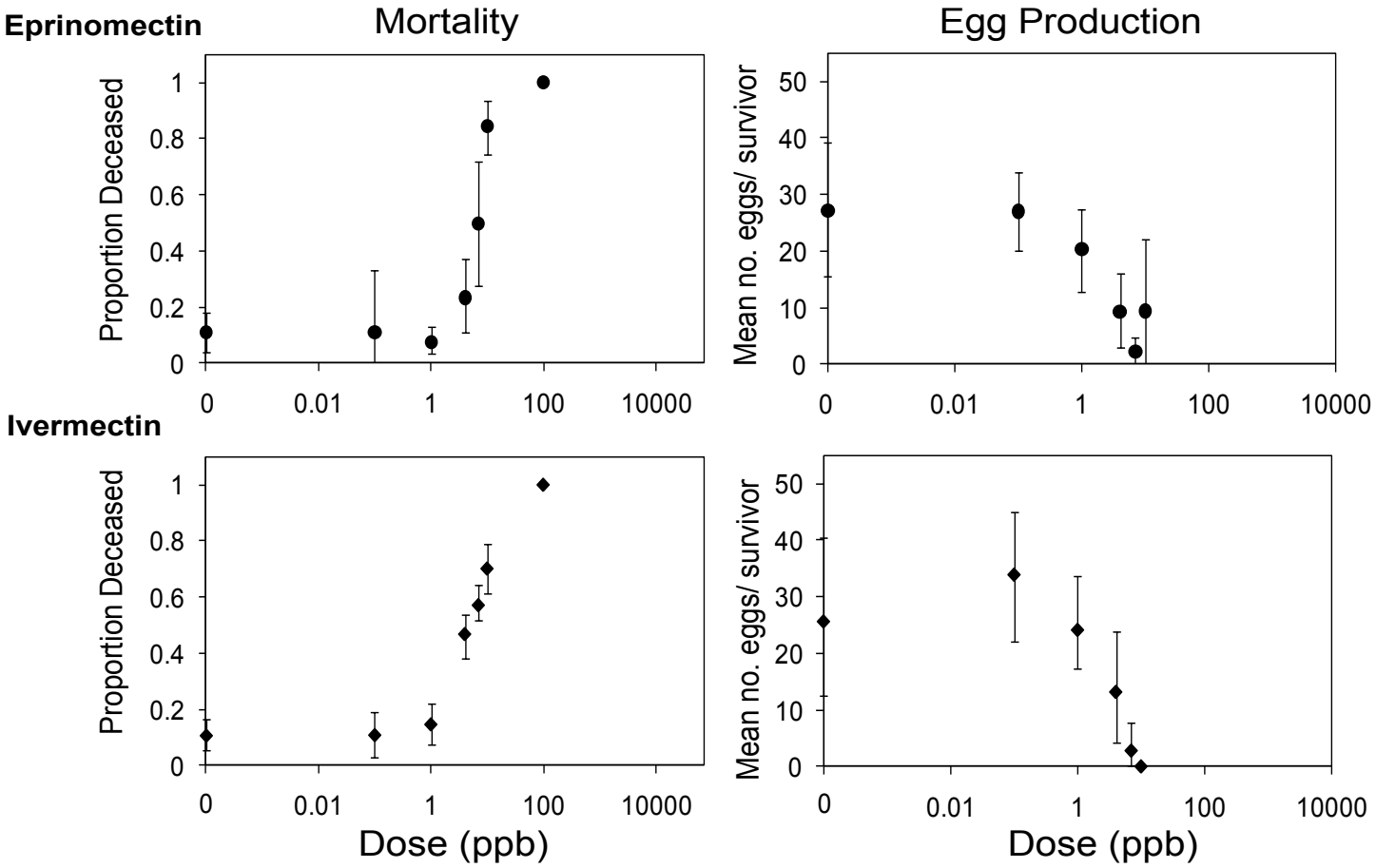

Figure 6.1. Uncorrected mean proportion of *Anopheles arabiensis* deceased (left) and production of eggs by survivors (right) within 9 d of feeding upon eprinomectin- and ivermectin-treated blood. Error bars represent bootstrapped 95% confidence intervals (N=1000).

