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QUANTITATIVE BEHAVIORAL ANALYSES OF THE INTERACTIONS OF ANOPHELES GAMBIAE S.S. GILES (DIPTERA : CULICIDAE) WITH INSECTICIDE-TREATED BEDNETS

presented by

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Date

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By

Fred Anangwe Amimo

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ABSTRACT

QUANTITATIVE BEHAVIORAL ANALYSES OF THE INTERACTIONS OF *ANOPHELES GAMBIAE* S.S. GILES (DIPTERA; CULICIDAE) WITH INSECTICIDE-TREATED BEDNETS

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Direct behavioral observations were conducted on *Anopheles gambiae* s.s. females interacting with Permanet-, Olyset- and Netprotect-Treated BedNets (ITNs). Conditions progressed in complexity from small static cages and a wind tunnel with a fully-untreated, fully-treated, and half-treated cage and screen nettings, respectively, to a house-scale four-choice arena in Western Kenya, fitted with treated vs. untreated bednets. In laboratory tests, each female mosquito was introduced in the arena during the last 2 h of photophase and observed continuously for 30 min; frequencies, durations, and locations of behavior were recorded using the Observer software (Noldus, http: www.noldus). Two hundred 2-3 day old female mosquitoes were released in a central courtyard of the house arena and their room occupancy, as well as 8 h and 24 h mortality was recorded. In the small arenas all ITNs elevated flying behavior. Mosquitoes showed no evidence that they could detect insecticide from a distance or avoid approaching it. In the wind tunnel they flew upwind with or without insecticide in the plume of host cues. Mosquitoes occupied rooms equally with or without treated nets. Both 8 h and 24 h mortality was significant. Overall, this research demonstrated that the mode-of-action of ITNs is lethality, not avoidance.

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CHAPTER 1

Bednets as a Tactic for Malaria Suppression: Literature Overview

Impact of Malaria in Africa

Malaria, HIV AIDS, tuberculosis, and childhood diarrhea are the four most important infectious diseases of humans worldwide (USAID 1997; Molyneux 2004). Over 70% of the world's 3 billion people live in the ca. 100 countries where malaria is endemic (Figure 1.1), and 18% live in epidemic-prone zones. An estimated 300-500 million clinical cases of malaria are reported annually; 90% of them occur in Sub-Saharan Africa (WHO 2000). Here, case fatality rates can be as high as 40%, and the annual continental death rate is typically 1.5 to 2.7 million.

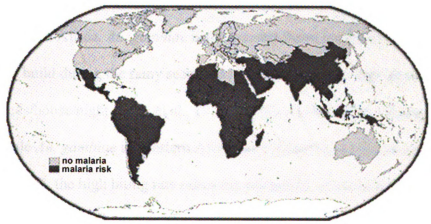


Figure 1.1. Global distribution of malaria risk. Note: Sub-Saharan Africa experiences the greatest risk of malaria epidemics, as well as the highest death rates. Source of Map: WHO.

Beyond human deaths, illness due to malaria impedes economic development. Per-annum estimated costs for Sub-Saharan Africa alone range from $3-12 billion and represent a drag on economic growth by as much as 1.3% each year (Gallup and Sachs 2001). Sachs and Mallaney (2002) observe that malaria prospers most were human societies prosper least, i.e., malaria is a correlate of poverty.

Mosquito Vectors of Malaria

All four known human malaria parasites, *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*, are transmitted between humans by female *Anopheles* mosquitoes (WHO 1983). Newly emerging adult mosquitoes are always parasite-free; they acquire malaria parasites only after feeding on the blood of infected humans. In high-burden areas of Africa, *P. falciparum*, the most dangerous species of human malaria, causes the majority of infections and the majority of deaths. The mosquitoes, *Anopheles gambiae* Giles and *An. funestus*, are the most efficient and prevalent vectors of malaria throughout Sub-Saharan Africa. *An. gambiae* is the greatest threat because its populations can quickly build during the rainy season to resting densities as high as several hundreds/house/night (Joshi et al., 1975; Lindsay et al., 1998). Although only about 3% of female *An. gambiae* in Western Africa have infective oocysts at a given time (Fabian et al., 2005), the high biting rate raises the probability of malaria infection to near certainty after just a few days of normal human exposure during the rainy season. Mosquitoes can easily enter and exit houses because screening of house eve-openings (Figure 1.2) necessary for heat dissipation via ventilation is unaffordable. Moreover, female anophiline mosquitoes can live for six weeks or more (Oketch et al., 2003). It is well known that vector age is highly and positively correlated with vectorial capacity

(Bockarie et al., 1995). In epidemic-prone zones, the combination of a dangerous pathogen efficiently vectored by high and widely distributed populations of long-lived mosquitoes results in nearly ubiquitous exposure of humans to malaria parasites. Tragically, ca. 1 million African children, mostly under 4 years of age, succumb to malaria each year (WHO, 1995).

Figure 1.2. Interior of a house typical of the Luo tribe near Kisumu, Kenya. The *ca.* 30-cm high opening at the top of the walls and under the roof overhang is continuous around the circumference of these houses. It offers mosquitoes unencumbered entrance into and exit from houses.

Tactics for Malaria Treatment and Suppression

The most common form of malaria remediation has been the administration of anti-malaria drugs to sick patients. Beginning with quinine, a long series of different pharmaceuticals has been employed as malaria treatments and prophylactics. Drug

shifting is necessitated by development of resistance by the parasite in given geographical regions (Caraballo and Rodriguez-Acosta, 1999). Currently the drugs sulfadoxine-pyrimethamine and artemisinin and its derivatives are widely used in Africa. The most prevalent prophylactics taken by visitors to high-risk areas are malarone and doxycycline; both are intended for temporary usage.

Widespread and on-going administration of anti-malaria drugs to malaria-endemic regions could theoretically reduce malaria incidence dramatically. However, this practice has not been adopted because: a) there are concerns that all effective drugs could be lost due to wide-spread selection for resistance, b) pharmaceutical companies have not shown an interest in manufacturing the necessary quantities of drugs, probably for monetary reasons, and c) the world has not rallied the political and economic will to commit to such an large-scale and continuing humanitarian endeavor.

Considerable research has been devoted to development of vaccines against malaria (Webster and Hill, 2003). Although various formulations have reached large-scale field tests, none has proven practical to date. The weak link has been that immunity lasts for a maximum of several months. Thus, the human population targeted for protection must be vaccinated repeatedly. This task has been deemed unrealistic due to the immense cost. Nevertheless, the search for better vaccines continues.

Encouragingly, anti-vector tactics can reduce human suffering and deaths from malaria. In principle, such tools should be inexpensive and implemented in a sustainable strategy with a public health rather than medical-treatment mentality. The first such successful program in Africa commenced in 1940s and it involved regular sprays of interior house-walls with DDT (Musawenkosi et al., 2004). This program ran for over 10

years and reduced malaria incidence and human suffering appreciably. Moreover, the environmental damage associated with this particular DDT application was low because of containment within the dwellings. However, this program was terminated before it reached its lofty goal of global malaria eradication (Sachs and Malaney, 2002). Key reasons were: a) stigma associated with use of DDT and other persistent insecticides, and b) the socio-economic retrenchment of Great Britain and other world leaders during the 1970s. Interestingly, house-sprays with DDT are being revisited (Curtis, 2002), this time driven by African rather than foreign governments.

Use of bednets for physical protection of humans from mosquito bites has developed over the past three decades as the most-favored anti-vector tactic for developing countries. The practice of fully surrounding the beds of sleepers with netting so as to preclude visits by insects has been known since the 6th Century BC (Lindsay and Gibson, 1988). However, the perceived potential for this approach rose dramatically in the 1930s with the realization that bednet fabrics could be safely treated with highly effective chemical insecticides. If Insecticide-Treated-bedNets (ITNs) (Figure 1.3) were to kill most mosquitoes alighting upon them, the benefits of such a lethal trap might extend to other sleepers in the house not under a net, or even to unprotected persons in nearby houses. This would reduce the density and mean age of local populations of mosquito vectors (Magesa et al., 1991), thus reducing vectorial capacity (Gary and Foster, 2001). ITN programs in epidemic-prone zones have become the center-piece of current malaria-suppression efforts, e.g., the global partnership under the Roll Back Malaria (RBM) program (Nabarro and Tayler, 1998). Results of such programs will be summarized following an introduction to ITN technologies.

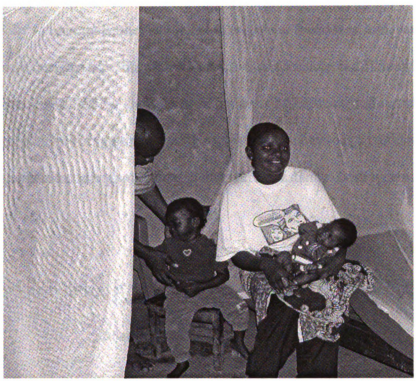


Figure 1.3. Insecticide-treated-bednets (ITNs) hanging over two different beds in a Western Kenyan home. A priority of ITN implementations is malaria-protection for young children and pregnant mothers; malaria can be transmitted *in utero*. The source of picture was CDC.

Pyrethroid Insecticides Incorporated Into ITNs

Pyrethroids are a group of stable synthetic insecticidal compounds, equivalent of the natural insecticide, pyrethrum, which are structurally derived from pyrethrins, naturally found in the flowers of *Chrysanthemum cinerariaefolium*. The first pyrethroid insecticide, allethrin, was synthesized in 1948 following the structural elucidation of six major insecticidal constituents in 1920s and 1930s (Casida, 1980) (Figure 1.4). Currently there are over 42 such compounds of commercial interest; they are either stereoisomers

or differ totally in their chemical structures (NPTN, 1998). While these insecticides have low toxicity to mammals, they are lethal to insects at low doses, and, toxicity increases as temperature decreases i.e. they have a negative temperature coefficient for toxicity (Chang and Plapp, 1983). Their mode of action is by blocking sodium channels so as to produce repetitive firing of neurons. Cross-resistance between pyrethroids and DDT has been demonstrated using some insects that were resistant to both types of chemical.

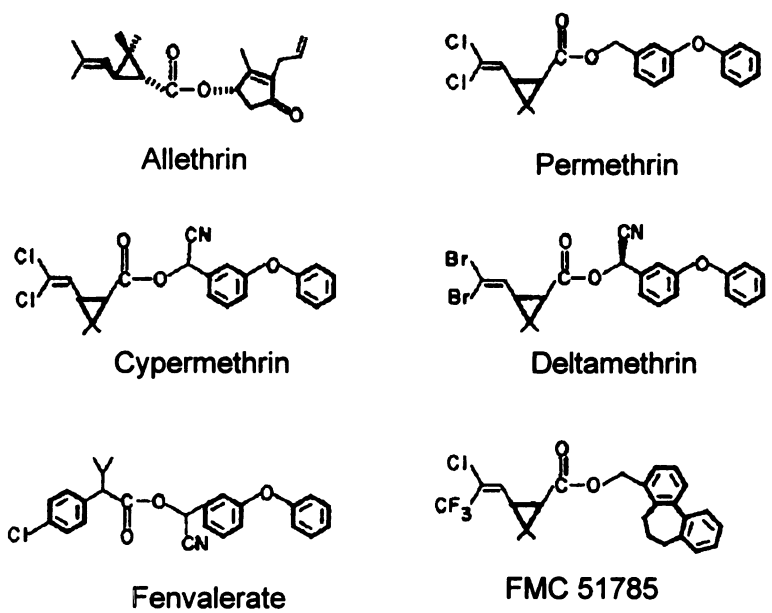


Figure 1.4. Chemical structures of six pyrethroid insecticides.

This has been attributed to insensitivity of the nervous system, a mechanism which is referred to as kdr (knockdown resistance) (Scott and Matsumura, 1981; Dong et al., 1998). Based on chemical structure, pyrethroids have been divided into Types I and II.

Type I pyrethroids (for example, permethrin, tefluthrin, and allethrin) do not contain an alpha-cyano group in their molecule. Their action is characterized by restlessness, incoordination, and inactivity in insects, and these insecticides cause tremors in mammals (T-syndrome). Type II pyrethroids (for example, deltamethrin, cypermethrin, and fenvalerate) contain an alpha-cyano group and produce ataxia, convulsions, hyporesponsiveness in insects and choreathetosis and salivation in mammals (CS-syndrome) depending on the animal model used (Aldridge, 1990; Tordoir et al., 1994; Soderlund et al., 2002). However, some toxicologists would rather not consider any given pyrethroid as Type I or Type II, but look at them as existing along a continuum, with certain ones eliciting primarily a Type I response (e.g. allethrin, permethrin) and others like deltamethrin, eliciting a Type II response.

Pyrethroids have been extensively employed for agricultural pest control. Recently, they have also played a major role in vector-control programs where over 520 tons of active ingredients are utilized annually on a global scale (Zaim & Jambulingam, 2004). This growing usage in public health is attributed to their high insecticidal potency at low dosages, rapid knockdown effects, and their relative safety for humans. Typical LD_{50} for vertebrates are 250-4000 mg/kg body weight. Unlike many other insecticides, pyrethroids do not significantly accumulate in the human body; 90 % of administered dosage is usually excreted in urine and faeces in the first week after exposure (Aldridge, 1990; Vijverberg & van den Bercken, 1990; IPCS, 2000b). However, when pyrethroids were introduced in the early 1980s in China, cotton growers who handled these compounds without taking precautions experienced deltamethrin and fenvalerate

poisoning (WHO, 1990). Since 1988 no clinical cases of occupational pyrethroid poisoning have been reported.

ITNs are impregnated with the pyrethroid insecticides deemed suitable under the recommendation of the WHO Pesticide Evaluation Scheme (WHOPES). So far, six pyrethroids have been so recommended: alpha-cypermethrin, cyfluthrin, deltamethrin and lambdacyhalothrin (alpha-cyano pyrethroids), and etofenprox and permethrin (non-cyano pyrethroids) (Hougard et al., 2003).

Initially, bednets were treated by the users, who steeped untreated nets in a solution of local water in which was dissolved tablets of an emulsified formulation of pyrethroid provided with an untreated bednet. Such formulations lasted only about 6 months and required regular re-treatment (frequently not done as prescribed). Impregnating the netting with insecticides during manufacturing was a major advance in this technology. The major steps for incorporating pyrethroids into netting in factory manufacture include: running suitable netting through an aqueous bath containing an emulsified formulation of pyrethroid, removing excess liquid by squeezing the fabric between rollers at a prescribed pressure, and quickly drying the treated fabric at a prescribed temperature. The treated netting is then cut into pieces and sewn into rectangular or tent-like ITNs of various sizes, the most common of which covers what westerners refer to as a "single bed." The current, most widely marketed factory-manufactured ITNs are Olyset™ (1 g permethrin/m^2 polyethylene fiber; Sumitomo, Inc.) and Permanet™ (55 mg wash-proof deltamethrin/m^2 polyester fabric; Vestegaard Frandsen, Inc.) (WHO, 2000; N'Guessan et al., 2001). These bednet brands are termed "long-lasting" because they are reputed to kill mosquitoes at sustained levels for more

than 3 years. However, even these ITNs eventually need to be re-treated or replaced, as any net becomes worn and tattered with constant use.

Tests of ITN Safety and Efficacy

ITNs were tested as a malaria-intervention tool throughout the 1980s. Initially, their safety for humans was assessed by longitudinal trials overseen by WHO. From the mid-1980s, clinical trials were carried out to assess ITN impact on malaria incidence. In a late 1980s study in a Gambian village where all nets were treated with permethrin, a remarkable 72% reduction in malaria parasitaemia was realized. Based on this encouraging outcome, field tests were extended to four large WHO/TDR trials (Lengeler, 2000) in Burkina Faso, Ghana, Kenya, and The Gambia, again with positive outcomes. The results led to the Abuja declaration at the Africa Summit on Roll Back Malaria (RBMCP 2000) calling for high levels (> 60%) of bednet coverage in malaria-prone regions. This goal was soon achieved in selected sites, commencing with a notable social-marketing program in an area of high malaria transmission in Tanzania (Schellenberg et al., 2001).

Further documentation of ITN efficacy was forthcoming. A series of randomized, controlled trials in four large areas of stable malaria transmission in The Gambia, Ghana, Burkina-Faso, and Kenya in the 1990's demonstrated a 20 - 25% reduction in infant deaths and improvements in the overall health of children (D'Alessandro et al., 1995; Binka et al., 1996; Nevill et al., 1996; Habluetzel et al., 1997). An average of 6 lives per 1000 children was reported to be saved in the age group 1-59 months each year (Lengeler 2000). Considering that there are about 80 million children under the age of five in Africa, over 400,000 deaths could be prevented annually. In a further trial conducted in

Coastal Kenya in the early 1990s, heavy ITN implementation halved the number of episodes of clinical malaria and cases of severe malaria arriving at the coastal hospitals (Nevill et al., 1996).

A notable ITN study anchored by the USA Centers for Disease Control has focused on long-term ITN effectiveness and impact at the community level. This Western Kenyan study site is located near the city of Kisumu (Figure 1.5), a region notorious for holoendemic malaria. Approximately 120,000 people have been under permethrin-impregnated bed nets in a 500-km^2 area centered on Asembo and Gem. Key outcomes relating directly to malaria were that transmission was reduced by over 90% in intervention villages (Gimnig et al., 2003a). Cross-sectional and longitudinal studies of children enrolled in the study demonstrated that children had a 19%, 39% and 44% reduced incidence of parasitemia, anemia, and clinical malaria, respectively (ter Kuile et al., 2003a). Furthermore, infants residing in intervention villages experienced better height and weight gain (mean difference in Z-score = 0.36) compared to those residing in control villages (ter Kuile et al. 2003b). All-cause mortality in children less than 5 years was reduced by 20%. This effect was strongest in children less than 1 year with an estimated 34 lives saved per 1000 infants protected by permethrin-treated bed nets (Phillips-Howard et al., 2003b).