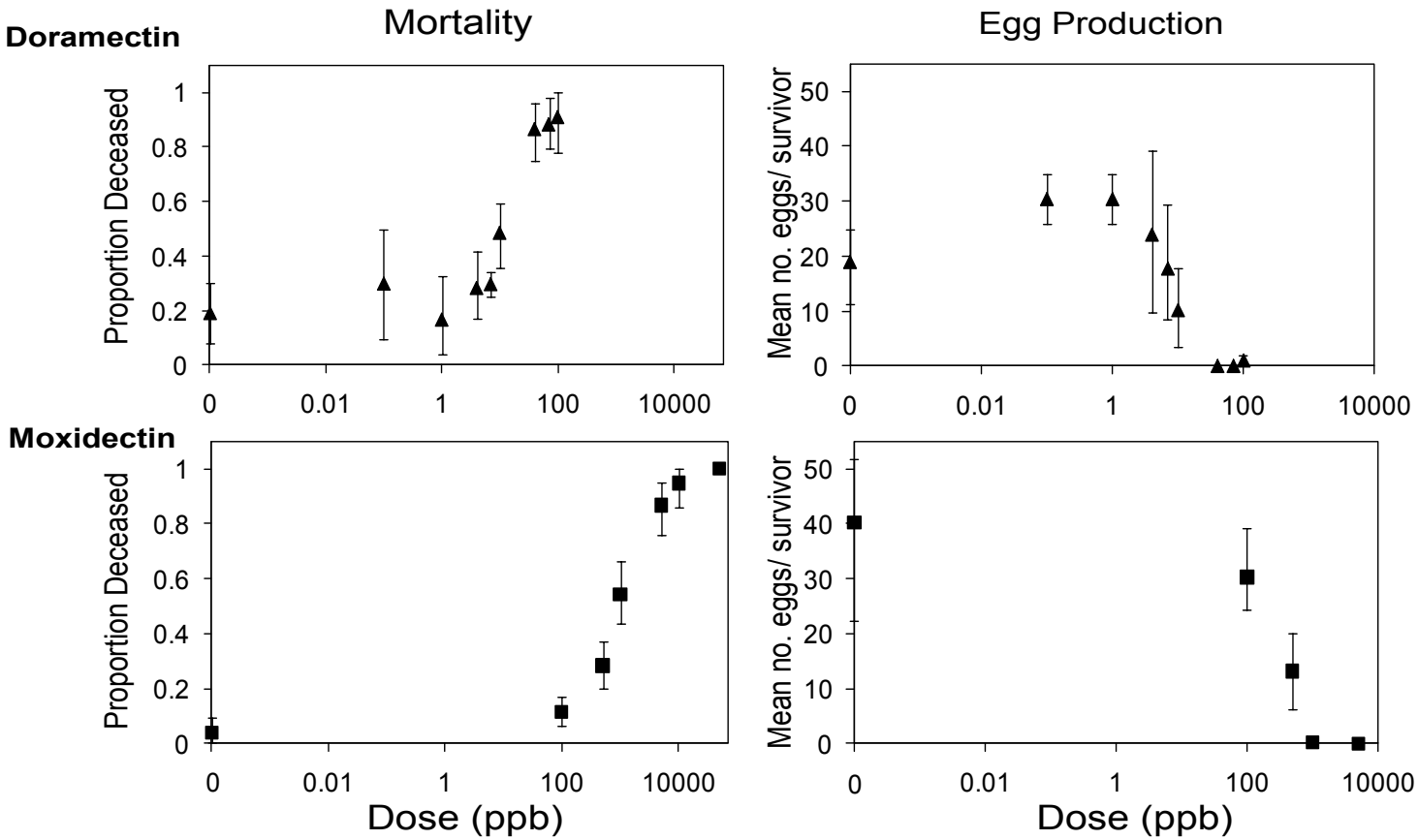

Figure 6.2. Uncorrected mean proportion of *Anopheles arabiensis* deceased (left) and production of eggs by survivors (right) within 9 d of feeding upon doramectin- and moxidectin-treated blood. Error bars represent bootstrapped 95% confidence intervals (N=1000). Doramectin dose-response curve was adjusted using HPLC analysis of final extraction product.

Table 6.3. Mean number of eggs per surviving female with bootstrapped 95% confidence intervals (N=1000). A significant p-value (denoted by *) signifies that increasing the drug dosage significantly reduces egg production in surviving *Anopheles arabiensis*.

Drug	N	χ^2 df	p-value	Dose	Mean no. eggs per survivor (95% CI)
Eprinomectin	38	1	0.6082	0	27.2 (15.5, 39.2)
				0.1	27.0 (20.0, 34.0)
				1	20.4 (12.9, 27.5)
				4	9.3 (2.9, 16.1)
				7	2.2 (0, 4.8)
				10	9.4 (0, 22.2)
Ivermectin	32	1	0.0003	0	25.7 (12.6, 40.6)
				0.1	34.0 (22.0, 45.0)
				1	24.2 (17.4, 33.6)
				4	13.2 (4.2, 24.0)
				7	2.8 (0, 7.8)
				10	0 (0, 0)
Doramectin	35	1	0.0042	0	23.93 (19.07, 30.02)
				0.1	19.0 (11.3, 24.8)
				1	30.5 (26.0, 35.0)
				4	24.0 (9.8, 39.2)
				7	17.8 (8.4, 29.4)
				10	10.2 (3.5, 17.8)
				40	0 (0, 0)
				70	0 (0, 0)
				100	1.0 (0, 2.0)
Moxidectin	26	1	0.0031	0	40.2 (22.4, 51.8)
				100	30.3 (24.5, 39.3)
				500	13.2 (6.3, 20.0)
				1000	0.2 (0, 0.5)
				5000	0 (0, 0)

Table 6.4. Mean proportion of eggs oviposited out of the total number of eggs produced (± bootstrapped 95% CIs; N=1000) by *Anopheles arabiensis* females surviving dosages lower than each drug's LC_{50} compared with respective controls.

Drug	Treatment	n	Mean proportion of eggs deposited (± 95% CI)	t-value	p-value
Eprinomectin	Treated below LC50	144	0.23 (0.06, 0.44)	0.37	0.71
	DMSO only	92	0.16 (0.01, 0.34)		
Ivermectin	Treated below LC50	120	0.52 (0.35, 0.69)	0.07	0.95
	DMSO only	91	0.69 (0.40, 0.93)		
Doramectin	Treated below LC50	155	0.27 (0.16, 0.39)	1.02	0.32
	DMSO only	82	0.32 (0.08, 0.58)		
Moxidectin	Treated below LC50	111	0.08 (0.01, 0.16)	-0.56	0.58
	DMSO only	51	0.05 (0, 0.15)		

those that fed upon blood treated with only DMSO (Figures 6.1and 6.2). While egg production tended to be lower for eprinomectin survivors than for mosquitoes fed upon DMSO-treated blood, this difference was not statistically significant (Table 6.3). *An. arabiensis* fed upon drug-treated *vs*. DMSO-treated blood deposited the same proportion of their eggs (Table 6.4). Therefore, capacity for egg deposition was not influenced by drug treatment.

Discussion

The activity of eprinomectin-treated blood against *An. arabiensis* is equal to ivermectin at low dosages (Table 6.2). It is highly active against *An. arabiensis*, and labeled for use in pregnant and lactating cattle, qualifying it as a potential tool for management of zoophilic malaria vectors. However, plasma availability of eprinomectin is low in zebu Gobra cattle (*Bos indicus*), compared with Holsteins (*B. taurus*) treated at the same rate, though the mechanism for this difference remains unknown (Bengone-Ndong *et al*. 2006). When treated topically at a rate of 200 µg/kg active ingredient (AI), Holstein cattle achieve eprinomectin plasma concentrations of 43-76 ng/mL (Alvinerie *et al.* 1999), while zebu Gobra only reach plasma concentrations of *ca*. 8 ng/mL (Bengone-Ndong *et al*. 2006). The milk/plasma ratio of 0.094 in eprinomectin-treated zebu Gobra (Bengone-Ndong *et al.* 2006) might permit an increase the amount of AI applied to zebu Gobra cattle to increase plasma deposition; the maximum concentration of eprinomectin permitted in milk is 30 ng/mL (Alvinerie *et al.* 1999). The effects of increased eprinomectin exposure on both zebu Gobra cattle health and safety, and meat withdrawal times should be thoroughly examined.

Interestingly, doramectin-treated blood was less lethal to *An. arabiensis* than ivermectin or eprinomectin, but caused egg production to severely decline at concentrations higher than 10 ppb. Thus it functioned well as a sterilant (Table 6.3). Conversely,

eprinomectin-treated blood was highly lethal to *An. arabiensis*, but did not reduce egg production as strongly in survivors. Moxidectin required higher dosages to reduce survivorship and egg production for *An. arabiensis*. These results suggest variation in mosquito target site sensitivity to each of the drugs. Furthermore, drug interaction with the target site, or in the case of doramectin, the target site itself, may vary according to drug. Some insecticides also modulate insect behaviors in addition to their lethal effects (Haynes 1988, Siegert *et al*. 2009). While it was beyond the scope of this study, it would be of interest to know whether, and for how long, the avermectins reduce host-seeking behaviors in mosquitoes that have imbibed sub-lethal doses.

Use of doramectin and eprinomectin is not restricted in pregnant animals, and pregnant and lactating animals respectively, whereas ivermectin has such restrictions. The broader applicability of both eprinomectin and doramectin make them better candidates for malaria mosquito control than ivermectin, against those more zoophilic vectors such as *An. arabiensis* whose primary host is often cattle. Field tests of doramectin- and eprinomectin-treated cattle should be the next step in the investigation of this novel vector control measure. The mass treatment of cattle populations with ivermectin, doramectin, or eprinomectin at the onset of the rainy season, in combination with ITN use, is a promising management technique for zoophilic vectors like *An. arabiensis*.

CHAPTER 7:

HOST UTILIZATION BY *ANOPHELES GAMBIAE S.S.* AND *ANOPHELES ARABIENSIS* IN AN AREA OF HIGH INSECTICIDE-TREATED BED NET COVERAGE IN WESTERN KENYA AS DETERMINED BY REVERSE DOT BLOT, DNA-DNA HYBRIDIZATION

Abstract

A DNA-DNA hybridization technique previously used in sand flies, reverse dot blot analysis (RDBA), was adapted to the *An. gambiae s.l.* system. Blood meal identification for *An. gambiae s.l.* via RDBA was comparable to conventional PCR and direct sequencing techniques. However, RDBA identified mixed blood meals, whereas PCR and direct sequencing could not. Blood fed *An. gambiae s.l.* collected in June and July 2008 in Kisian, Kenya were 58% *An. arabiensis*, and 24% *An. gambiae s.s*. Eighteen percent could not be identified to species via qPCR. Blood meals imbibed by *An. arabiensis* were mostly (>90%) bovine in origin, whereas *An. gambiae s.s.* fed upon humans > 90% of the time. Two *An. arabiensis* of 160 took blood meals from more than one host species. Recent insecticide-treated bed net (ITN) use in Kisian village has likely caused the shift in the dominant vector species from *An. gambiae s.s*. to *An. arabiensis*. We conclude that vector populations will not decline to levels whereby malaria transmission is eliminated until the cattle-mosquito connection is broken. Additional management tools targeting cattle-feeding vectors are badly needed.