Entomological evaluation during this experiment was paired with laboratory and behavioral studies to assess differences in the effects of bednets on different mosquito species and to assess the importance of prompt re-treatment and actual use of bednets each night (Gimnig et al., 2003a). The proportion of *An. gambiae* s.s. (feeds mainly on humans) relative to *An. arabiensis* (feeds mainly on cattle) was higher in ITN - villages

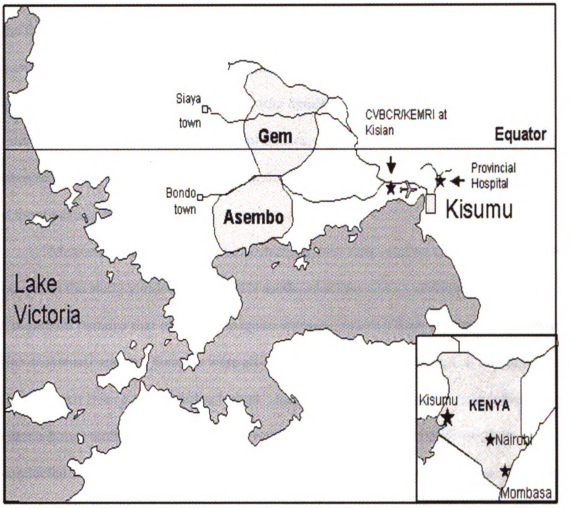
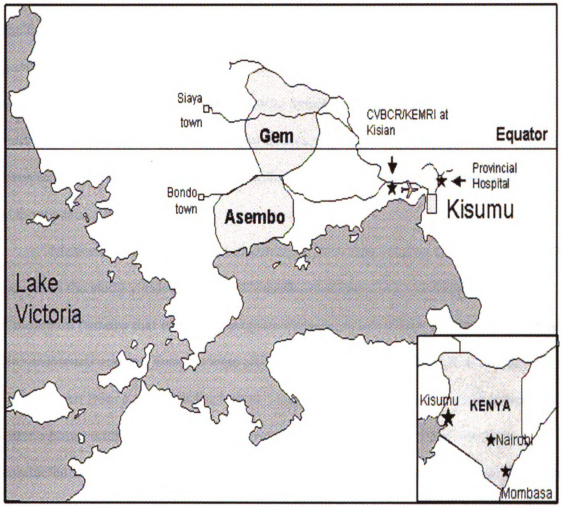


Figure 1.5. Location of a long-running CDC and Michigan State University ITN study site relative to the location of the city of Kisumu. Kenya. This map shows the Center for Vector Biology and Control Research / Kenya Medical Research Institute in Kisian where most of the research reported in this dissertation was conducted. This map was obtained courtesy of the U.S.A. Centers for Disease control.

(55 %) than control villages (45 %). This shift is most parsimoniously attributed to a steeper reduction in *An. gambiae* than *An. arabiensis* populations in the ITN-zone. An.



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funestus populations were suppressed even more than *An. gambiae* populations because the former species was much more susceptible to permethrin than the latter. For *An. gambiae*, bednets were most effective when re-treated every 6 months and when used every night. For *An. funestus*, indoor resting densities were consistently 85-90% lower in intervention houses compared to control houses, regardless of whether the bednets had been retreated within the previous 6 months or properly deployed the night before (Gimnig et al., 2003a).

Measuring mosquito spatial distributions over time relative to ITN- vs. non-ITN houses in the study yielded clues to ITN mode-of-action when combined with use of Colombian curtains that estimate mosquito entrance and exit from houses. *An. gambiae*, *An. arabiensis* and *An. funestus* were all less likely to obtain a blood meal and more likely to exit from a house with a bednet. Additionally, *An. funestus* was less likely to enter a house with a bednet (Mathenge et al., 2001). All-night biting collections conducted in intervention and control villages yielded little evidence for a shift in biting times of either *An. gambiae* or *An. funestus* (Mathenge et al., 2001).

In addition to demonstrating significant personal protection afforded to bednet users, spatial analyses of the CDC data supported a community-wide benefit from bednets. Fifty eight per cent fewer anophelines were collected in houses within 300 m of an intervention relative to a control village. The suggested interpretation was that presence of bednets suppressed mosquito populations sufficiently to reduce biting pressure on occupants of nearby unprotected houses, and that this phenomenon could occur over appreciable distances (Gimnig et al., 2003b). Spatial analysis of child morbidity and mortality provided further support of this hypothesis. A strong protective

effect (Relative change in Odds ratio of 7.1,7.8,11.7,20.0 and 4%) was detected in control houses lacking bednets but located within 300 meters of intervention villages for child mortality, clinical malaria, moderate anemia, high-density parasitemia, and hemoglobin levels respectively (Hawley et al., 2003a). This "community effect" on nearby houses without nets was nearly as strong as the effect observed within villages with bednets. Together, these results indicate that, for optimal efficacy, insecticide-treated bednets must cover a high proportion of households, be retreated at adequate intervals, and be deployed every night (Hawley et al., 2003b).

Collectively, the case is compelling for widespread implementation of high-density ITN use throughout Sub-Saharan Africa and beyond. ITNs, as they exist today, have the capability for saving millions of human lives. Moreover, this life-saving potential of ITNs should not be squandered.

Concerns about Resistance of Mosquitoes to ITNs

The hope of any pest management program is that the tools effecting pest suppression will remain effective over time – i.e. sustainability. History has repeatedly taught entomologists that continual use of a single control tactic (e.g. a given insecticide or class of insecticides) is likely to soon lead to resistance in the pest population, be it metabolic, target site modification, or behavioral. Thus, despite the confidence with which ITN programs are being implemented in developing countries, forethought and planning need to be given with respect to how to manage ITN use to maximize sustainability.

Resistance to pyrethroid insecticides is already well-known in insects. It can occur via mutations in the sodium channel, the target site of these insecticides (knockdown resistance or *kdr*) (Dong et al., 1998; Liu et al., 2002), and increased rates of insecticide

detoxification due to up-regulation of certain detoxifying enzymes such as cytochrome P450s (Hemingway and Ranson, 2000). Alternatively, the pyrethroids incorporated into ITNs might cause mosquitoes to begin avoiding treated surfaces or treated houses, perhaps at the expense of unprotected sleepers. It is not out of the question that blood-feeding by mosquitoes capable of detecting and avoiding ITNs could shift from night-time to the evening and morning, when occupants of houses are not under ITNs. However, one study (Mathenge et al., 2001) failed to detect this effect. Several authors have already sounded cautions about evolution of resistance of *Anopheles* mosquitoes to pyrethroids (Snow et al., 1997; Smith et al., 2001). Lines (1996) argues that resistance studies should be a research priority, whether field-resistance is high or not. Moreover, foci of pyrethroid resistance are known to occur in *An. gambiae,* in both East and West Africa (Vulule et al., 1996; Chandre et al., 1999), and in *An. funestus* in South Africa (Hargreaves et al., 2000). Data from experimental huts documented that pyrethroid-resistant mosquitoes from West Africa were less irritated by pyrethroids, and hence tended to acquire a higher dose of insecticide than the susceptible insects (Chandre et al., 2000). Somewhat counter-intuitively, mortality was higher in the resistant population under exposure to pyrethroid-treated surfaces. Scientists at WHOPES (WHO Pesticide Evaluation Scheme) have recognized the potential for evolution of mosquito resistance to ITNs and have offered bi-treated bednets (two different types of insecticides on differing parts of one bednet) and alternative pyrethroids such as bifenthrin as solutions to resistance development (Guillet et al., 2001; Hougard et al., 2003). However, before such measures are taken to side-step development of metabolic and target site resistance, it seems important that the possible and actual modes of action of ITNs be delineated.

Possible ITN Modes-of-Action and Means for Distinguishing among Them

Surprisingly little is settled about the mechanisms whereby ITNs actually protect human sleepers. Few researchers have focused on the detailed behaviors of mosquitoes as they interface with ITN surfaces. The possibility initially favored by researchers in this field was that ITNs enveloping human sleepers act as lethal traps. The chemical (CO_2 and body odors) and physical (heat) cues emanating from houses and humans were envisioned to attract hungry mosquitoes into houses and onto the treated netting adjacent to a sleeper. After spending sufficient time on treated netting, it was assumed that enough toxicant would be absorbed for knock-down (falling over due to inability to maintain normal body stance) and kill soon thereafter. Some mosquitoes might recover from knockdown, but their lifespan might be subsequently shortened by the stress of the encounter with toxicant. Indeed, death and shortening of life-span is evident when mosquitoes are confined within small plastic cones and forced to sit mainly on insecticide-treated netting (WHO, 1983). However, evidence for ITN lethality to mosquitoes in houses under normal circumstances is sparse to non-existent, probably because of: a) the difficulty in visually detecting dead mosquitoes on the normally cluttered house floors, and b) presence of scavengers like ants that can remove dead mosquitoes.

An alternative class of explanations for ITN mode-of-action is that presence of insecticide-contaminated surfaces alters mosquito behavior so that lethality is avoided. Furthermore, sleepers in a house might experience some behaviorally-mediated protection, even if they are not under a bednet. Because the literature in medical entomology is fraught with ambiguities and miss-uses of the terminologies elsewhere

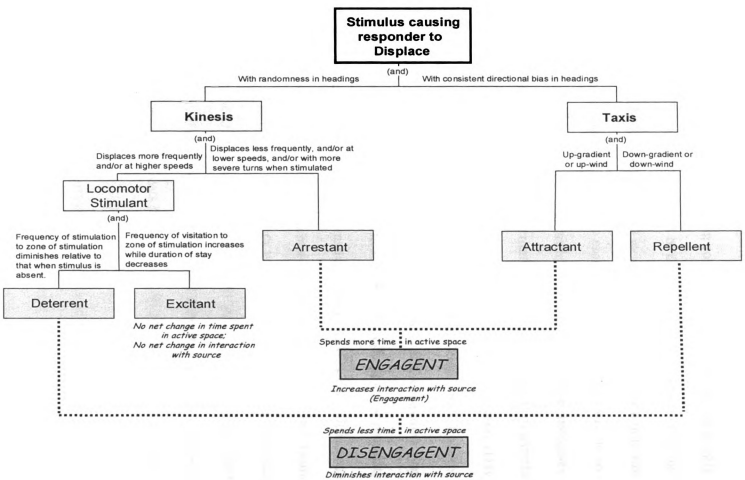
codified by formal behaviorists, we offer a new set of behavioral terms and definitions for use in medical entomology (Figure 1.6). These terms and definitions will be used consistently throughout this dissertation. Insofar as possible, the non-standard and sometimes confusing terminologies often used by mosquito researchers will be translated into the terms of Figure 1.6.

As specified in Figure 1.6, there are diverse mechanistic possibilities whereby behavioral effectors like the insecticides in ITNs might influence mosquito locomotory behaviors. They range from simply increasing the frequency of un-directed behaviors normally expressed in the non-stimulated state (locomotor stimulant), to highly directed behaviors, e.g., consistently navigating away from the source of stimulation (repellent). The terms and definitions in the top half of Figure 1.6 are consistent with precedents set by Dethier (1960) and Kennedy (1977). Overall outcomes are indicated in the bottom half of our scheme. The novel terms *avoidant* and *engagent* are proposed for effectors that decrease and increase interactions of responders with the source of stimulation, respectively. Use of these inclusive, results-based terms is recommended when the end-result of stimulation is known, while the actual mechanisms mediating that outcome are unknown. Conversely, we recommend that the mechanistically specific terms be used only when evidence is obtained that they apply.

ITN Behavioral Effects on Mosquitoes

Although not yet offering mechanistic explanations as precise as those invited by Figure 1.6, there is mounting evidence that ITNs elicit the spectrum of effects represented in the left half of this diagram. For example, Lines et al., (1987) and Snow et al., (1987)

Figure 1.6. Expansion of Dethier et al.'s (1960) terminology for effects of chemicals on the locomotor behaviors of insects, now structured to show the relationships among various types of effectors. The upper part of this figure having Arial font and solid lines refers to movement mechanisms (causes) and their measurable characteristics. The lower part of this figure having dotted lines and *Comic Sans font* indicates behavioral outcomes (effects). In the interest of simplicity, This scheme does not cover all theoretically possible mechanisms and outcomes. Movers are expected to shift mechanisms through time, as well as in response to varying stimulus sets. Active space refers to the area or volume where the concentration of stimulus is detectable by the mover.


Stimulus causing responder to Displace
(and)
With randomness in headings
With consistent directional bias in headings
Kinesis
Taxis
(and)
(and)
Displaces more frequently and/or at higher speeds
Displaces less frequently, and/or at lower speeds, and/or with more severe turns when stimulated
Up-gradient or up-wind
Down-gradient or down-wind
Locomotor Stimulant
(and)
Frequency of stimulation to zone of stimulation diminishes relative to that when stimulus is absent.
Frequency of visitation to zone of stimulation increases while duration of stay decreases
Arrestant
Attractant
Repellent
Deterrent
Excitant
No net change in time spent in active space;
No net change in interaction with source
Spends more time in active space
ENGAGENT
Increases interaction with source
(Engagement)
Spends less time in active space
DISENGAGENT
Diminishes interaction with source
(Avoidance)

suggest that ITNs function by driving visiting mosquitoes out of houses via physical irritation. By our interpretation, such irritation could increase house-exiting simply by increasing non-directed locomotion (excitant or deterrent). By analogy, increasing the temperature of a gas in a leaky container will increase gas exit, albeit by a non-directed mechanism. Molecules of the gas continue to move randomly, but, they will do so with greater velocity. Hence, their probability of hitting a gap in the container increases relative to slower-moving molecules. Such irritative or excitatory effects of pyrethroids, along with responses causing dispersal are widely recognized in the literature (Davidson 1953; Mouchet & Cavalie, 1961; Lockwood et al., 1984; Threlkeld, 1985; Roberts and Andre 1994; Chareonviriyaphap et al., 1997; Rutledge et al., 1999; Chareonviriyaphap et al., 2001; Roberts et al., 2002), Attempts at measuring these effects are appropriately included in standard bioassays of mosquito responses to ITNs (Coluzzi 1963; Rachou et al. 1963; Shalaby 1966, WHO 1970; Bondareva et al. 1986; Pell et al., 1989; Quinones and Suarez 1989; Ree and Loong 1989; WHO 1998). More precise research is needed to distinguish whether these effects represent only locomotor stimulants, or also deterrents (reduce the rate of return of responders to the zone of stimulation) or true repellents. We also need to know whether such effects involve only contact stimuli, and/or odors (volatiles).

Recent field research using arrays of experimental huts housing sleepers under ITNs has shifted prevailing notions of ITN mode of action from the lethal-trap hypothesis to avoidance. For example, Miller et al., (1991) reported that ITNs impregnated with permethrin reduced house-entry by 60%. Likewise, Mathenge et al., (2001) found that permethrin-impregnated ITNs reduced or prevented house-entry of *An. funestus*, and

increased the exit rates of *An. gambiae* and *An. funestus*. No difference was apparent in the rates of blood feeding and exiting of *An. arabiensis*. A number of other studies offer similar findings and interpretations (Lindsay et al., 1991; Takken 2002, Gimnig et al., 2003). Some authors interpret these effects as being odor-mediated and repellency. However, evidence for such interpretations is arguable. Lindsay et al., (1991) caution that such avoidance may be caused by the solvent vapors or some other ingredients associated with the pyrethroids used in some ITNs rather than with the given pyrethroids themselves. Because of their high molecular weights (see Figure 1.4), pyrethroids tend to have very low vapor pressures. Pauluhn (1999) have noted that some formulations of pyrethroid insecticides have airborne effects due to movement of dust particles bearing toxicant. The speculation can be added that pyrethroid molecules, once evaporating, may rapidly condense upon dust particles and other solid surfaces. Gut et al., (2004) have documented such adsorptive behaviors by insect pheromone molecules of high molecular weight.

The oldest studies of mosquito behavioral responses to insecticides are still informative and worth considering. They were conducted using DDT. Anopheline and culicine mosquitoes resting on surfaces sprayed with DDT flew away after 5-10 minutes (WHO, 1963). Mean elapsed time to first departure decreased as DDT concentrations on paper increased (Brown, 1958; Ungureanu & Teodorescu, 1963). In a study by Shalaby (1965) excitability/deterrency of a DDT-resistant strain of *An. culicifacies* Giles did not differ from that for a DDT-susceptible strain. In contrast, what was reported as DDT deterrency by an Indian *An. stephensi* strain resistant to DDT was less than that for a susceptible strain (Choudhury & Rahman, 1967). Current research (Roberts and Alecrim,

1991; Chareonviriyaphap et al., 1997; Roberts et al., 2000) is re-exploring the pronounced ability of DDT to cause mosquitoes to avoid DDT-treated surfaces; however, little progress has been made in explaining these effects in terms of Figure 1.6.

A major conclusion of this literature review is that, despite the importance of ITNs and the considerable research attention they have drawn to date, sizable gaps remain in our knowledge of how they actually function and whether formulations should maximize avoidance vs. lethality or vice versa, or be some compromise between the two. With respect to prevention of transmission of non-zoonotic diseases like malaria, diversion of mosquitoes from humans to alternate hosts may be a desirable outcome of any intervention. Interestingly, Bogh et al., (1998) reported that, following introduction of permethrin-impregnated bed nets in villages in Kwale District, near the coast of Kenya, the human blood index (proportion of blood meals taken from humans vs. other animals) dropped from 99.5% to 87.5% in *An. gambiae* s.s. sample specimens. This is a remarkable result, since *An. gambiae* s.s. is a highly anthropophilic mosquito reported to rarely feed on hosts other than humans (Takken & Knols 1999). On the other hand, direct kill of mosquitoes vectoring malaria directly so as to reduce density and mean vector age may be a more robust outcome because this reduces vectorial capacity. Research helping to differentiate among these possibilities should ultimately prove useful to improving and sustaining malaria suppression via ITNs.

Rationale

ITNs are currently making a major contribution to malaria prevention. Knowledge of the mode-of-action of ITNs will be critical to predicting possible response of *An. gambiae* populations in response to heavy ITN implementation.

Hypotheses

This study postulates that quantitative analyses of malaria mosquitoes interacting with ITNs will reveal behaviors not entirely explained by toxicosis. This study also postulates that attraction to host cues and repellence from ITNs will have offsetting effects on mosquito foraging for a blood meal resulting in continued interaction and leading to toxicosis.

Research Objectives

The core objectives of this dissertation research are to: characterize, quantify, and classify the behaviors of blood-seeking *An. gambiae* interacting with untreated vs. long-lasting pyrethroid-treated -bednetting currently being deployed in Sub-Saharan Africa. This research aims for: a) a level of behavioral resolution surpassing that of previous studies, and b) differentiation among the behavioral responses outlined in the top half of Figure 1.6. The insights resulting from this study are expected to significantly increase understanding of the mode-of-action of ITNs. In turn, such knowledge will hopefully enable leaders of long-term programs for malaria vector suppression to make informed decisions about how ITNs might be managed to maximize sustainability.

CHAPTER 2

Quantitative Analyses of Behavioral Interaction of Female *Anopheles gambiae* s.s. Giles with Insecticide-Treated Netting in Small Static Cages and a Wind-tunnel

INTRODUCTION

Insecticide-treated-bednets (ITNs) with formulations of deltamethrin (Permanet)™ or permethrin (Olyset™) lasting up to five years currently serve as a key component of the malaria-suppression programs being implemented across Sub-Saharan Africa, where malaria has been killing nearly 1 million people annually (WHO, 2000). It has been proposed that ITNs protect sleepers by several of the following mechanisms: a) restraining mosquitoes from approaching sufficiently close to human skin to take a blood-meal, b) killing mosquitoes sitting on the nets sufficiently long to pick up a lethal dosage (Vijverberg & Van den Bercken, 1990; Bogh et al., 1998), and c) causing mosquitoes to leave treated nets or avoid approaching treated nets (Lindsay et al., 1991; Miller et al., 1991; Rutledge et al., 1999), or even avoiding entering rooms or houses containing ITNs (Gimnig et al., 2003b; Mathenge et al., 2001). Evidence is mounting that, under dense deployment, ITNs can offer some protection to nearby persons not actually under a bednet (Gimnig et al., 2003a & b). However, the degree to which the various alternative explanations above are contributing to such positive end results has been unclear and the subject of some debate (Habluetzel et al, 1997; Roberts et al., 2002). Definitive answers to this question could inform managers of vector-control programs about how the ITN tactic might be optimized as well as sustained by avoidance of resistance, be it metabolic or behavioral.