Introduction

Host-selection patterns influence transmission in vector-borne disease systems, and increase efficiency of pathogen transmission among host populations when vectors exhibit high host fidelity (McCall and Kelly 2002). Analyzing vector populations for patterns of host-selection achieves several objectives: 1) identifies hosts important to pathogen amplification (Molaei *et al*. 2006a, Hamer *et al*. 2009), 2) determines the relative contribution of vector species to disease transmission (Apperson *et al*. 2004, Molaei *et al.* 2006b), and 3) elucidates seasonal host-use shifts (Edman and Taylor 1968, Kilpatrick *et al.* 2006). Tools used to examine mosquito host-selection include immunological- and DNA-based technologies to analyze the gut contents of mosquitoes post-blood meal (Symondson 2002). Enzyme-linked immunosorbent assays (ELISA) largely replaced the precipitin and related immunological methods to assess host-selection patterns (Beier *et al.* 1988, Scott *et al*. 1993, Rubio-Palis *et al.* 1994, Bogh *et al.* 1998, Diatta *et al*. 2001, Mwangangi *et al*. 2003, Apperson *et al*. 2004, Lefèvre *et al*. 2009). However, immunological methods suffer from several drawbacks including lack of specific reagents, and low sensitivity especially for older blood meals (Symondson 2002). More recently, DNA-based techniques have been successfully used for vector blood meal analysis (Lee *et al.* 2002, Cupp *et al.* 2004, Kent and Norris 2005, Kilpatrick *et al.* 2006, Molaei *et al.* 2006a, Molaei *et al.* 2006b, Hamer *et al*. 2008, Hamer *et al.* 2009, Kent 2009). In many studies (Kilpatrick *et al.* 2006, Molaei *et al.* 2006a, Molaei *et al.* 2006b, Hamer *et al.* 2008, Hamer *et al.* 2009), DNA from the gut contents of mosquitoes is extracted, amplified via PCR, directly sequenced and compared with a reference data-base for host identification. While DNA-based assays are efficient and the results more reliable than those of ELISA, tools used for direct sequencing are not widely available in field laboratories. Additional tools for analyzing host-selection in field settings would be useful.

The mosquitoes *Anopheles gambiae sensu stricto* and *An. arabiensis* are highly important to the perpetuation of the human malaria transmission cycle in Sub-Saharan Africa. While host-selection by *An. gambiae s.s.*(hereafter *An. gambiae*) tends toward anthropophily (White and Rosen 1973, White 1974, Coluzzi *et al.* 1979), and *An. arabiensis* tends toward zoophily (Highton *et al.* 1979, Duchemin *et al.* 2001), regional differences in host-selection by *An. gambiae* (Diatta *et al.* 1998, Bøgh *et al.* 2001) and *An. arabiensis* (Tirados *et al.* 2006, Kent *et al*. 2007), as well as phenotypic plasticity (Lefèvre *et al*. 2009) have recently been described. Vector control measures executed within human dwellings, such as insecticide-treated bed nets (ITNs) preclude human blood meal acquisition (Molineaux and Gramiccia 1980, Gimnig *et al.* 2003, Killeen and Smith 2007), thereby causing *An. gambiae* populations (White 1974, Bayoh *et al*. 2010) and malaria transmission (Lindblade *et al*. 2004) to decline. Some studies have examined the shifts in *An. gambiae* and *An. arabiensis* abundance post-ITN distribution (Bayoh *et al.* 2010) but the influence of ITNs on host-selection by these vectors remains understudied. In the present study, we adapted a novel blood meal analysis tool, reverse dot blot analysis (RDBA), previously used in Phlebotomine sand flies (Abbasi *et al.* 2009) to the *An. gambiae s.l*. system, and examined host-selection in an area of high ITN use. We postulated that the acquisition of human blood meals by *An. gambiae* and *An. arabiensis* following a mass distribution of ITNs in Kisian in 2006, would decline, and the proportion of blood meals taken from non-human hosts would increase. Moreover, we postulated that the diversity of hosts utilized by *An. gambiae* and *An. arabiensis* would increase as human hosts become unavailable.

Methods

Study site

Sampling was conducted in Western Kenya, in the village of Kisian (Nyanza Province), during the months of June and July in 2008. Lindblade *et al*. (2004) previously described the demography, physiography, and geography of this locale. ITNs were owned by *ca.* 68% of households within the sampling area (Hightower *et al.* 2010).

Mosquito collection and species identification

Blood fed mosquitoes were collected indoors from the walls of human dwellings, and outdoors from clay pots (Odiere *et al.* 2007) via mouth aspirator. The clay pots had been placed by local residents to collect roof water and were typically situated near the houses. Mosquitoes were collected on 16 dates from 57 housing compounds. After collection, mosquitoes were frozen, and then morphologically identified as *An. gambiae s.l.* Engorged abdomens were separated from the thorax with a scalpel, then placed individually in vials and dried in a desiccator containing Drierite for transport to Michigan State University. There, DNA was extracted from the mosquito abdomen (DNeasy Tissue Kits; Qiagen, Valencia, CA) using sterile technique. Extracted DNA could be used in the identification of both host and vector depending on the primers used for amplification. To identify whether the vector was *An. gambiae* or *An. arabiensis*, extracted DNA was amplified via quantitative PCR according to Walker *et al.* (2007). Taqman mastermix (Applied Biosystems P/N 4304437) and *An. gambiae s.l.* universal primers (Forward: 5'-GTGAAGCTTGGTGCGTGCT-3', and Reverse: 5'-GCACGCCGACAAGCTCA-3') amplified a *ca.* 150 bp region of the *An. gambiae s.l.* intergenic spacer region (IGR) of sample DNA. Fluorecently-labeled species-specific Taqman probes (*An. gambiae*: 5'VIC – CGGTATGGAGCGGGACACGTA-3', *An. arabiensis*: 5'FAM - TAGGATGGAGAAGGACACTTA) were coupled to a minimum

groove binding ligand and a 3'quencher, and were added to the reaction. Results were identified via spectrophotometer, after the 5' nuclease activity of the Taq polymerase released the fluorescent label, causing it to fluoresce. Amplification and detection were accomplished with an ABI Model 7900 HT workstation (Applied Biosystems, Foster City, California, USA).

Blood meal identification via direct sequencing

Host DNA was initially identified using an established protocol for blood meal identification following Hamer *et al.* (2009). Extracted DNA used in mosquito identification was amplified via PCR, purified and directly sequenced (ABI Prism 3700 DNA Analyzer; Applied Biosystems, Foster City, CA). For host identification, sequences were queried in GenBank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) with a BLAST search. Host identity was accepted for a sample when the following criteria were met: 1) the sample produced an amplicon after PCR, and 2) the sample sequence was matched with >95% similarity according to the BLAST search.

Host DNA amplification with Cyto primer pair

The forward and reverse primers from Abbasi *et al.* (2009) (hereafter, "Cyto" primers) are universal primers that amplify a 344 bp segment in a conserved region of the mitochondrial cytochrome b gene. A biotin group was added to the 5' end of Cyto primer 1 (5' – CCA TCA AAC ATC TCA GCA TGA TGA AA-3') and Cyto primer 2 (5' – CCC CTC ATA ATG ATA TTT GTC TCT-3'), forward and reverse primers respectively. Biotin-labeled DNA hybridized to probes on the membrane could be detected using a Biotin Chromogenic Detection Kit (Fermentas International Inc., Burlington, Ontario, Canada) These primers were examined for *An. gambiae* complex host identification suitability.

Extracted host DNA was amplified in a 100 µl reaction (4 µl DNA extract with 3.3µl of 25 *p*M primer per sample) using the Failsafe PCR system (Epicenter Biotechnologies, Madison, WI), under the following reaction conditions: an initial denaturation step of 5 min at 94°C, followed by 35 cycles at 94°C for 30 sec, 55°C for 30 sec, and an elongation step at 72°C for 1 min. Electrophoresis (E-gel system; Invitrogen, Carlsbad, CA) revealed amplicons that would be submitted for identification via direct sequencing and RDBA. Amplicons were visualized and scored based upon band intensity (0 = no product and 4 = bold product). An aliquot (45 µL) of samples yielding a visible amplicon was purified (QIAquick PCR Purification Kit; Qiagen), submitted for direct sequencing, and identified as above. The remaining 45 µL of amplified and unpurified host DNA was directly subjected to RDBA.

Reverse dot blot analysis

Candidate hosts were selected based upon 1) Organism-specific oligonucleotide probes (Table 7.1) were diluted with Tris-EDTA buffer to a concentration of 5 *p*M/µL, and imprinted onto a nylon membrane in a double line. Probes were covalently linked at the 5' amino modified end to Pall Biodyne B nylon membranes (Nagle Nunc International, Rochester, NY) using a 96 pin high-density replicating tool on a Biomek 2000 robot (Beckman Coulter, Inc.). Printed membranes were baked for 30 min at 80°C to fix the probes.

Membranes were cut into strips containing each probe (Fig. 7.1), and strips placed individually into a 15 mL conical tube. Membrane strips were washed with 3mL of 2xSSC [0.15 M NaCl, 0.015 M sodium citrate, 0.1% sodium dodecyl sulfate (SDS)] solution in preparation for hybridization with DNA samples. Tubes were held at 45°C for 30 min, and gently rocked to allow the prehybridization solution to wash over the membranes. Amplified

Table 7.1. Oligonucleotide probes imprinted upon membrane strips for reverse dot blot procedure.

	Species	Oligonucleotide sequence
1	Bovine 1 *(Bos indicus)*	ATT ATG GGT CTT ACA CTT T
2	Bovine 2 *(Bos taurus)*	ATT ACG GGT CTT ACA CTT T
3	Brown rat *(Rattus rattus)*	CAG TCA CCC ACA TCT GC
4	Goat *(Capra hircus)*	ATA CAT ATC GGA CGA GGT CTA
5	Sheep *(Ovis aries)*	TCC TAT TTG CGA CAA TAG CTT CCT
6	Domestic mouse (*Mus musculus)*	TGG AGT ACT TCT ACT GTT CGC AGT
7	Domestic cat *(Felis domesticus)*	CAT TGG AAT CAT ACT ATT
8	Human *(Homo sapiens)*	ATG CAC TAC TCA CCA GAC GC
9	Domestic chicken *(Gallus gallus)*	CAT CCG GAA TCT CCA C
10	Domestic dog *(Canis familiaris)*	CAG ATT CTA ACA GGT TTA
11	General Avian 2	GCC TCA TTC TTC TTC AT
12	General Avian 1	TAC ACA GCA GAC AC