The current study, employing modern techniques for quantifying animal behavior in high detail, was part of a research program whose objective was to quantify the relative contributions of the above possible mechanisms to permethrin- and deltamethrin-impregnated ITN mode-of-action against *Anopheles gambiae* Giles, the major vector of malaria in Sub-Saharan Africa. Here we report results of laboratory studies conducted at a small spatial scale, in: a) cages fashioned from netting and experiencing little air movement (static), and b) a small wind-tunnel where air-flow was controlled through treated and un-treated netting. Results at the house scale will follow in Chapter 3.

MATERIALS AND METHODS

Mosquitoes

The Kisumu laboratory strain *of An. gambiae* s.s. was used throughout. Mated-female stock for this culture was house-collected between 1990 and 1992 from multiple sites near the Kenya Medical Research Institute (KEMRI) at Kisian (Vulule et al, 1994). Eggs were deposited on moist paper at 28 ± 1^0C under a photoperiod LD12:12 h. After 1 day of incubation, they were transferred to plastic containers measuring 27 x 19.4 x 9.5 cm and holding 700 - 800 ml of tap water for larval rearing. Most of the lid area of the rearing containers was covered with 0.25 mm polyester nylon mesh (American Home and Habitat, Squires, MO, U.S.A.). Light at approximately 700 lux was provided at the water surface by fluorescent bulbs (Lumichrome 40 W, 122 cm T12, 5000K, CRI96, M & M Lighting Co., Ronan, MT, U.S.A.) mounted directly over the rearing containers. Larvae were reared on ground and sieved (100 mesh) food mixture (2:1) fish food (TetraMin(R), Blacksburg, VA, U.S.A.): brewers' yeast (Bio-Serve). Amounts served were: 5 mg on day 1, 28 mg on day 2, 36 mg on day 3, 120 mg on day 4, 200 mg on day 5 and subsequent

days before pupation. Pupae were collected from the containers each day and transferred into mosquito pupal breeders (21 cm height 12 cm diameter) consisting of 2-L clear styrene containers (BioQuip Products, Gardena, CA, U.S.A.). The bottom portion of each pupal breeder held 250 ml water with several hundred pupae. A plastic lid between the two portions contained a clear, vinyl funnel through which emerging adults flew into the upper portion ventilated at the top with aluminium screening (5 cm diameter). Eclosed adults were transferred into rearing and supply cages.

Adult female mosquitoes were fed via an artificial blood-feeder (Huang et al., 2005) consisting of a water bath, fountain pump, Tygon(R) tubing (Saint-Gobain Performance Plastics, Beaverton, MI, U.S.A.), and blood reservoir was used. The reservoir was a metal jar lid (6 cm diameter x cm height) hot-glued to a flattened copper tube connected to Tygon tubing. Warm water (44°C) was pumped from the water bath through the tubing (1.8 cm diameter) and through the copper tube, to heat the blood in the reservoir to 36°C. At each feeding, 15 ml of defibrinated horse blood (Lampire Biological Laboratories, Pipersville, PA, U.S.A.) was poured into the feeder reservoir and sealed with two layers of very thinly stretched Parafilm™. The feeder was placed membrane-down on top of the rearing cage, arranged so its top was flat. A small piece of dry ice and four paper towel strips (2 x 25 cm) soaked in Limburger cheese solution (23% w/w, stored overnight at 4°C) were placed near the blood reservoir as attractants and stimulants (Knols et al., 1997). Mosquitoes were allowed to engorge for 1-2 h every other day. Sugar feeders, comprised of a cotton wick connected to a 20 ml reservoir of 10% honey solution, were continuously present on the cage floor (Huang et al., 2005).

Experimental Series 1 – Tests of Insecticide-Treated and Untreated Netting in Small Static Cages

These tests were conducted in 20 cm diam. x 20 cm tall cylindrical cages (Fig 2.1). The cage top and sides were constructed of standard polyester 100-denier 156-mesh bed netting material (Siam Dutch Ltd, Thailand) sewn together and attached with Velcro™ to external wire framing for support. The floor was clear plastic, a substrate upon which *An. gambiae* do not normally prefer to sit. Mosquitoes were inserted or withdrawn from cages via an aspirator inserted through a small slit in a fabric seam, sealed with Velcro™. Three types of static cages were tested in a walk-in environmental chamber (12L: 12D, 28°C, RH 75-85%): 1) all untreated fabric, 2) all ITN fabric, and 3) half-untreated and half-ITN. An individual female mosquito was introduced into an arena during the last 2 h of photophase and observed continuously for 30 min, a period sufficient for evaluation of ITN efficacy (Hougard et al., 2002). Using a lap-top computer operating Version 5.0 of the Observer (Noldus, http://www.noldus.com) behavioral quantification software, the following data were recorded: frequency and duration of flight; number of contacts with cage walls; time and location of landings; and elapsed time sitting and walking on cage surfaces. Also recorded were timing and duration of behaviors associated with toxicosis: postural changes; tremors; leg autotomy (Moore and Tabashnik 1989a, b); knockdown; and loss of responsiveness to tactile stimulation. Mosquitoes in advanced toxicosis were placed in 10 ml glass vials and monitored for time of death or recovery. Test A of this series compared an untreated cage to fully-treated Permanet™ (Vestegaard Frandsen; 55mg wash-proof deltamethrin/m^2 impregnated into polyester) (WHO, 2000), Olyset (Sumitomo; 1 g Permethrin/m^2 of polyethylene fiber (N'Guessan et al., 2001), and

Netprotect (Intelligent Insect Control) cages (10 replicates of each); Test B compared an untreated cage with half-treated Permanet, Olyset, and Netprotect cages (10 replicates of each). The experimental design for treatments within a test was randomized complete block; replicate blocks were accumulated over time.

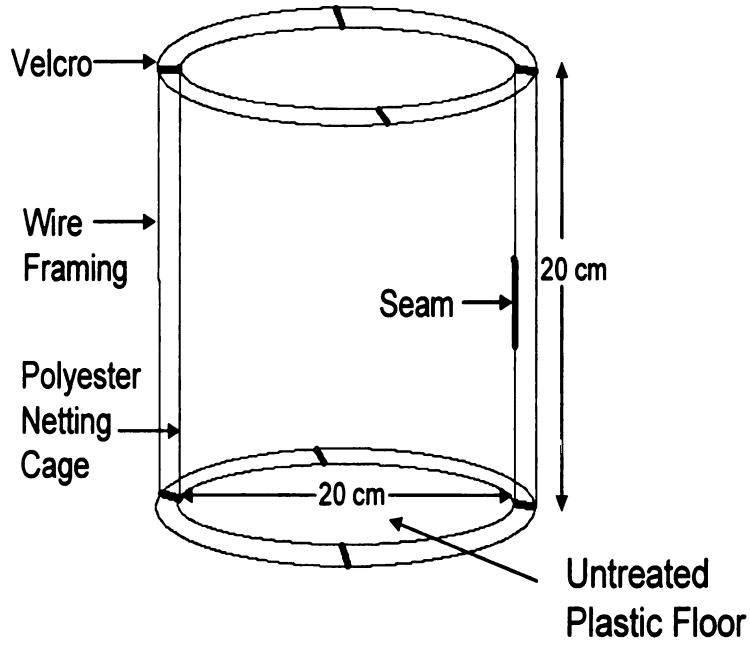


Figure 2.1. Representation of a 20 cm diam. x 20 cm tall static cage used for observations of *Anopheles. gambiae* females responding to ITNs. Cages were either constructed: 1) entirely of untreated polyester netting, 2) of half-untreated netting and the other half of Permanet, or 3) half-untreated netting and the other half Olyset. Halved cages were split vertically.

Experimental Series 2 – Tests of Untreated and Insecticide-Treated Netting in a Wind Tunnel with Host Cues

A wind-tunnel (Figure 2.2) suitable for quantifying the behaviors of flying mosquitoes in the vicinity of insecticide-treated vs. control screens was constructed after the designs of Miller and Roelofs (1978), Gillies and Wilkes (1981), and Miller and Gibson (1994). A variable-speed electric blower was joined by flexible plastic sheeting to a wind-laminator, consisting of a 60 cm high x 70 cm wide x 15 cm deep wooden frame having 3 layers of stretched sheer fabric (*ca.* 10 mesh/cm) stapled to each face. Attached in series to the laminator were two 90 cm long x 70 cm wide x 60 cm high chambers of

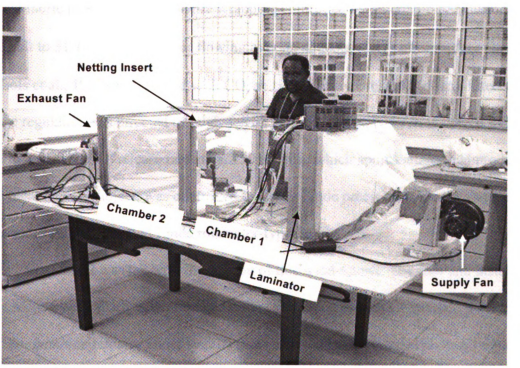


Figure 2.2. Wind tunnel for testing responses of *Anopheles gambiae* females to insecticide-treated bednet materials placed downwind of host cues.

clear Plexiglas™ (see Figure 2.2), joined by a wooden frame slotted so as to permit insertion of exchangeable panels of bet-netting, stretched and supported by independent frames. Types of netting panels used were: untreated netting, all Permanet™, all Olyset™, or half-untreated joined to half-treated deltamethrin (25 mg/m²) or permethrin (500mg/m²) panels with a vertical seam at their midpoint. Chamber 1 housed sources of host cues released as one centered set when a panel of netting was homogeneous, or as two sets centered on each half-panel when two types of netting were tested simultaneously. Heat approximating that from human skin was provided by mounting a 4 cm diam hexagonal head of a 7 cm long x 2.5 cm diam steel bolt against the upwind side of the fabric panel. A resistive-wire wrapping controlled by a variable transformer heated the bolt to 31°C. A stream of air flowing over an aqueous slurry of limburger cheese (Knols et al., 1997) and infused with CO_2 released from a pressurized tank fitted with a 2-stage regulator, was directed through Tygon tubing, 3.2 mm (i.d.) so it flowed over the warm bolt. The CO_2 release rate at ca. 250 ml/min, which approximates that produced by a typical human subject, was controlled by using Linde precision control valves and measured using calibrated Linde rotameter-type flowmeters.

Chamber 2 was the working section of this wind-tunnel. The roof and distal end of this section were fitted with untreated polyester netting to provide suitable perching sites for *An. gambiae* females under test. Its downwind end was closed with metal window screening, in which openings were made to receive 11 cm long x 5 cm diam plastic cages with removable screened ends, for releasing individual female mosquitoes (release cages) within a stimulus plume. An air-scoop connected to a second blower fan exhausted all stimulus fumes from the laboratory. Beneath the floor of Chamber 2 was white paper on

which fifty 4-cm diam black circles were randomly positioned to provide a strong stimulus for optimotor responses (Knols et al., 1994). Wind flowed laminarly through the working section at 0.2 m/s when all hinged doors permitting access to the chambers were closed. The flight chamber was illuminated by a 40W fluorescent light bulb suspended above the tunnel. Light was diffused through layer of 3 mm opaque, white acrylate and another of white filter paper reduced light intensity to 6.5 ± 1.5 lux in the chamber. No other illumination was present in the room.

Wind tunnel procedure

Individual 2-3 day-old female *An. gambiae* mosquitoes reared as above and having previous access to sugar solution but not blood were gently transferred by aspirator into a release cage. Cages were positioned in clean air on the floor of the wind-tunnel working-section for 5 - 10 min to allow for acclimation to the flowing wind. To initiate a run, a release cage was inserted through the end-screen and into the plume of cues; the upwind end of the release cage was then gently removed. Continuously for the next 30 min, a researcher sitting beside the working-section of the wind-tunnel keyed the following behaviors into a laptop computer running Observer™ software: sitting (stationary state with at least two pairs of legs in contact with surface); walking (forward or sideway alternate movements of legs on surface); flying (free airborne movements on the wing); net-flying (flying while mouthparts and front legs kept in near constant contact with the net); hopping (short leaps of flight interspersed with short periods of sitting); labella tapping (occasional pressing of mouthparts on net surface and through openings in the net); leg lifting (alternating rapid movements on and off the net or other surface while in a sitting position); grooming (rubbing/cleaning legs, wings and mouthparts using

a pair of legs); antennae raising (elevating antennae beyond normal position); jerking (twitching motions of whole body or parts of it), leg dropping (legs detaching from main body), erratic movements (staggering rapid motions of flying, walking and sitting), knockdown (loss of normal posture and locomotion) and wing spread (wings remain outstretched while sitting or in knockdown state). The position where each behavior occurred in the arena was noted and recorded as: Upwind treated or untreated (section of a treated or untreated screen in the netting insert); Downwind mesh (metal window screening at downwind end); Netted side (vertical wall side of Chamber 2 opposite an observer fitted with untreated netting); Netted roof (ceiling side of chamber 2 fitted with untreated netting); Floor (bottom area of Chamber 2); Front plexiglas (immediate transparent vertical side facing observer); Treated/Untreated heat (nut heads placed behind treated or untreated screen, respectively); Intersection (exposed frame positions and other locations not defined above). After the 30 min, the specimen was placed into a holding cup for 24 h with access to 10% sugar solution and water, for assessment of leg autotomy and mortality. A total of 25 replicates was accumulated for each type of net-treatment to be reported. Tests were blocked so that all ITN types were successively tested in random order at one setting.

Data Analysis

Before analysis, sets of data were examined under the Descriptive Statistics/Basic Statistics feature of Systat for levels of kurtosis and skewness. Data were transformed as necessary, usually to $(x + 0.5)^{1/2}$, and occasionally to $\log_{10}(x + 0.5)$. ANOVA was performed using SAS version 9.1 (2000) or SYSTAT Version 10 and means were separated using Fisher's LSD test. Time-event tables were generated within Observer and

various plots of the data were examined to identify outcomes justifying statistical analyses. Lag sequential analysis was carried out to visualize the temporal structure of sequence of events. It allowed for calculation of frequencies of transitions between pairs of events within a certain lag in a time series. The first event of each pair was designated the criterion event and the second as the target event. The lag was therefore the time separating the criterion and target events.

Transition matrices of pre-knockdown and post-knockdown behaviors, with the diagonals treated as logical zeroes (no behavior could follow itself), were constructed as per Walker and Archer (1988). Only analyzable first order transitions were considered. A 2 x 2 contingency table analysis was conducted on each cell to test that the before/after transition frequency differed from chance. The main point of interest was whether the transition was higher than expected. Kinematic graphs were helpful in visualizing behavioral transitions and frequencies in untreated and treated-net scenarios and in pre-knockdown and post-knockdown periods.

RESULTS

Experimental Series 1 – Tests of Insecticide-Treated and Untreated Netting in Small Static Cages

Test A – Untreated cage vs. fully-treated Permanet, Olyset, and Netprotect cages

The average times *An. gambiae* allocated to various behaviors during the combined 30-min observations while interacting with untreated and fully-treated Permanet, Olyset, and Netprotect ITN static cages are shown in Figure 2.3. On average, the duration was 12% for flying, 82 % for sitting, and 6% for walking on the wall, roof, and floor of an untreated control (Figure 2.3). Flying within fully-treated Permanet,

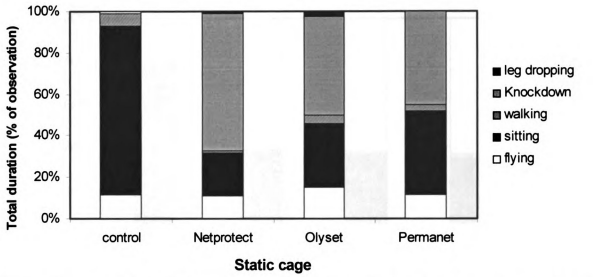


Figure 2.3. Average percent time *Anopheles gambiae* allocated to various behaviors during the 30-min observations of single females in untreated static cages or cages fully-treated with deltamethrin (Netprotect and Permanet) and permethrin (Olyset).

Olyset, and Netprotect ITN cages constituted 11, 12, and 10% of observation time, respectively. A mean of 80% of the time was spent on the walls of the untreated cage (Figure 2.3). However, sitting duration was 37, 28 and 18% of the time for the fully-treated Permanet, Olyset, and Netprotect ITN cages, respectively. Time spent on the floor for *An. gambiae* females was 63, 62 and 60% for fully-treated Permanet, Olyset, and Netprotect ITN cages, respectively (Figure 2.4). However, average time allocations are of less interest behaviorally than shifts in behaviors over time.

Mosquitoes in control cages were consistent in their behaviors through time. No statistical differences across time were found for: types of behavior (Figure 2.5 A, C, E), durations of behavior (Figure 2.5 B, D, F), positions of behaviors (Figure 2.6 A, C, E), and durations of behaviors at specified positions (Figure 2.6, B, D, F). However, presence

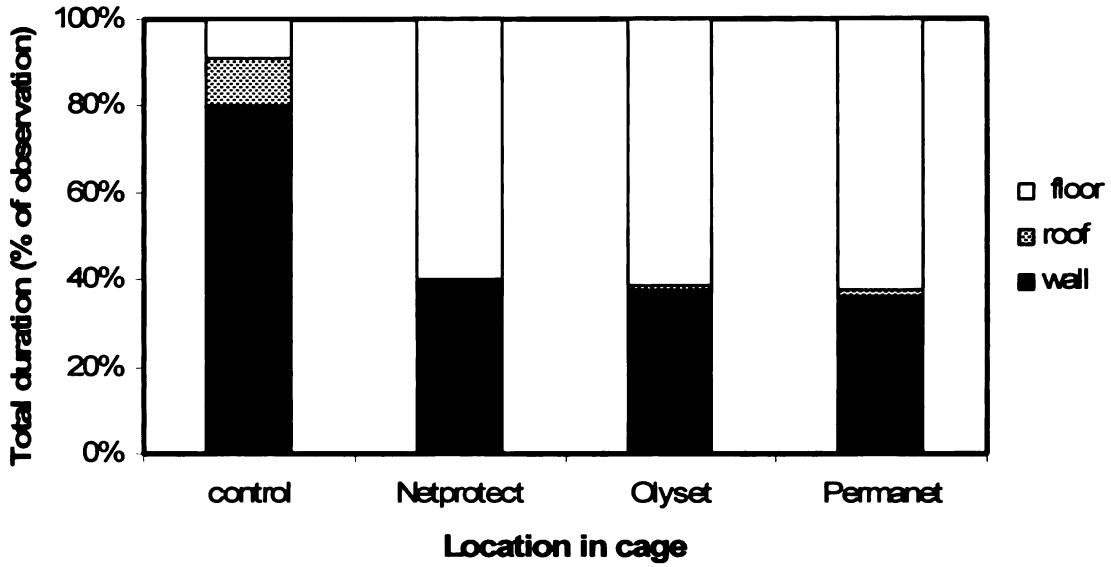


Figure 2.4. Total time spent in given locations in cage during 30-min observations of *Anopheles gambiae* females in static cages treated with three types of ITN fabric

of insecticides caused major shifts in behaviors through time. Initially, all ITNs significantly elevated frequency (Figure 2.5 A) and duration (Figure 2.5 B) of flying. Flight durations under Permanet exposure were not as high as those for Netprotect and Olyset. Also elevated initially was frequency (Figure 2.5 C) but not duration (Figure 2.5 D) of sitting events. Since every flight ends with a new sitting event, it is likely that sitting was elevated only because it must follow flight. This is why Figure 2.5 A is virtually identical with Figure 2.5 C. The mean frequency (Figure 2.5 E) and mean duration (Figure 2.5 F) of walking were never elevated by insecticide exposure.

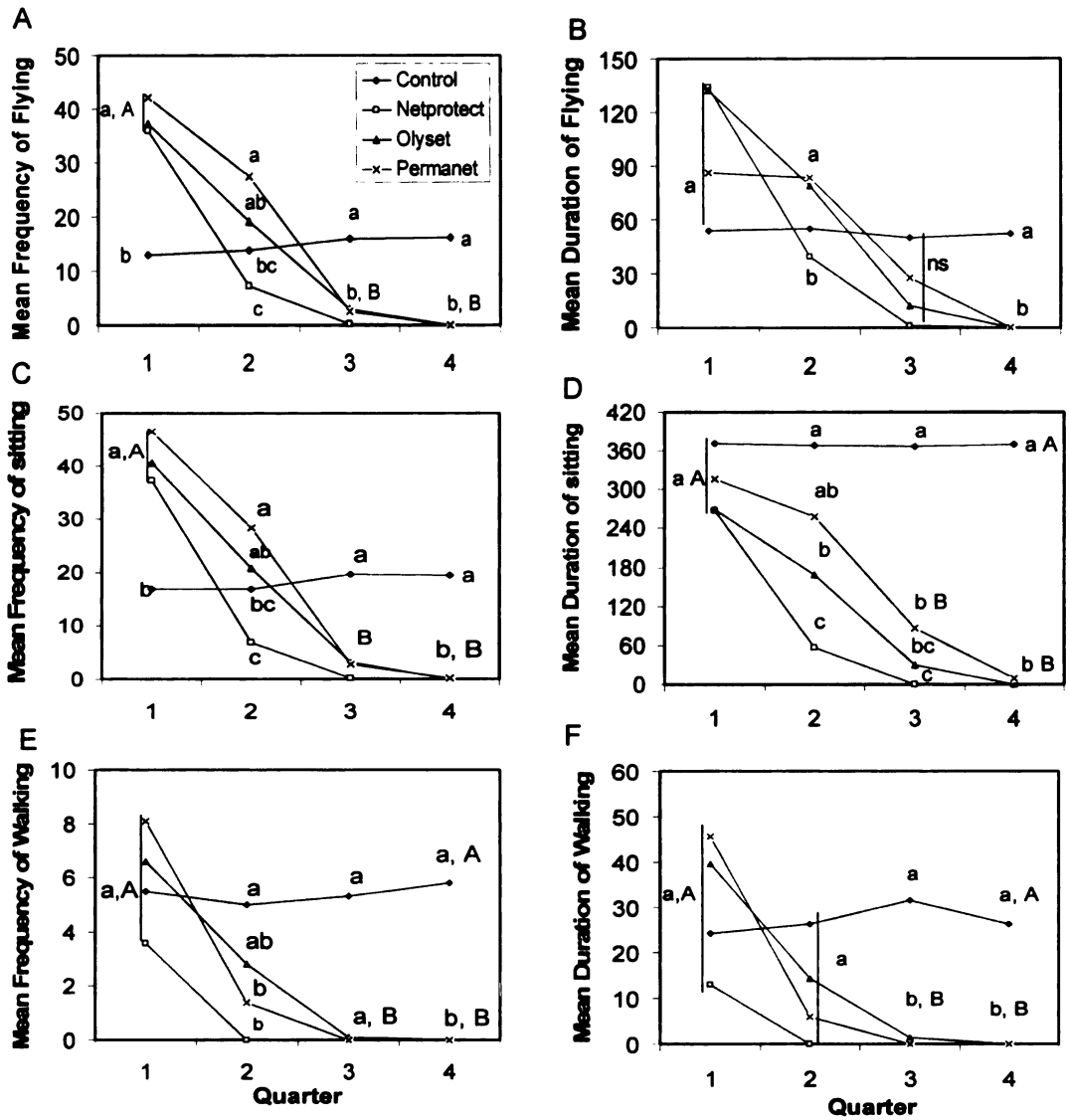


Figure 2.5. Mean total frequencies and mean total durations for behavioral responses of *Anopheles. gambiae* females broken out per quarter of the 30 min observation period. This test was conducted using either fully-untreated cage walls and roof or fully-treated cage walls and roof. Data within a column sharing the same lower-case letter are not statistically significant; data across quarters sharing the same capital letter are not statistically different.

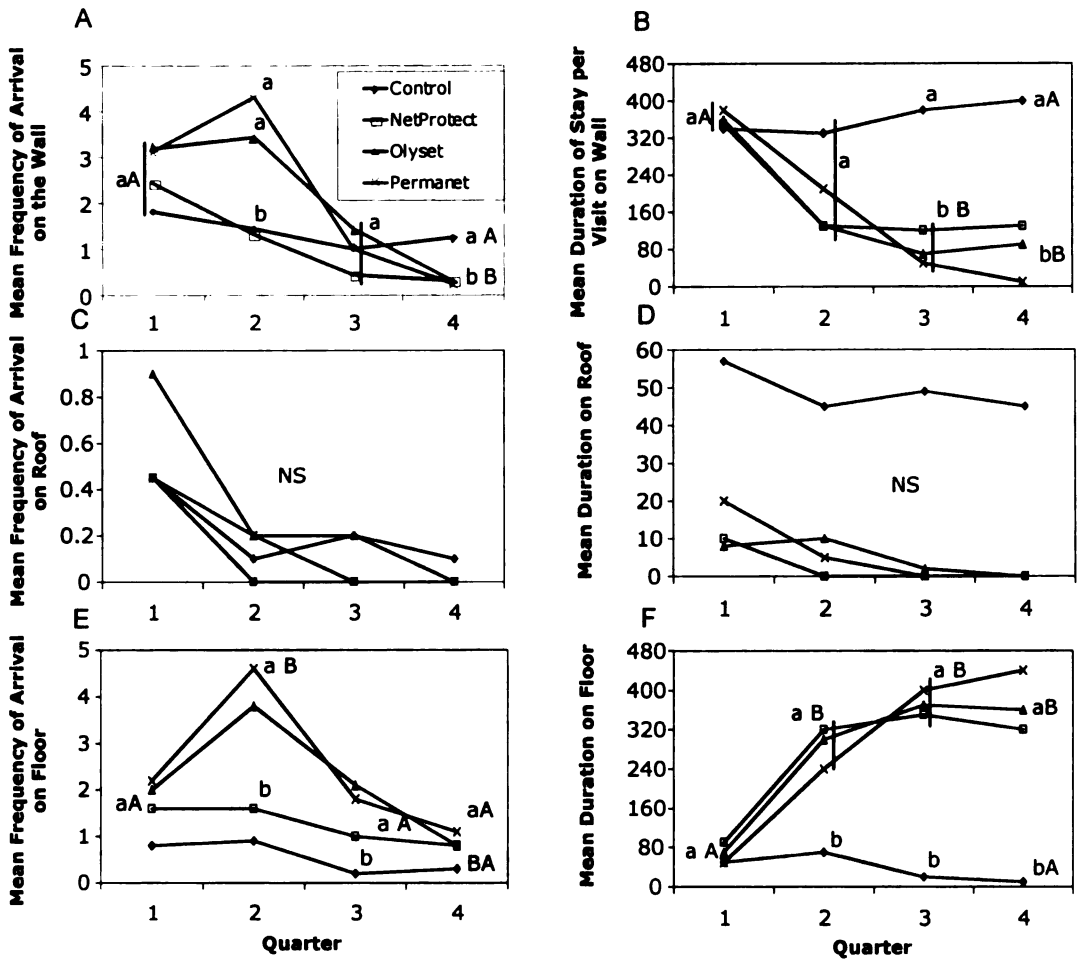


Figure 2.6. Mean total frequency of arrival at particular positions and mean total durations of stay at those locations for female *Anopheles gambiae* in static cages, as broken out by quarter of the 30 min observation period. This test was conducted using either fully-untreated cage walls and roof or fully-treated cage walls and roof. Data within a column sharing the same lower-case letter are not statistically significant; data across quarters sharing the same capital letter are not statistically different.

The mean elapsed time for first knockdown for Netprotect, Olyset, and Permanet are given in Table 2.1. Elapsed time for first knockdown by Netprotect was significantly shorter than that for Permanet. Olyset fell between and was not significantly different from Permanet and Netprotect. At about the time of first knockdown, the stimulatory effects of the ITNs begin to diminish as frequency of erratic movements increased (Figure 2.7 A). Also at this time labella tapping and grooming frequencies diminished. Initially labella tapping had been elevated for Olyset and grooming had been depressed by Permanet and Olyset. By quarter 3, flying, sitting, and walking were all significantly reduced below levels for mosquitoes in control cages (Figure 2.5). Knockdown (Figure 2.8) shifted mosquito presence from the walls to the floor (Figure 2.6 B vs. F). By the final quarter of the test, virtually all mosquitoes were lying on the floor, incapable of flying, walking or sitting (Figure 2.5 A, E, C).

Test B – Untreated cage vs. half-treated Permanet, Olyset, and Netprotect cages

Figure 2.9 shows the average times during 30-min observations that *An. gambiae* allocated to various behaviors while interacting with untreated small static cages and half-treated Permanet, Olyset and Netprotect ITN cages. Flying within half-treated Permanet, Olyset, and Netprotect ITN cages constituted 15, 19 and 10% of observation time, respectively. Sitting occupied 48, 46, and 45% of the time for the half-treated Permanet, Olyset, and Netprotect ITN cages, respectively. The percentage of time *An. gambiae* sat on the floor of half-treated Permanet, Olyset, and Netprotect cages vs. the control cage was 18, 20, and 19 vs. 46%, respectively, (Figure 2.10). Consistencies and changes in behaviors in these cages during 30-min observations follow.

Table 2.1. Comparison of elapsed time for first knockdown event for *Anopheles gambiae* females in small cages fully- or half-treated with ITN fabrics. Means followed by a common letter at any place in the table are not significantly different at p = 0.05.

	Elapsed Time (Min ± SEM) for First Knockdown		
Cage Treatment	Netprotect	Olyset	Permanet
Untreated Netting	No knockdown	No knockdown	No knockdown
Fully-treated Cage	8.1 ± 1.0 c	11.7 ± 1.3 bc	14.8 ± 1.5 ab
Half-treated Cage	14.7 ± 1.2 ab	14.1 ± 1.2 ab	17.8 ± 1.3 a

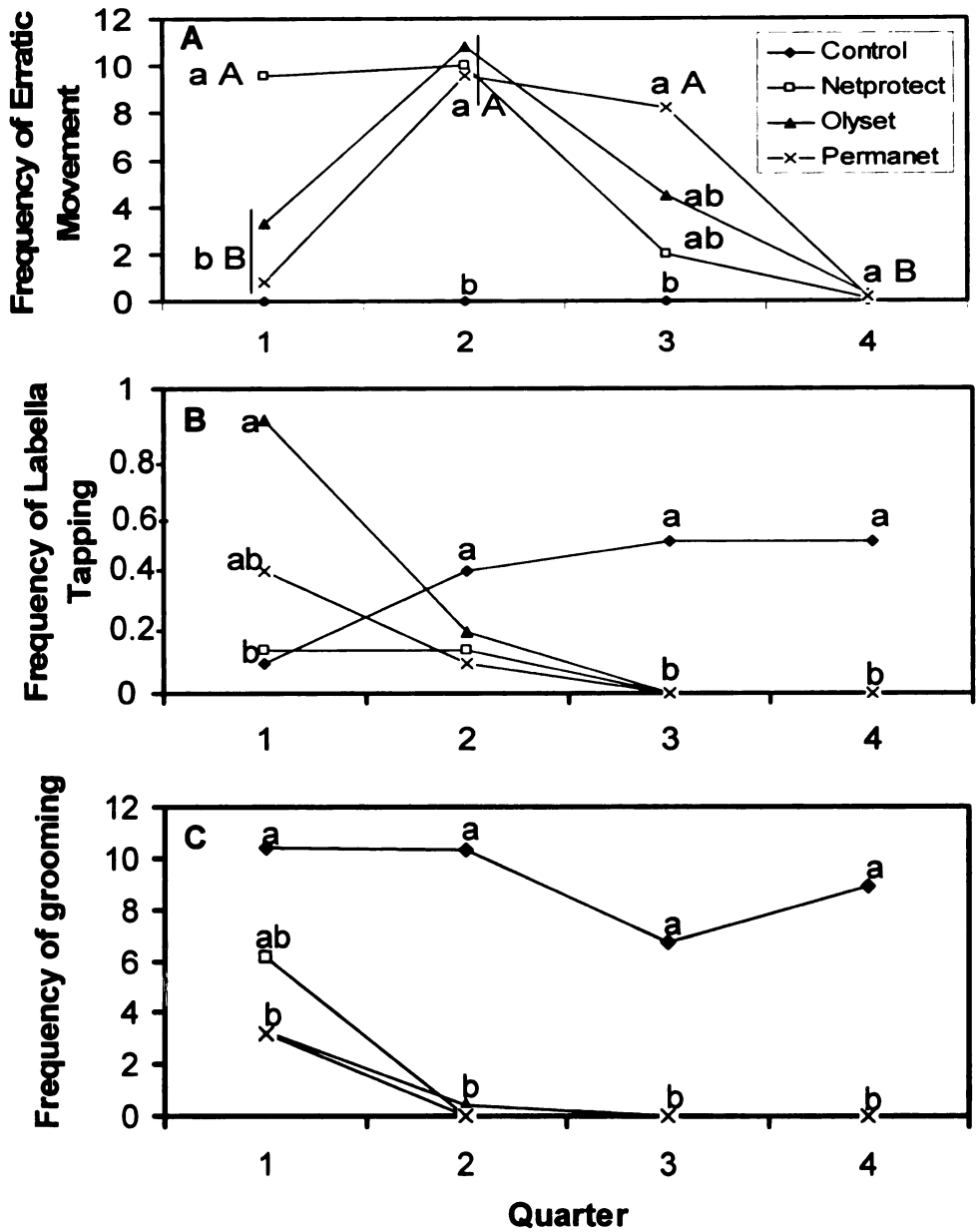


Figure 2.7. Mean total frequencies for behavioral responses of *Anopheles gambiae* females broken out per quarter of the 30 min observation period. This test was conducted using either fully-untreated cage walls and roof or fully-treated cage walls and roof. Data within a column sharing the same lower-case letter are not statistically significant; data across quarters sharing the same capital letter are not statistically different.

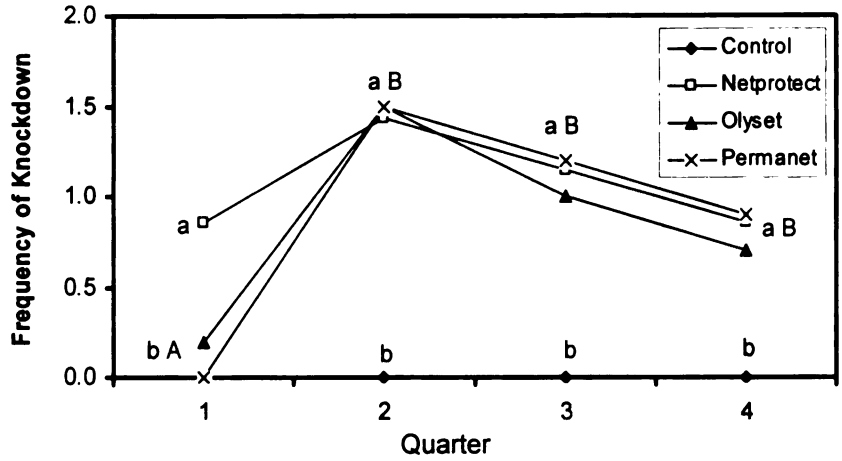


Figure 2.8. Mean total frequencies for knockdown of *Anopheles gambiae* females broken out per quarter of the 30 min observation period. This test was conducted using either fully-untreated cage walls and roof or fully-treated cage walls and roof. Data within a column sharing the same lower-case letter are not statistically significant; data across quarters sharing the same capital letter are not statistically different.

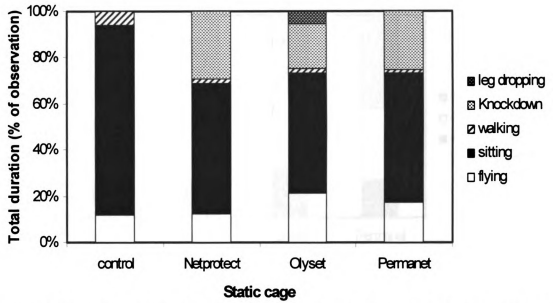


Figure 2.9. Average time *Anopheles gambiae* allocated to various behaviors during the 30-min observations of single females in untreated static cages or cages half-treated with deltamethrin (Netprotect and Permanet) and permethrin (Olyset).

The same consistencies reported above for mosquitoes in control cages were again evident (Figure 2.11). Moreover, the repeatability both for controls and ITN treatments between these full-treated and half-treated cage experiments was striking. Figures 2.11 and 2.5 are a near match when overlaid. Because cages in the latter experiment were only half-treated with insecticide-treated netting, there was less consistency in the timing in the first insecticide exposure. For example, a female landing and sitting on untreated netting would absorb no insecticide until leaving and alighting on treated netting. Thus, it is not surprising that elapsed times for first knockdown were delayed for half-treated cages relative to fully-treated cages (Table 2.1).