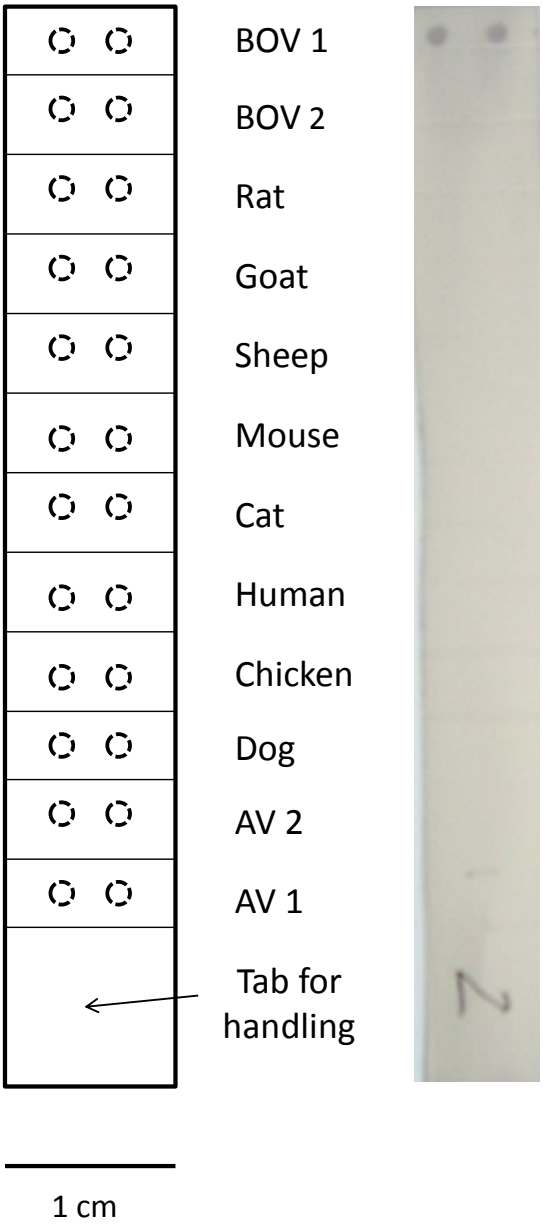

Figure 7.1. Schematic of a membrane strip used for reverse dot blot analysis, including placement of the probes. On the right, a membrane strip used in a test reveals that the unknown host was bovine.

host DNA, suspended in a total volume of 45 μL, was heated to 94°C for 5 min as a melting step, and then added to the conical tube for hybridization. Hybridization of host DNA to the membrane took place at 43°C for 1 hr. Following hybridization, the membrane was washed with 2 mL of 0.7xSSC solution for 20 min at room temperature. A Biotin Chromogenic Detection Kit (Fermentas International Inc., Burlington, Ontario, Canada) was used for visual detection of the results.

Tests of membrane strip accuracy for detecting host DNA were first conducted using DNA samples extracted from 5 μL blood samples of known origin. Blood was also combined from two hosts in proportions ranging from 1:7- 7:1, and DNA was extracted and amplified. Mixed DNA treatments were then subjected to RDBA to determine the sensitivity with which this method detects a mixed blood meal. The remaining 45ul of amplified DNA from unknown samples were identified via RDBA, and a Pearson's chi-squared test was used to compare success rates of RDBA and direct sequencing.

Results

In total, 299 blood-fed anopheline mosquitoes were collected in June and July of 2008. Molecular identification of the species established that *An. arabiensis* comprised 58% of the mosquitoes collected, while 24% were *An. gambiae*. The remaining 18% could not be identified to species, but were morphologically *An. gambiae s.l.* *Culex* spp. were discarded and *Anopheles funestus* was absent. *An. gambiae* were four times more abundant from indoor resting sites than outdoor resting sites, whereas *An. arabiensis* were twice as abundant from outdoor resting sites (Table 7.2).

In 46 of the samples, there was insufficient DNA available for both Reverse Dot Blot Analysis (RDBA) and direct sequencing. In these cases, only blood meal analysis via direct

sequencing was performed. By at least 1 of the 3 methods described above, hosts were identified for *ca*. 75% of the mosquitoes.

Only 3 host animals were fed upon by *An. gambiae* and *An. arabiensis*: humans, cattle, and birds. Nearly eighty percent of the total blood meals identified were bovine. *An. arabiensis* fed upon cattle 50 times more frequently than upon humans, while most *An. gambiae* fed upon humans (Table 7.3).

Identification of visible amplicons by direct sequencing and RDBA were equivalent ($p = 0.327$). Direct sequencing identified the utilized hosts in 147 of the field collected samples, whereas RDBA identified 139. Furthermore, analysis of field samples identified by both RDBA and direct sequencing with Cyto primers always yielded identical results. DNA from mixed blood meals was identifiable by RDBA, even when 1 blood type represented as little as $1/4^{th}$ of the total blood meal (Figure 7.2). Three of the field samples analyzed via RDBA revealed mixed blood meals: 2 *An. arabiensis* fed upon both human and bovine hosts, and 1 unidentified *An. gambiae s.l.* individual fed upon human and avian hosts. The avian host was identified by BLAST to a *Turdus* species.

Discussion

The proportion of *An. gambiae* relative to *An. arabiensis* collected in June and July of 2008 suggests a shift in the predominant *An. gambiae s.l.* complex vectors present in human dwellings. Prior to 1998, *An. gambiae* comprised between > 70% of the total *An. gambiae* complex population in Kisian village (Joshi *et al*. 1975, Service *et al.* 1978, Petrarca *et al.* 1991, Githeko *et al.* 1994), yet comprised < 25% in the present study. Widespread ITN use is likely contributing to this phenomenon, which has been demonstrated in this part of Western Kenya (Bayoh *et al*. 2010).

Table 7.2. Resting sites from which *An.gambiae s.s.* and *An. arabiensis* were collected. Indoor resting sites included the sides and underside of furniture, and the interior walls of houses. Outdoor resting sites were the interior of clay pots used for rain catchment outside of houses. Individuals classified as *An. gambiae s.l.* could not be identified to species level via qPCR.

Species	Resting Site	
	Indoor	Outdoor
An. gambiae s.s.	58	14
An. arabiensis	67	105
An. gambiae s.l.	40	15

Table 7.3. *An. gambiae s.s.* and *An. arabiensis* hosts were identified by at least 1 method of blood meal analysis. Individuals whose blood meals were mixed or avian were classified as "other".