Although significant differences were not found between half and fully-treated Permanet and Olyset cages when each was analyzed separately, statistical significance

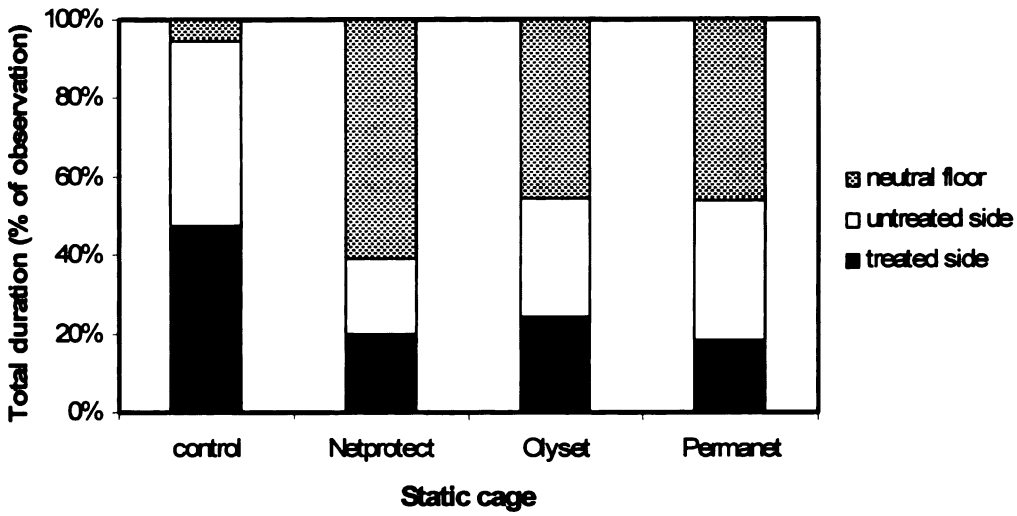


Figure 2.10. Average times *Anopheles gambiae* females spent in particular locations in untreated vs. half-treated static cages as averaged over the whole 30 min observation period.

was obtained at P = 0.03 when paired data for all three net types were combined. It follows that the depressions in frequencies and durations of behaviors seen in Figure 2.11 slightly lag those in Figure 2.5. Overall, the main effect of the insecticides was to stimulate flying until knockdown commenced. The behavioral repertoire became progressively dominated by erratic movements (Figure 2.14) and knockdown events (Figure 2.15), until the females accumulated on the cage floor as knockdown became permanent.

No evidence was found that *An. gambiae* females detected and avoided insecticide-treated sections of half-treated cages. This is shown in Figure 2.12, where the profiles for treated and untreated cage halves were statistically identical for all cages

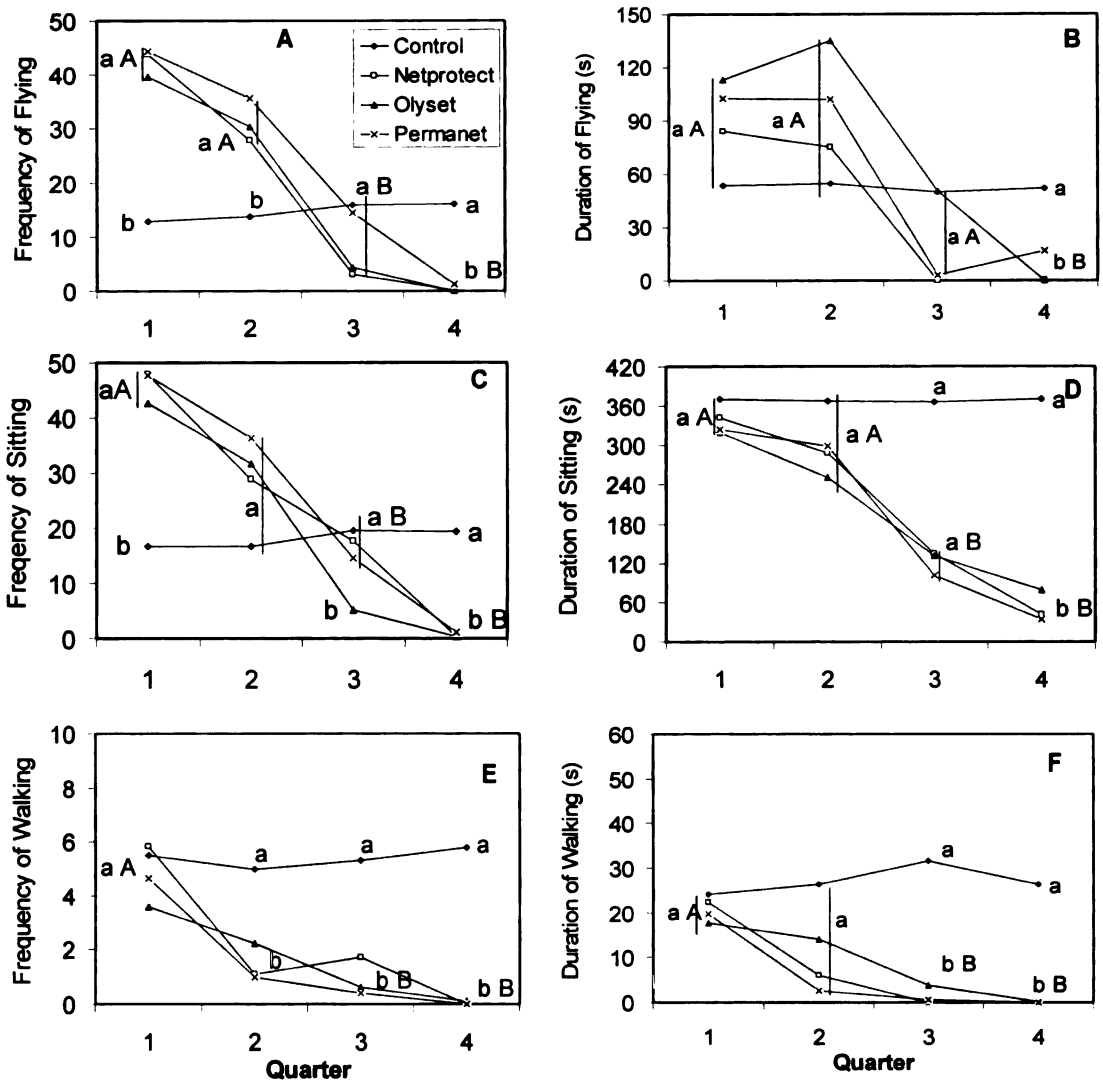


Figure 2.11. Mean total frequencies and mean total durations for behavioral responses of *Anopheles gambiae* females broken out per quarter of the 30 min observation period. This test was conducted using either fully-untreated cage walls and roof or half-treated cage walls and roof. Data within a column sharing the same lower-case letter are not statistically significant; data across quarters sharing the same capital letter are not statistically different.

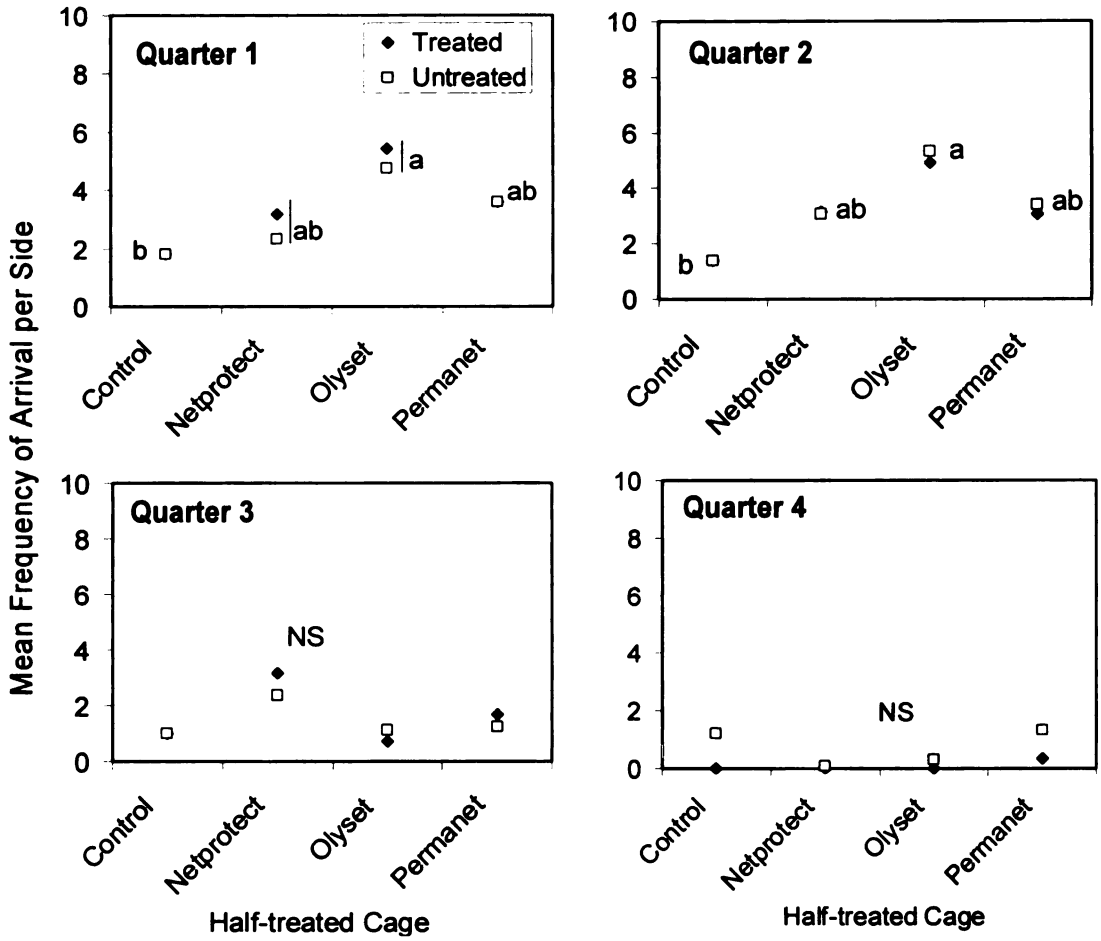


Figure 2.12. Mean total frequencies of arrival per side by *Anopheles gambiae* females broken out per quarter of the 30 min observation period. This test was conducted using either fully-untreated cage walls and roof or half-treated cage walls and roof. Data within a column sharing the same lower-case letter are not statistically significant.

containing insecticide. This was true throughout all four quarters of the 30-min tests. In quarters 1 and 2, Olyset elevated frequency of arrival on both untreated and treated cage walls over the values obtained for fully-untreated cages (Figure 2.12 A and B). This effect disappeared in quarters 3 and 4 (Figure 2.12 C and D). Durations of stay on untreated vs. treated halves of half-treated cages were identical across all 4 quarters and for all three ITN types (Figure 2.13).

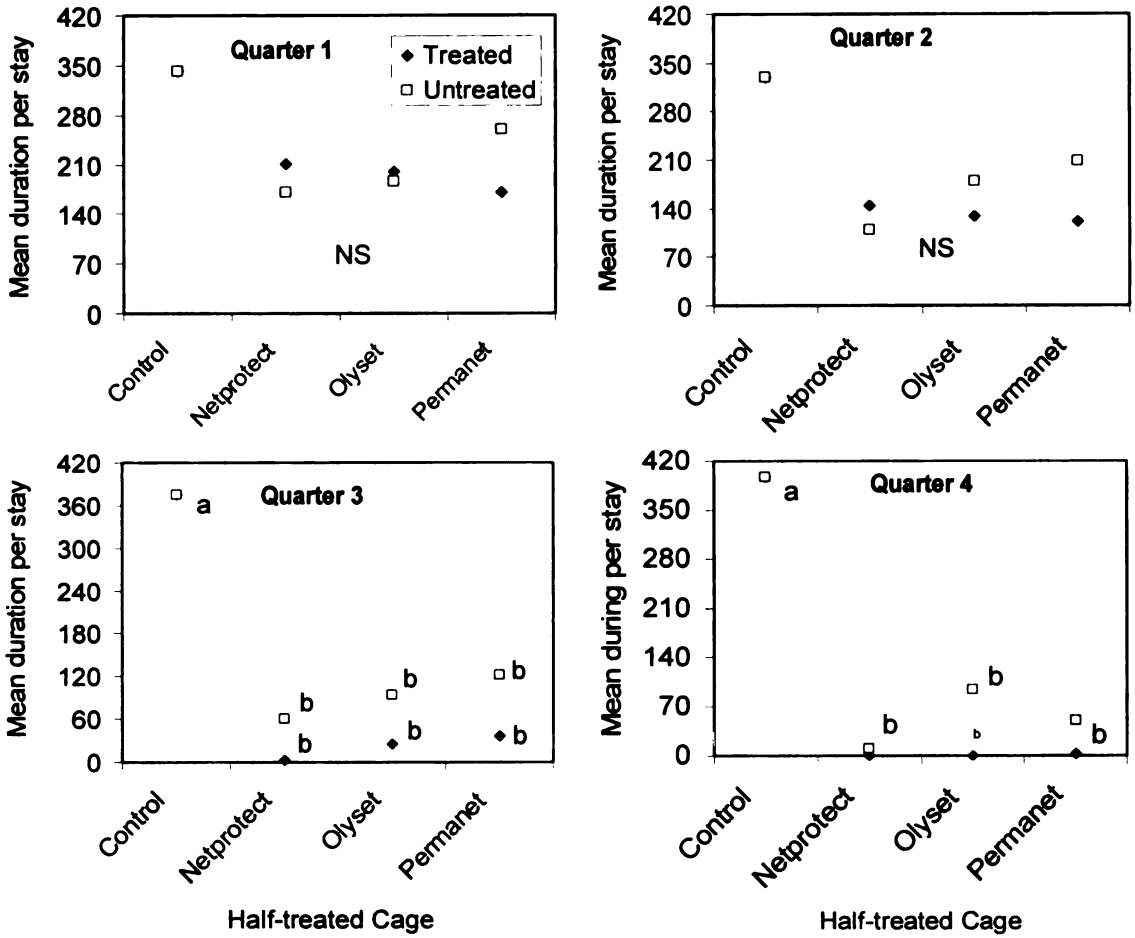


Figure 2.13. Mean total duration of stay by *Anopheles gambiae* females on treated vs. untreated sides of half-treated cages broken out per quarter of the 30 min observation period. Data within a column sharing the same lower-case letter are not statistically significant.

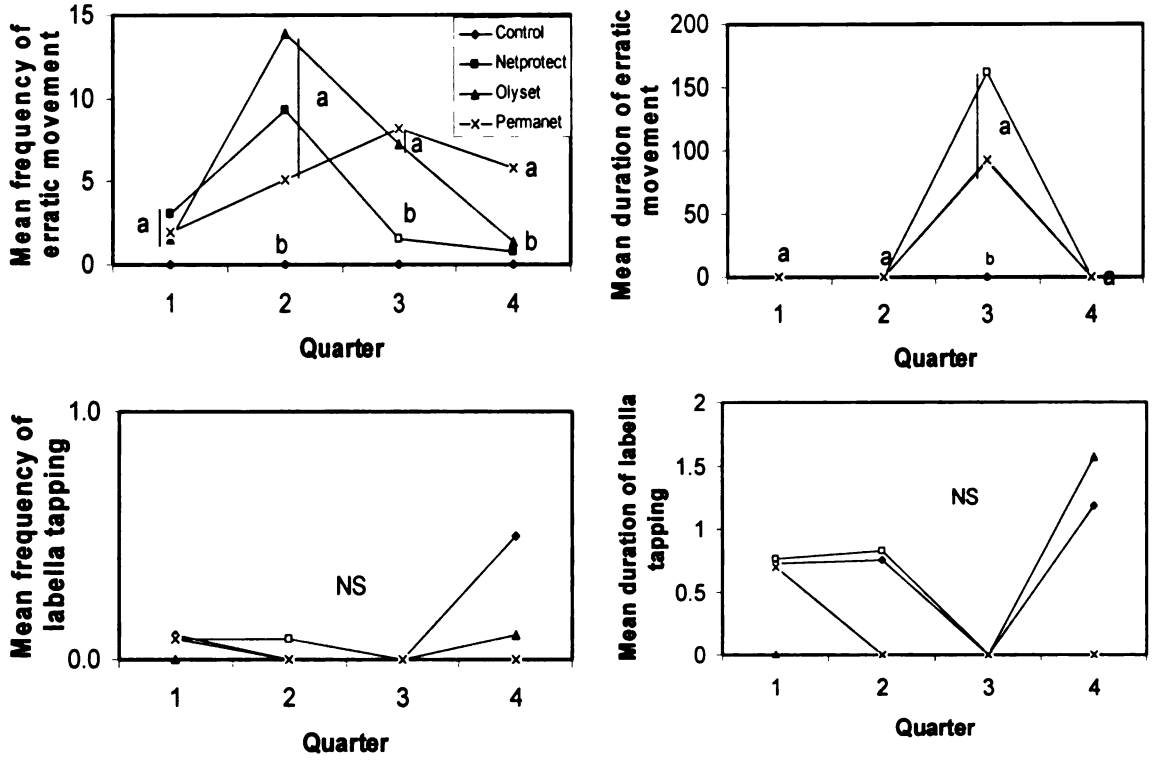


Figure 2.14. Mean total frequency and duration of behaviors by *Anopheles gambiae* females on treated vs. untreated sides of half-treated cages broken out per quarter of the 30 min observation period. Data within a column sharing the same lower-case letter are not statistically significant.

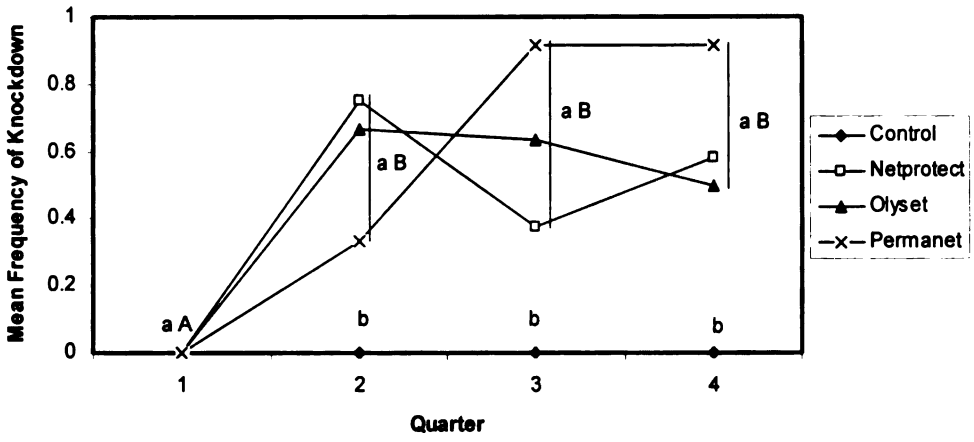


Figure 2.15. Mean total frequency of knockdowns by *Anopheles gambiae* females on treated vs. untreated sides of half-treated cages broken out per quarter of the 30 min observation period. Data within a column sharing the same lower-case letter are not statistically significant; data across quarters sharing the same capital letter are not significantly different.

Experimental Series 2 – Tests of Untreated and Insecticide-Treated Netting in a Wind Tunnel with Host Cues

Test A – Untreated cage vs. fully-treated Permanet, and Olyset screens

The average times *An. gambiae* allocated to various behaviors during the 30-min observations when exposed to untreated and fully-treated Permanet, and Olyset ITN screens at the upwind end of the wind tunnel are shown in Figure 2.16. When no insecticide was present, 74% of the time was spent sitting (Figure 2.17), of which 50% occurred on the upwind screen (Figure 2.17). When the upwind screen contained Permanet, females on average spent 48% of the total time sitting, of which 26% occurred on the upwind screen. When Olyset was present, females spent 42% of the total time

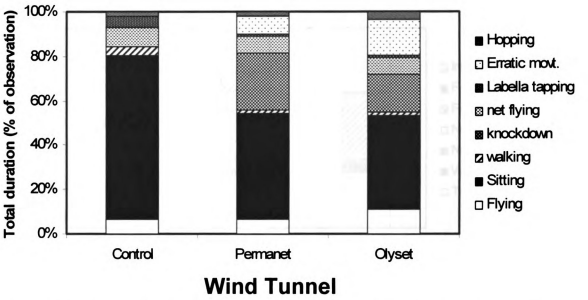


Figure 2.16. Average time *Anopheles gambiae* allocated to various behaviors during the 30-min observations of single females in a wind tunnel with untreated and fully ITN-treated upwind screens, as averaged over the full 30-min observational period.

sitting, of which 28% was spent on the upwind screen. The overall pattern in time budget within the wind tunnel was more similar to that within the half-treated static cage (Figure 2.9). Duration of stay on the floor for Permanet and Olyset was double that of stay on the floor for untreated screen (Figure 2.17). The behaviors expressed by *An. gambiae* females in the wind tunnel under no exposure to insecticide-treated netting were less consistent through time than were behaviors in static cages.

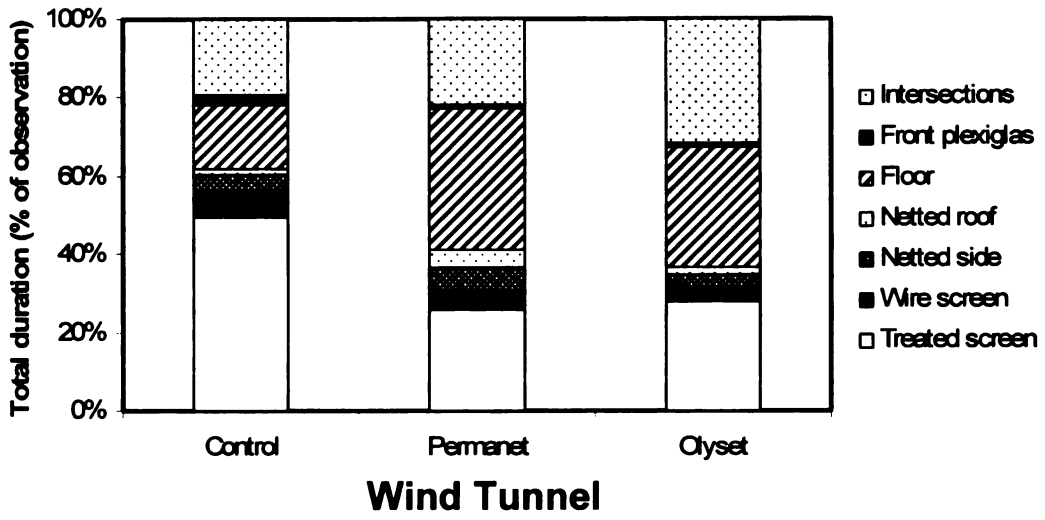


Figure 2.17. Average times *Anopheles gambiae* spent in particular locations during the 30-min observations of single females in a wind tunnel with untreated and fully ITN-treated upwind screens, as averaged over the full 30-min observational period.

Frequency and duration of flying by control mosquitoes in the wind tunnel tended to rise over time (Figure 2.18 A, B). The rise through time was significant for mean frequency of flying (Figure 2.18 A), and close to significant (P = 0.057) for mean frequency of walking (Figure 2.18 E). An upward tendency through time was observed for various other behaviors by control insects (Figure 2.19; Figure 2.20).

As in the static cages, insecticide-treated fabrics at the upwind end of the wind tunnel elevated some behaviors of exposed *An. gambiae*. Both Olyset and Permanet significantly increased frequency (Figure 2.18 A) and duration (Figure 2.18 B) of flying; and, Olyset was significantly more stimulating than was Permanet. Thus, both Olyset and

Permanet functioned as locomotor stimulants (Figure 1.6). However, these insecticides did not elevate walking (Figure 2.18 E) or labella tapping (Figure 2.19).

Mean elapsed time until first arrival on the upwind screen of the wind tunnel for control, Permanet, and Olyset was 4.2 ± 1.4, 3.4 ± 1.1, and 3.2 ± 0.8 min, respectively; differences not significant by ANOVA on data transformed to $(x + 0.5)^{1/2}$ ($P = 0.98$). This suggests there was no repellent effect of these insecticides, i.e. no tendency to fly downwind when exposed to air flowing over insecticide. Mean elapsed times for the first knockdown event by Olyset and Permanet in the wind tunnel for the experiment were 18.5 ± 1.2 and 17.3 ± 1.4 min, respectively, differences not significant. These values were only slightly greater than for elapsed time until the first knockdown in half-treated static cages (Table 2.1). Perhaps the presence of moving air and host cues in the wind tunnel led to speedy delivery of a lethal dose of insecticide in the wind tunnel, even though it was much larger than a static cage.

Beginning in the second quarter and until the end of the tests, the frequency and duration of erratic movements increased dramatically (Figure 2.19 E, F), and first knockdown soon followed early in the third quarter. Thereafter, behaviors such as flying (Figure 2.18 A,B), sitting (Figure 2.18 C,D), and walking (Figure 2.18 E,F), labella tapping (Figure 2.19 A, B), and net flying (Figure 2.19 C, D) all diminished progressively until 90 and 80% of females exposed to Olyset and Permanet, respectively, were continuously confined to the floor, beginning at 9.7 ± 0.9 vs. 3.4 ± 1.4 min, respectively (difference significant at $P = 0.035$ by ANOVA on untransformed data).

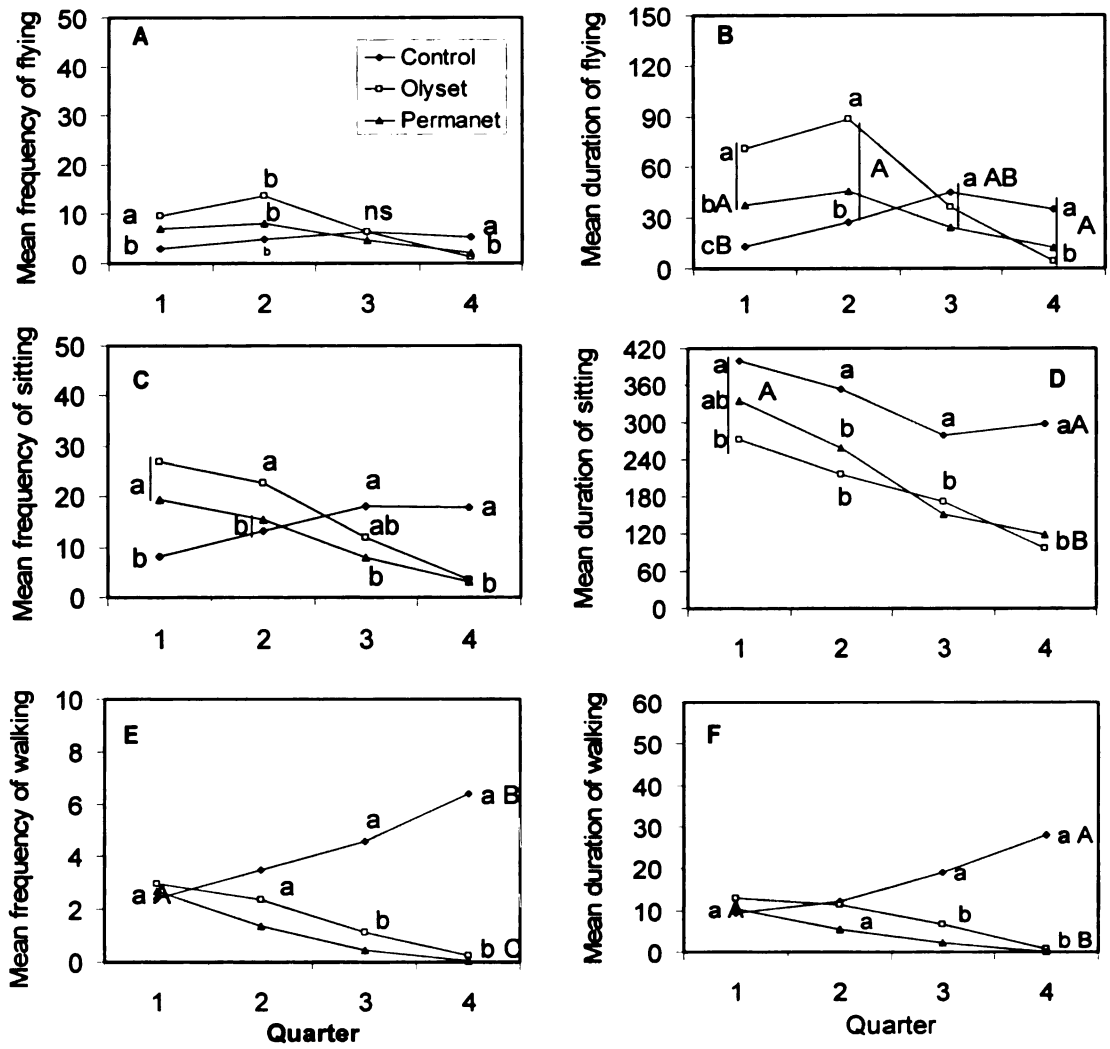


Figure 2.18. Mean total frequencies and mean total durations for behavioral responses of *Anopheles gambiae* females broken out per quarter of the 30 min observation period. This test was conducted in a wind tunnel using either fully untreated or fully ITN-treated upwind screens. Data within a column sharing the same lower-case letter are not statistically significant; data across quarters sharing the same capital letter are not statistically different.

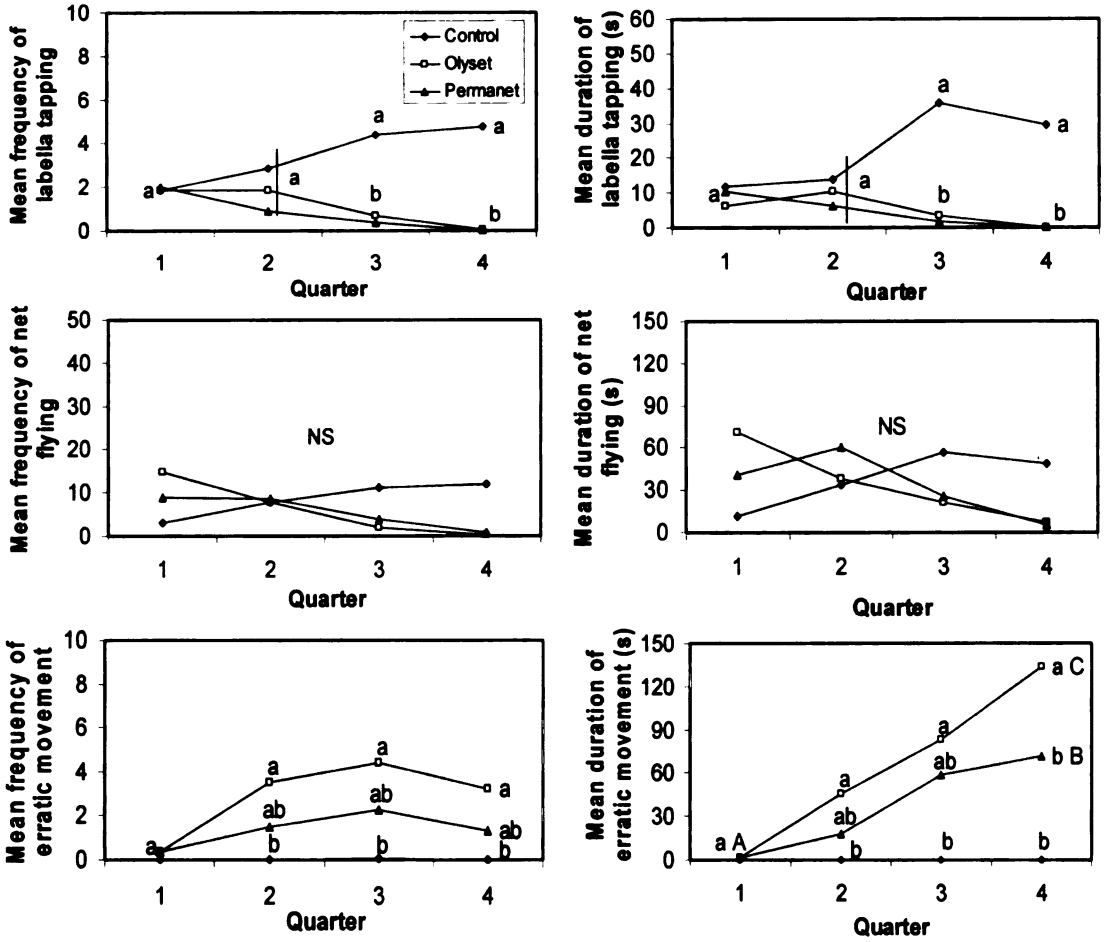


Figure 2.19. Mean total frequencies and mean total durations for behavioral responses of *Anopheles gambiae* females broken out per quarter of the 30 min observation period. This test was conducted in a wind tunnel using either fully untreated or fully ITN-treated upwind screens. Data within a column sharing the same lower-case letter are not statistically significant; data across quarters sharing the same capital letter are not statistically different.

Working in a wind tunnel where air flow was controlled, enhanced ability to characterize the behavioral mechanisms mediating ITN effects in the terminology of Figure 1.6. It has already been noted that neither Permanet nor Olyset diminished the frequency of flying upwind in air flowing over these insecticides. Thus the Permethrin and Deltamethrin dosages used here were not repellents i.e., repellents cause responders to move downwind or out of stimulus plumes by maneuvers always directed away from the source of stimulation.

Frequency of arrivals on the upwind screen of the wind tunnel was significantly elevated for Olyset over the control (Figure 2.20), as would be expected for a Locomotor Stimulant (Figure 1.6). But, the rate of stay on the treated screen was not reduced relative to that for the untreated screen (Figure 2.20). Rather, mosquitoes stayed somewhat longer (although not significantly so (Figure 2.21)) than their counterparts on an untreated screen. Nor can Olyset be considered a Deterrent (Figure 1.6) in the present context, as the frequency of visitation to the Olyset source increased slightly from quarter 1 to quarter 2, rather than decreased relative to the visitation rate to the control (Figure 2.20). Thus by the terminology of Figure 1.6, Olyset can be termed locomotor stimulant. Permanet was likewise documented to be a locomotor stimulant because it increased flying frequency in the wind tunnel (Figure 2.18 A). However, this effect by Permanet was not sufficiently strong so as to significantly increase visitation rate to its source on the upward screen (Figure 2.20). Like Olyset, Permanet proved to be only a locomotor stimulant, albeit a weaker one than Olyset. In quarters 3 and 4, visitation rates to the upwind screens and duration of stay (Figure 2.21) diminished as mosquitoes became increasingly poisoned. Frequencies of visitation and duration of stay on the downwind

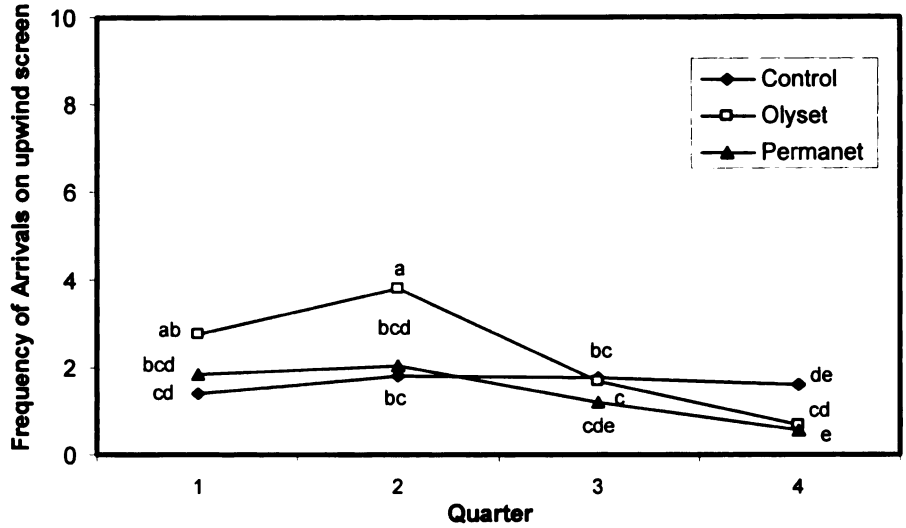


Figure 2.20. Mean total frequencies of arrival by *Anopheles gambiae* females on the upwind screen of a wind tunnel, broken out per quarter of the 30 min observation period. This test was conducted in a wind tunnel using either fully untreated or fully ITN-treated upwind screens. Data within a column sharing the same lower-case letter are not statistically significant.

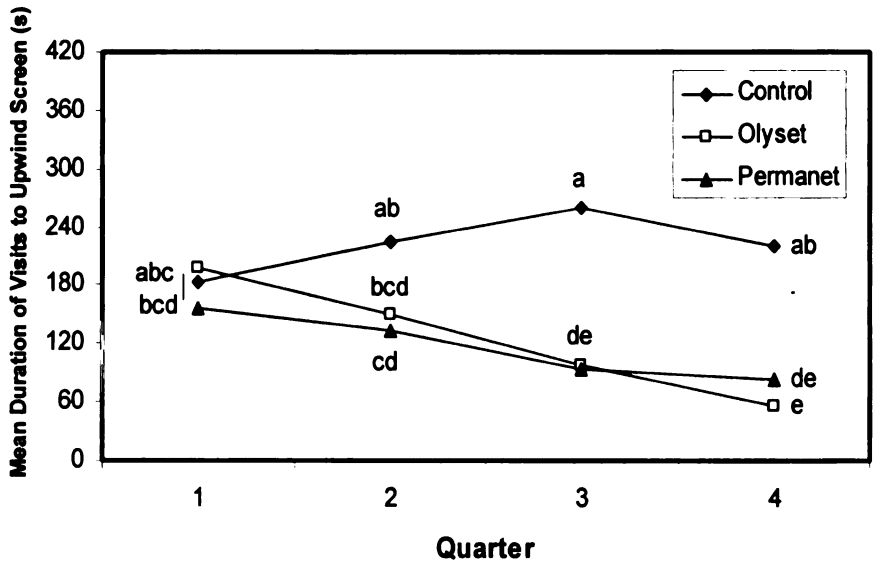


Figure 2.21. Mean duration of visits by *Anopheles gambiae* females to the upwind screen of a wind tunnel, broken out per quarter of the 30 min observation period. This test was conducted in a wind tunnel using either fully untreated or fully ITN-treated upwind screens.

screen of the wind tunnel did not change significantly for either Olyset or Permanet across all four quarters (Figure 2.22).