Species	Host	Resting Site	
		Indoor	Outdoor
An. gambiae s.s.	Bovine	2	1
	Human	29	4
	Other	0	0
An. arabiensis	Bovine	49	105
	Human	1	2
	Other	1*	2**
An. gambiae s.l.	Bovine	14	6
	Human	4	1
	Other	0	1***

* *Turdus* spp.
** Mixed bovine and human blood meals
*** Mixed human and avian blood meal

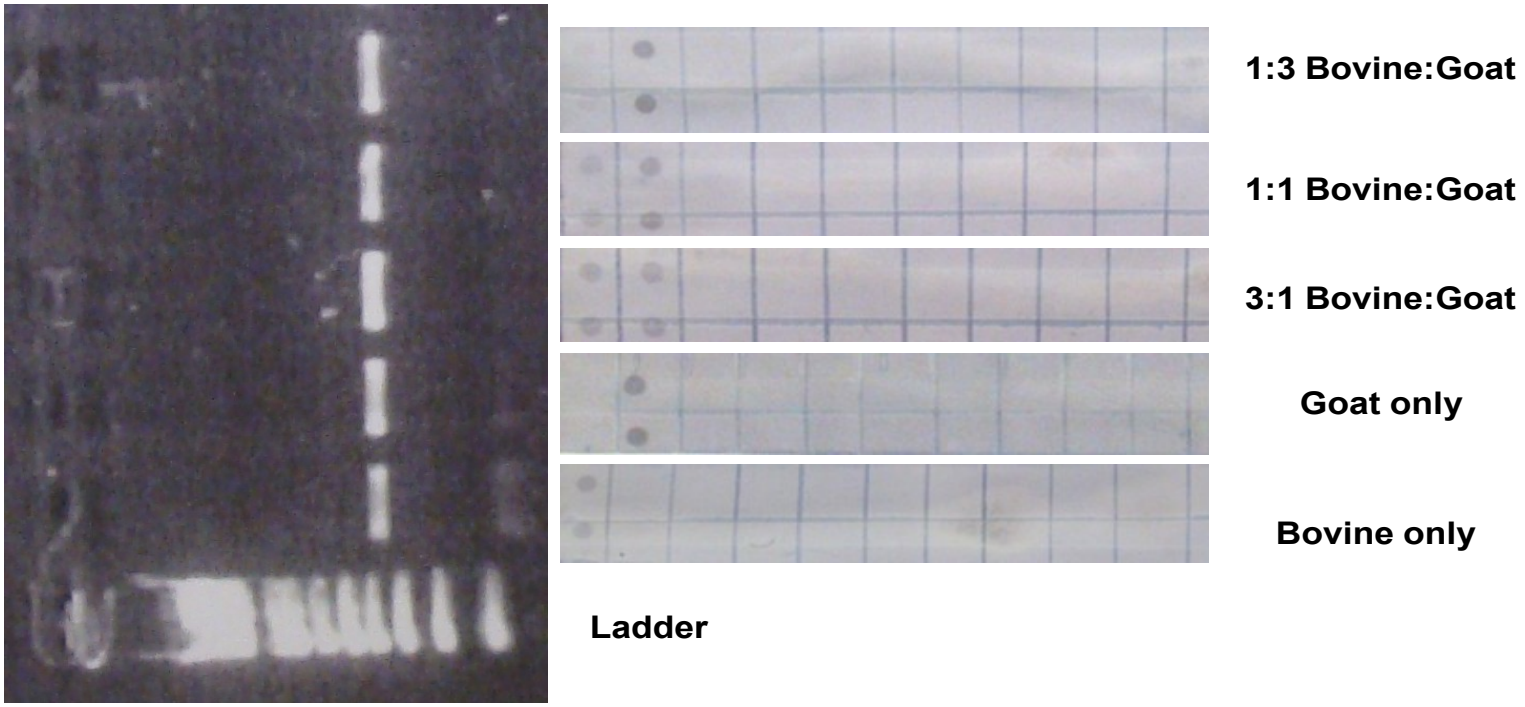

Figure 7.2. Relative visualization of probes in a mixed blood meal based upon the proportion of DNA from each host. Gel bands demonstrate a roughly equal quantity of DNA per samples, yet relative intensity of the RDBA probe varies based upon the proportion of DNA from each host. In this figure, background brightness for some strips was adjusted to create equivalent background brightness across all strips.

Surprisingly, increased ITN coverage does not appear to have shifted host-use by *An.gambiae*. Neither *An. gambiae* nor *An. arabiensis* expanded their host range, despite the reduced availability of human hosts. Over 90% of *An. gambiae* fed upon humans, yet ITNs had been present in nearly 70% of human dwellings since 2006. These results are consistent with those of Fornadel *et al.* (2010), which demonstrated that host-selection of an anthropophilic *An. arabiensis* population remained unchanged in the two years following mass ITN distribution. In Burkina Faso, long-term inaccessibility of human hosts (Robert *et al.* 1991) has led to the evolution of phenotypic plasticity, such that nearly 90% of *An. gambiae* feed upon cattle, though preference for human odor-baited traps is maintained in the population (Lefèvre *et al.* 2009). Perhaps the latency between ITN distribution and the present study, household ITN ownership, nightly ITN use, or a combination of these was insufficient to generate selection pressure for phenotypically plastic host-use in the Kisian *An. gambiae* population.

In the present study, the proportion of blood meals taken from cattle reveals their importance for malaria control. In some areas where ITN use is widespread, the *An. gambiae* population declines, but malaria transmission persists (Bayoh *et al.* 2010). We postulate that *An. gambiae s.l.* populations persist by feeding on cattle when deprived of human hosts, yet feed on humans whenever the opportunity arises.

Additional vector control measures may be necessary to break the cattle-mosquito connection if the goal of malaria eradication is to be achieved. Treatment of cattle with the systemic insecticides, such as ivermectin (Foley *et al.* 2000, Fritz *et al.* 2009), or topical insecticides, such as pyrethroids (Hewitt and Rowland 1999) has been demonstrated as an effective way to reduce zoophilic vector longevity. Further research is necessary to determine whether deprival of both human and cattle hosts in a field setting, using ITNs and cattle treatment, could reduce *An. gambiae s.l.* populations and thereby improve malaria control.