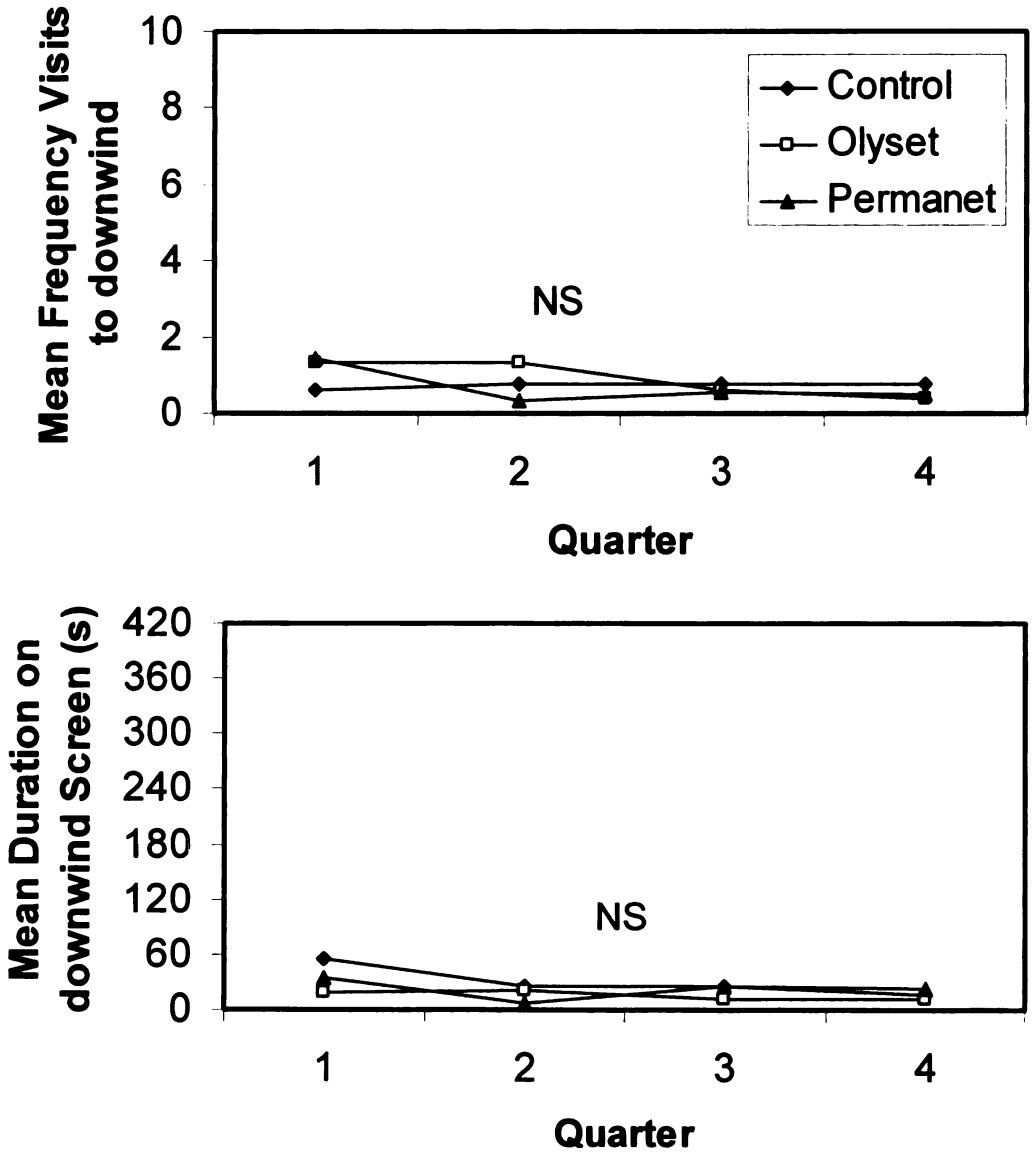


Figure 2.22. Mean total frequencies of arrival by *Anopheles gambiae* females on the down-wind screen of a wind tunnel, broken out per quarter of the 30 min observation period. This test was conducted in a wind tunnel using either fully untreated or fully ITN-treated upwind screens

The sequential organization of behaviors expressed by *An. gambiae* in this wind tunnel test was analyzed as transitional matrices. Three 10 x 10 transition matrices were constructed for behaviors of *An. gambiae* in the vicinity of untreated and fully-Permanet and Olyset screens, respectively (Table 2.2, 2.4, and 2.6). Three other 7 x 7 transition matrices were constructed for positions in the wind tunnel including the screens upwind (Table 2.3, 2.5, and 2.7).

Table 2.2. A 10 x 10 transition matrix of behavioral responses of *An. gambiae* when untreated netting covered the upwind end of a wind tunnel.

Behavior	flying	walk	sitting	k-down	n-flying	l-tapping	Jerking	Erratic-m	Hopping	w-spread	SUM
flying	-	0	**254**	0	**135**	1	0	0	73	0	463
walk	17	-	50	0	86	**226**	0	0	17	0	396
sitting	**242**	207	-	0	**515**	85	3	1	**217**	1	1271
k-down	0	0	0	-	0	0	0	0	0	0	0
n-tapping	110	21	**652**	0	-	5	0	0	14	0	802
l-tapping	**26**	**163**	54	0	45	-	0	0	32	0	320
jerking	2	0	0	0	0	0	-	0	1	0	3
erratic-m	0	0	0	0	1	0	0	-	0	0	1
hopping	**61**	3	**288**	0	7	3	0	0	-	0	362
w-spread	0	0	0	0	1	0	0	0	0	-	1
SUM	458	394	1298	0	790	320	3	1	354	1	3619

Bolded and underlined values are significantly higher than expected by chance, while underlined values are lower than expected by chance. Bolded only values are moderately higher than expected by chance, after Bonferroni correction. Values in normal font are not significant. *Italized* values had expected value < 5 and no chi square test was calculated. K-down = knockdown; n-flying = net flying; l-tapping = labella tapping; erratic-m = erratic movements; w-spread = wings spread.

Table 2.3. A 7 x 7 transition matrix of positions of *An. gambiae* behaviors when untreated netting covered the upwind end of a wind tunnel.

Position	t-screen	wirescreen	n-side	n-roof	plexiglas	floor	intersection	SUM
u-treated	-	10	5	2	5	**49**	36	107
d-mesh	10	-	4	0	*9*	16	27	66
n-side	3	3	-	0	1	7	6	20
n-roof	2	0	0	-	2	1	0	5
plexiglas	**55**	16	5	0	-	0	41	117
floor	2	*11*	0	0	0	-	4	17
refuge	33	24	4	0	3	**47**	-	111
SUM	105	64	18	2	20	120	114	443

Bolded and underlined values are significantly higher than expected by chance, while underlined values are lower than expected by chance. Bolded only values are moderately higher than expected by chance, after Bonferroni correction. Values in normal font are not significant. *Italized* values had expected value < 5 and no chi square test was calculated. u-screen = untreated screen; n-side = netted side; n-roof = netted roof

Table 2.4. A 10 x 10 transition matrix of behavioral responses of *An. gambiae* when Permanet covered the upwind end of a wind tunnel.

Behavior	flying	walk	sitting	k-down	n-flying	l-tapping	Jerking	erratic-m	hopping	w-spread	SUM
flying	-	1	**271**	6	**106**	1	0	**35**	33	1	454
walk	7	-	18	0	20	*42*	2	1	17	0	107
sitting	**292**	**70**	-	8	**297**	*29*	**30**	13	**172**	15	926
k-down	1	0	1	-	0	0	4	26	0	5	37
n-tapping	**65**	6	**362**	1	-	0	0	10	14	2	460
l-tapping	12	25	22	0	6	-	0	1	10	0	76
jerking	9	4	1	6	4	1	-	4	4	4	37
erratic-m	*22*	0	25	22	13	0	1	-	7	11	101
hopping	24	1	**228**	3	5	3	0	1	-	1	266
w-spread	8	0	5	6	0	0	4	12	0	-	35
SUM	440	107	933	52	451	76	41	103	257	39	2499

Bolded and underlined values are significantly higher than expected by chance, while underlined values are lower than expected by chance. Bolded only values are moderately higher than expected by chance, after Bonferroni correction. Values in normal font are not significant. *Italized* values had expected value < 5 and no Chi square test was calculated. k-down = knockdown; n-flying = net flying; l-tapping = labella tapping; erratic-m = erratic movements; w-spread = wings spread.

Table 2.5. A 7 x 7 transition matrix of positional placement of *An. gambiae* when Permanet covered the upwind end of a wind tunnel.

Position	t-screen	wirescreen	n-side	n-roof	plexiglas	floor	intersection	SUM
treated	-	6	9	1	4	**39**	**37**	96
d-mesh	5	-	1	1	2	19	**30**	58
n-side	**8**	0	-	0	1	6	2	17
n-roof	3	0	2	-	1	1	1	8
plexiglas	**28**	19	3	0	-	0	**39**	89
floor	1	*6*	1	1	0	-	5	14
refuge	**42**	**26**	3	1	3	**37**	-	112
SUM	87	57	19	4	11	102	114	394

Bolded and underlined values are significantly higher than expected by chance, while underlined values are lower than expected by chance. Bolded only values are moderately higher than expected by chance, after Bonferroni correction. Values in normal font are not significant. *Italized* values had expected value < 5 and no chi square test was calculated. u-screen = untreated screen; n-side = netted side; n-roof = netted roof

Table 2.6. A 10 x 10 transition matrix of behavioral responses of *An. gambiae* when Olyset covered the upwind end of a wind tunnel.

Behavior	flying	walk	sitting	k-down	n-flying	l-tapping	Jerking	Erratic-m	Hopping	w-spread	SUM
flying	-	0	**416**	4	**136**	0	0	**107**	60	16	739
walk	25	-	49	0	12	*50*	1	4	15	0	156
sitting	**413**	**99**	-	16	**385**	**52**	**69**	26	**396**	33	1489
k-down	2	0	0	-	1	0	8	*40*	5	9	65
n-tapping	**130**	3	**427**	2	-	1	2	6	15	9	595
l-tapping	17	40	20	0	12	-	4	0	14	0	107
jerking	30	10	2	6	12	2	-	63	28	6	159
erratic-m	46	0	103	46	23	0	14	-	12	9	253
hopping	49	3	**483**	1	13	1	2	21	-	2	595
w-spread	3	0	2	5	1	0	5	10	4	-	30
SUM	715	155	1502	80	595	106	105	277	549	84	4168

Bolded and underlined values are significantly higher than expected by chance, while underlined values are lower than expected by chance. Bolded only values are moderately higher than expected by chance, after Bonferroni correction. Values in normal font are not significant. *Italized* values had expected value < 5 and no Chi square test was calculated. K-down = knockdown; n-flying = net flying; l-tapping = labella tapping; erratic-m = erratic movements; w-spread = wings spread.

Table 2.7. A 7 x 7 transition matrix of positions of *An. gambiae* behaviors when untreated netting covered the upwind end of a wind tunnel.

Position	t-screen	wirescreen	n-side	n-roof	plexiglas	floor	intersection	SUM
treated	-	17	12	9	10	**<u>74</u>**	**<u>68</u>**	190
d-mesh	16	-	4	2	3	26	**31**	82
n-side	**16**	6	-	2	2	9	4	39
n-roof	**9**	1	4	-	0	2	2	18
plexiglas	6	7	1	1	-	8	4	27
floor	**<u>70</u>**	21	13	4	9	-	**<u>70</u>**	187
refuge	**<u>65</u>**	19	6	10	14	**<u>61</u>**	-	175
SUM	182	71	40	28	38	180	179	718

Bolded and underlined values are significantly higher than expected by chance, while underlined values are lower than expected by chance. Bolded only values are moderately higher than expected by chance, after Bonferroni correction. Values in normal font are not significant. *Italized* values had expected value < 5 and no chi square test was calculated. t-screen = treated screen; n-side = netted side; n-roof = netted roof

Based on a 2 x 2 contingency table analysis, nine before/after transition frequencies of behaviors on untreated screen were highly significantly elevated over values expected by chance and two before/after transitions were moderately significantly higher than expected by chance (Table 2.2). Transitions in position from Plexiglas to the untreated screen, from the untreated screen to the floor, and from intersections to the floor were pronounced (Table 2.3). This indicated that the relevant transitions of these frequencies of behaviors required further organization. A kinematic graph (Figure 2.23) was constructed revealing four major sequences and two minor sequences of *An.gambiae* female behavior on an untreated screen in a wind tunnel: a) *flying and sitting sequence* occurred mainly between the untreated screen and the floor, intersections and the floor, and Plexiglas and the floor; b) a *net-flying (tapping) and sitting sequence* was confined on the upwind untreated screen and it occurred at the highest frequency; c) *a walking* and

labella tapping sequence was most evident after sitting on the untreated screen; d) a *hopping* and *sitting* sequence was evident on the screen and on the floor; e) a one-way *labella tapping* to *flying* sequence was just significant on the untreated screen; and f) a one-way *hopping* to *flying* sequence was intermittently mingled with other sequences. Other sequences represented in Figure 2.23 were insignificant.

When the wind tunnel contained a Permanet screen, nine before/after transition frequencies of behaviors were significantly higher than expected by chance, and two before/after transitions were moderately significantly higher than expected by chance (Table 2.4); they underwent further organizational analysis. Transitions in position from the Permanet screen to the floor, from the downwind, metal screen to intersections, and from intersections to the Permanet screen were significantly higher than expected by chance (Table 2.4), and so were organized as explained below.

A kinematic graph (Figure 2.23B) was constructed revealing four major sequences and one minor sequence of *An. gambiae* female behavior on the Permanet screen in a wind tunnel: a) a *flying and sitting sequence* occurred mainly between the Permanet screen and downwind metal screen, Permanet screen and the floor and intersections, and Permanet, b) a *net-flying (tapping) and sitting sequence* was confined on the Permanet screen and occurred at lesser frequency than on an untreated net, c) a *sitting jerking sequence* was most evident on the Permanet screen, d) a *hopping sitting sequence* was evident on the Permanet screen and on the floor, e) a *net flying (tapping) flying sequence* was modestly significant both ways on the Permanet screen, and f) a one-way *hopping* to *flying* sequence was intermittent with other sequences.

The Olyset screen yielded nine before/after transition frequencies of behaviors that were significantly higher than expected and three before/after transitions, which were moderately significantly higher than expected by chance (Table 2.6). Transitions in position from the Olyset screen to the floor, from the Olyset screen to intersections and back to the Olyset screen , and from the floor to the Olyset screen, were significantly higher than expected (Table 2.7) and were therefore were organized into a kinematic graph for determination of sequences of behavior.

A kinematic graph (Figure 2.23C) was constructed revealing five major sequences and *gambiae* female behavior in a wind tunnel presenting an Olyset screen: a) a *flying and sitting sequence* occurred between the Olyset screen and the floor, Olyset screen and intersections, b) a *net-flying (tapping) and sitting sequence* was confined on the Olyset screen and occurred at lesser frequency than on the untreated net but more than on the Permanet screen, c) a *sitting jerking sequence* was most evident on the Olyset screen, d) a *hopping* and *sitting sequence* was evident on the Olyset screen and on the floor, e) a *net flying (tapping)* and *flying sequence* was slightly significantly both ways on the Olyset screen, f) a *one-way flying to erratic movements* sequence was prominent, g) a one-way *sitting* and *labella tapping* and *jerking* sequence occurred mainly on the Olyset screen, and h) a one-way *sitting to walking* sequence was significant on the Olyset screen.

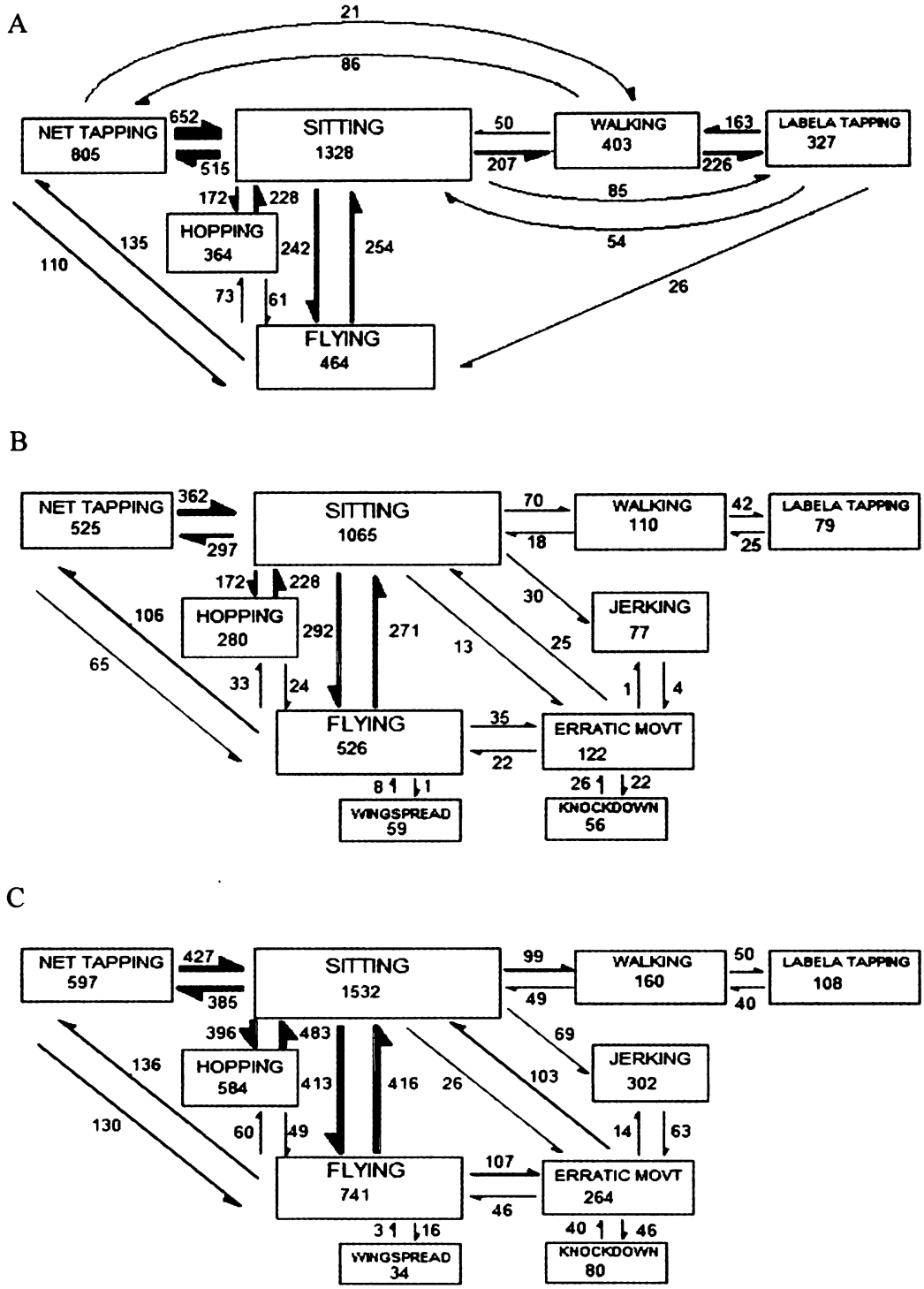


Figure 2.23. Kinematic graphs of behavioral sequences of *Anopheles gambiae* in a wind tunnel with upwind screens configured immediately downwind of host cues.