RDBA was as successful at identifying host DNA, as direct nucleotide sequencing, and the two methods consistently returned identical results for our field collected samples. One major advantage of RDBA is that it can detect mixed blood meals, while direct sequencing cannot. While the present study only addresses *An. gambiae* interspecies host-use, questions remain about how mosquitoes interact with, and feed upon multiple human hosts present in a single dwelling. RDBA could be extended to address this question; unique probes could be identified for individuals living in the same household, then imprinted on a membrane strip and used for blood meal identification. While RDBA appears to be an accurate and sensitive blood meal identification tool, it is best suited for systems in which host-use by vectors is restricted to relatively few host species (Abbasi *et al.* 2009). In the current study, the RDBA probes represented potential hosts that were most abundant around human dwellings. Furthermore, direct sequencing did not reveal any additional host animals that could not be detected via RDBA around Kisian village. However, blood meals from vectors with a broad range of hosts are still most accurately identified via direct nucleotide sequencing.

FINAL CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

Part I: Ovipositional flexibility of *An. gambiae*

An. gambiae is highly flexible in its ovipositional behavior. Previous work reveals a lack of stimulatory chemical cues for oviposition. While darkness and wetness are key physical stimuli for gravid females locating an ovipositional resource (Huang *et al*. 2007), *An. gambiae* accept a broad range of moistures, textures, and substrates onto which they deposit their eggs. The present studies demonstrate that *An.gambiae* oviposition peaks at dusk, and to a lesser extent, dawn, yet egg deposition is not restricted to any particular time of day. These ovipositional peaks are attributable to multiple individuals that deposit all their eggs at different hours of the night. Furthermore, oviposition can occur in the middle of the day if gravid females are previously deprived of an ovipositional resource.

Most *An.gambiae* deposit eggs while sitting horizontally on a moist substrate, but gravid females are also capable of flying and perching on a vertical structure near a water source and raining onto the water below. Understanding the ovipositional flexibility of this species is key to understanding its distribution and potential for management. The present, as well as previous studies suggest that *An. gambiae* is an ovipositional generalist, capable of broadcasting eggs over a broad range of aquatic environments (Minakawa *et al.* 1999, Minakawa *et al.* 2004, Huang *et al.* 2005, 2006a, 2006b, 2007, Miller *et al*. 2007, Omlin *et al.* 2007). This behavior would reduce the effectiveness of ovitraps for control of *Ae. aegypti* (Sithiprasasna *et al.* 2003) and other container-breeding mosquitoes. Furthermore, small larval populations may be distributed beyond the small, sunlit pools devoid of vegetation previously described as larval habitats for *An. gambiae*. During the rainy season, daily heavy rainfalls that cause water to flow over the ground surface may carry broadly cast eggs and larvae to low-lying areas where water collects. I speculate that flowing rain water may

facilitate generalistic ovipositional behavior in this important vector species. To date, no study has examined how flowing water during the rainy season influences the distribution of *An. gambiae* eggs and larvae. Further research on this aspect of the ecology of *An. gambiae* is critical to successful management of this important vector species.

Part II: *An. arabiensis* control using eprinomectin

In areas of where ITNs are broadly distributed and used to cover sleeping humans, *An. arabiensis* populations persist. We conclude that Western Kenyan populations of *An. arabiensis* still remain in close proximity to human dwellings, and continue to bite cattle despite *ca.* 70% ITN coverage. Interestingly, in the present studies, we demonstrate that *An. gambiae* and *An. arabiensis* are highly sensitive to the parasiticide, ivermectin. Although ivermectin-treated blood significantly reduces survivorship and fecundity of *An. gambiae s.l.* that feed upon it, strict milk and meat withdrawal times, as well as restrictions against its use in pregnant animals preclude widespread cattle treatment for malaria control under current registration guidelines. The present studies demonstrate that eprinomectin is an acceptable alternative to ivermectin, causing equivalent lethality in *An. arabiensis* that feed upon it. To determine the feasibility of this promising vector control measure, investigation of eprinomectin-treated cattle and their effects on *An. arabiensis* should expand to the village level. *An. arabiensis* population growth could be quantified in villages where cattle were treated with eprinomectin at the onset of the rainy season and compared to *An. arabiensis* population growth in villages with untreated cattle. If used in combination with ITNs, I suggest that eprinomectin could significantly reduce vector abundance. The results generated from such research would be important not only to the scientific community, and our understanding of vector control, but could also have life-saving consequences for people living in malaria endemic areas.

APPENDIX

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.: 2011-03

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

INVESTIGATIONS OF THE AFRICAN MALARIA MOSQUITO (*ANOPHELES GAMBIAE S.L.*, DIPTERA: CULICIDAE): OVIPOSITIONAL BEHAVIOR AND TOXICITY OF AVERMECTINS

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

Entomology Museum, Michigan State University (MSU)

Investigator's Name(s):

Megan L. Fritz

Date: August 12, 2011

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

APPENDIX 1.1
Voucher Specimen Data

Investigators Name: Megan L. Fritz
Date of deposition: August 12, 2011
Voucher No. 2011-03

The following specimens have been received by the Michigan State University Entomology Museum.

No. specimens:	Other		
	Adult males	10	
	Adult females	10	4
	Pupae		
	Larvae		
	Eggs		
	Label data for specimens collected or used and deposited	MSU colony	MSU colony
	Species or other taxon	*Anopheles gambiae* sensu stricto	*Anopheles arabiensis*

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