Arrows represent the transitions between behaviors; numbers next to the arrows refer to frequencies of transitions. Boxes represent behavioral states and numbers inside the boxes refer to frequencies of behavior. A = untreated screen: B = Permanet screen: C = Olyset screen.

Test B – Untreated cage vs half-treated Deltamethrin, and Permethrin screens

There was no evidence that *An. gambiae* females detected and avoided Deltamethrin- and Permethrin-treated sections of half-treated cages. The profiles for the treated and untreated halves of the screen in the wind tunnel were statistically identical for all screens containing insecticide (Figure 2.24). This was true throughout all four quarters of the 30-min tests. Lack of significant difference in frequency of arrival on either side of the half-treated screens during all four quarters was previously reported for half-treated cages (Figure 2.11 and 2.12). The inability of *An. gambiae* to differentiate between the sides of the half-treated wind tunnel treated with WHO recommended dosages of Deltamethrin and Permethrin argues strongly against repellency by these insecticides.

Duration of stay after arrival per half of upwind screens was significantly lower for the Permethrin-treated side relative to a fully-untreated screen in the wind tunnel. However, *An. gambiae* did not discriminate between the untreated half of Permethrin treated screens (Figure 2.25). The significantly shorter stay on Permethrin half-screens relative to the control screen argues for some perception of chemical presence, possibly via some irritant effect.

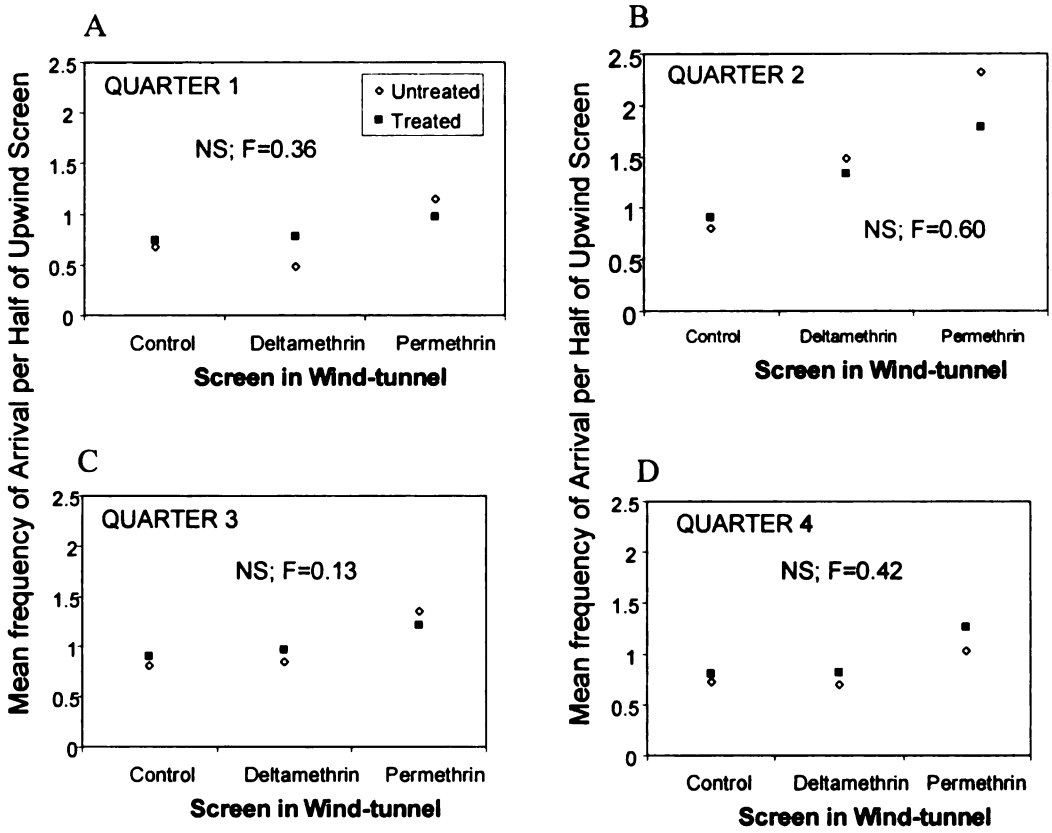


Figure 2.24. Mean total frequency of arrivals by *Anopheles gambiae* on upwind screen in a wind tunnel having screens either fully untreated or half-treated with deltamethrin or permethrin, as broken out by quarters of the 30-min observational period.

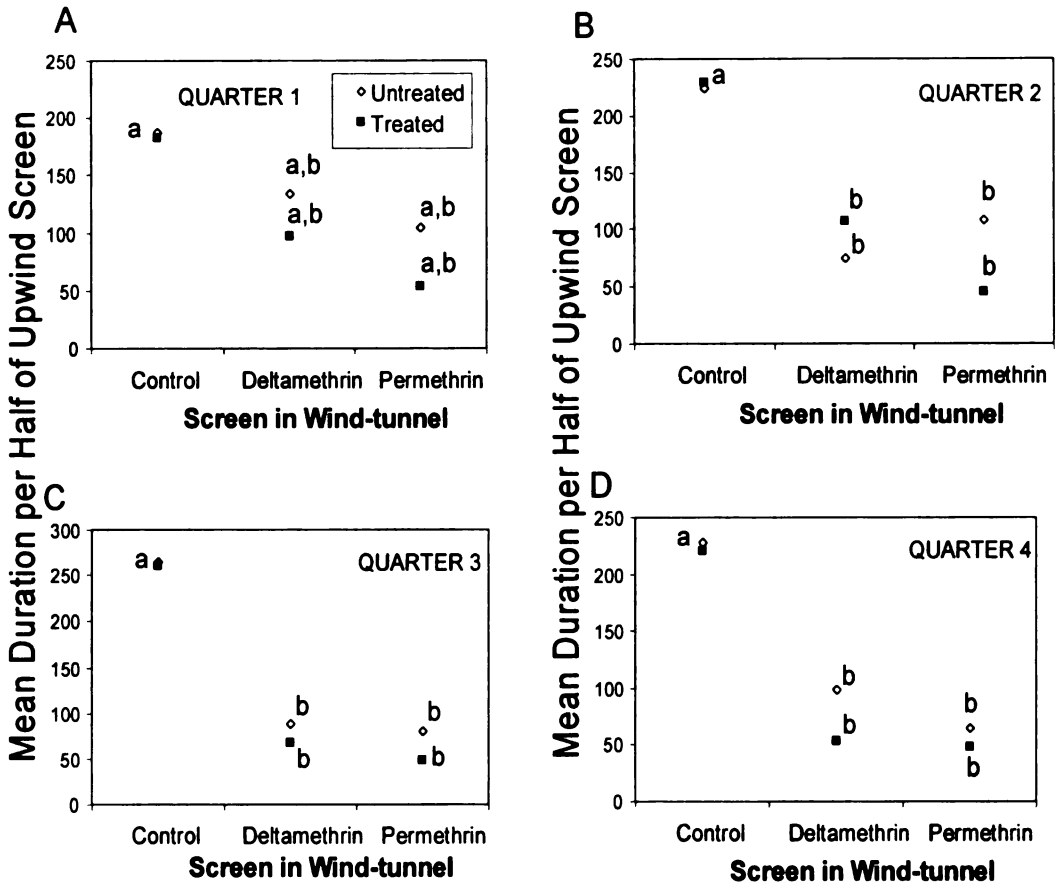


Figure 2.25. Mean total durations of visits by *Anopheles gambiae* on upwind screen in a wind tunnel having screens either fully untreated or half-treated with deltamethrin or permethrin, as broken out by quarters of the 30-min observational period.

DISCUSSION

Main pre-knockdown effects of ITNs on An. gambiae *behaviors*

Whether in fully-treated or half-treated static cages, or in a wind tunnel with a fully-treated or half-treated upwind screen, ITNs profoundly affected the behaviors of *An. gambiae* females within 15 min and killed many of them by 30 min. This was expected based upon the large body of previous toxicological data (Bondareva et al., 1986; Aldridge, 1990; Kolaczinski and Curtis, 2000; Mathenge et al., 2001; Hougard et al., 2003). However, the main interest of the current study was on changes in behavior occurring especially in the first but also in the second quarters of the 30 min observations, before first knock-down. If a mosquito were to adjust its behaviors so as to escape the lethal effects of ITNs, such adjustments would need to come early rather than late in the sequence.

Seemingly, the most effective behavioral adjustment *An. gambiae* females could make to avoid the lethal effects of ITNs would be to avoid alighting upon treated surfaces or to depart quickly when contacting insecticide. However, no such evidence was found. Foregoing data indicate that unexposed females alighted on treated surfaces as soon as and as frequently on treated as on untreated surfaces (Figure 2.12 and 2.24). They reduced their sitting time (Figure 2.18D) and females having some exposure to the ITNs likewise did not avoid landing upon treated surfaces (Figure 2.13 and 2.25). Indeed, no evidence was obtained that *An. gambiae*, at any time, was capable of discriminating between treated vs. untreated surfaces. This inability was particularly telling in the wind tunnel tests where the frequency of flying upwind and alighting on treated netting equaled that for untreated netting (Figure 2.24).

Once mosquitoes had alighted upon ITN-treated surfaces and absorbed some insecticide, behavioral effects were noted well before first knockdown. Chief among them was an elevation in the frequency of flight initiation (Figures 2.5A, 2.11A, and 2.18A). Notably, mosquitoes exposed to insecticides in both static cages and the wind tunnel and destined to spend a large proportion of the total 30 min in knockdown, managed to accumulate as much flying time as did the comparable controls in their full 30 min with no knockdown (Figures 2.3, 2.9, and 2.16). Although, this locomotor stimulant effect (Figure 1.6), did not extend to walking (Figures 2.5E, 2.11E, and 2.18E), excitation was evidenced in elevated labella tapping (Figure 2.7).

These pre-knockdown behavioral effects of ITNs were subtle rather than dramatic. This is reflected in the high similarities rather than dramatic differences between the kinematic graphs for untreated controls (Figure 2.24A) vs. mosquitoes exposed to Olyset or Permanet ITNs (Figure 2.24B and C). Rather than revolutionize the structure of behaviors, ITN exposure only shifted the frequency of transitions among pre-knockdown behaviors and then added new behaviors associated with toxicosis.

Any evidence for avoidance?

No evidence at all was found for avoidance of ITNs in the static cage or wind tunnel apparati, all of which were small and impermeable to mosquitoes. Had the static cages and wind tunnel contained ports for escape, it is possible that the exit rate relative to that for control mosquitoes could have been elevated due to the locomotor stimulant effect of the ITNs. Such an effect would be similar to raising the temperature of a volume of gas molecules contained in a leaky vessel; a rise in temperature would increase the rate of efflux over that at a lower temperature. However, in a closed container, the rate of

interaction of gas molecules with all walls of the container would rise with temperature. But because escape from a closed container would be blocked, the only overall response would be an increase in pressure.

This line of reasoning suggests that expression of avoidance by an indirect mechanism like the action of a locomotor stimulant could be context dependent. The current experiments only addressed the situation where behavioral arenas were closed. In this context, there was no evidence of avoidance mediated by either taxes (repellency) or kineses (locomotor stimulant or deterrent) (Figure 1.6).

Mode of action of ITNs

In the absence of any behavioral avoidance in static cages and the wind tunnel, all evidence supports lethality as the only mode-of-action of ITNs in these given arenas.

Fit of current data with previous reports

The current data conflict with a number of previous reports suggesting that ITNs function mainly by causing mosquitoes to avoid entering houses equipped with ITNs (Lines et al., 1987; Snow et al., 1987; Lindsay et al., 1991; Chandre et al., 2000; Takken 2002; Gimnig et al., 2003) or by raising exit rate (exophily) once mosquitoes encountered ITNs inside houses (Lockwood et al., 1984; Threlkeld 1985; Rutledge et al., 1999; Chareonviriyaphap et al., 2001; Mathenge et al., 2001). Several reasons can be suggested for the differences in outcomes of the current study relative to those reported previously: 1) the pyrethroids used here were highly pure (not accompanied by solvents or any other carriers) and of larger rather than smaller molecular weight, 2) the long-lasting net formulations used here, where insecticide is deeply imbedded in the fabrics by factory processes, may reduce the surface concentrations and rates of evaporation of insecticide

from ITNs as compared to nets treated only by dipping in insecticide solution, 3) detection of avoidance might require presence of sizable openings in the apparatus through which mosquitoes can readily exit, and 4) all mosquitoes in the current study were naïve and never previously exposed to any insecticide; perhaps pronounced avoidance requires learning.

Finally, we note that some other studies report results similar to those reported here. For example, Cuzin-Ouattara et al. (1999) and Maxwell et al. (1999) reported repeated contact of many mosquitoes with ITNs resulting in mass killing as expressed by a significant reduction in mosquito densities in treated areas. Miller et al. (1991) reported different killing abilities by different ITNs and Guillet et al. (2001) showed that *An. gambiae* that entered huts with nets treated with only deltamethrin (Permanet.) or bifenthrin 50mg a.i./m2 gave high mortality-rates (81-86%). The above observations point out that interpretation of the effects of ITNs will likely require careful documentation of both the mosquitoes being used in a study, as well as the spatial details of the apparatus. Answering the central question of what is the mode-of-action of ITNs in actual house settings will require that the tests be done under conditions exactly reflecting the houses in a given area where ITNs are deployed. The next chapter of this dissertation attempts to reflect housing conditions of the Luo people of Western Kenya near the city of Kisumu.

CHAPTER 3

Quantifying Redistribution and Survivorship of *Anopheles gambiae* in a Multi-Room Choice Arena Presenting Human Sleepers under Insecticide-Treated vs. Untreated Bed-Nets

INTRODUCTION

Insecticide-treated bed nets (ITNs) have proven efficacious in reducing malaria transmission, and in lessening morbidity and mortality in children in Sub-Saharan Africa (D'Alessandro et al. 1995; Binka et al. 1996; Nevill et al., 1996; Habluetzel et al., 1997; Lengeler et al., 2000; Schellenberg et al., 2001; Gimnig et al., 2003a, Phillips-Howard et al., 2003b). Entomological evaluations have documented population suppression of *Anopheles gambiae* Giles and *An. funestus* in the intervention areas (Gimnig et al., 2003a), and a positive community-wide improvement in health of occupants in nearby houses without ITNs (Hawley et al. 2003b). ITNs have been adopted by the World Health Organization (WHO) as efficient tools for malaria vector control and are now utilized on an increasingly large scale in the Roll Back Malaria initiative (WHO, 1993; RBMP, 2001). In the face of projected widespread and long-term distribution of ITNs, attention is shifting from immediate efficacy to sustainability, with emphasis on new and long lasting net formulations.

Surprisingly, knowledge is limited as to how ITNs affect mosquitoes, which is one of the key issues in addressing their long-term impact. Yet understanding how to manage ITNs for sustainability requires understanding their mode of action, including the behavioral interactions between mosquitoes and ITNs.

Two main explanations have been postulated for ITN mode-of-action. First is a killing effect, whereby the density and mean age of local populations of vectors is reduced and transmission drops accordingly (Magesa et al., 1991; Guillet et al, 2001). Mosquitoes visiting ITNs while host seeking were envisioned to absorb sufficient dosages of toxicant to cause knock-down and death, even after they may have flown off the net and displaced to other locations inside or outside a house. This outcome is revealed in "cone tests" (Kolaczinski and Curtis, 2000; Hougard et al., 2003), where mosquitoes are confined in small plastic cones clipped onto ITNs. However, in actual African houses, finding dead mosquitoes on the dirt floors is difficult, given the considerable visual interference and presence of scavengers such as ants. Thus, definitive evidence for direct lethality of ITNs under house-use has been weak, and its demonstration required forced contact assays, that may not reflect what happens normally.

Other researchers contend that the primary effect of ITNs is to cause avoidance of the treated net, typically measured by demonstration of contact or non-contact "excito-repellency" and/or "contact irritancy", as reflected by reduced entrance into houses or rooms in which ITNs are hung, and correspondingly increased exophily or exit rates from these domiciles (Lines et al., 1987; Lindsay et al., 1991; Mathenge et al., 2001). The terminology used to describe the consequences of exposure to ITNs by direct contact or by proximity but non-contact is diverse and sometimes confusing. Vocabulary is often influenced by the device used to conduct a test, and has a long historical context extending to evaluation of indoor spraying of insecticides for malaria control. The term "avoidance" is a reasonable word used to capture this non-lethal effect. The various

apparatus for these studies have included tunnel tests and hut studies, both attempting to model small and large-scale mosquito behaviors in response to treated nets in the presence of host cues. For hut studies, the protocols have varied between East and West Africa. In West Africa, entrances are narrow slits located at window-height; exit traps are conical and placed within doorframes. In East Africa, entrances are the house eaves; exit traps are placed in front of window frames. In both settings, test designs have generally used multiple houses arranged in rows, and have relied on visitations by wild mosquitoes, usually in locations where adult production is heavy, such as from rice fields. Variation can be imparted by: heterogeneous spatial distribution of mosquitoes, shifts in breeding sites during a test, subtle differences between huts, as well as hut structural designs.

Whether avoidance, lethality, or a combination of the two is most desirable for optimal and sustained ITN function is still unclear. Both may operate in implementation programs, but the degree of one effect or the other could be a function of formulation of insecticide on the net and time. Additional studies are needed that focus on the newer, long-lasting ITNs (Long Lasting Impregnated Nets or LLINs) that are and will be increasingly deployed for the next decades.

This study reports on tests using a new, house-like apparatus, where conditions are well-defined. Mosquitoes are released into a common courtyard connected to four sleeping rooms only by eave-like openings at 2 m height. This type of design permits manipulation of number, age, and strain of mosquitoes. Also, care can be taken that rooms are equivalent. The study addressed interactions of *An. gambiae* with Permanet, Olyset, and NetProtect ITNs. The objective was to determine whether the data supported

avoidance vs. lethality of ITNs deployed in a house-like apparatus with large eve-openings typical of Luo housing in Western Kenya.

MATERIALS AND METHODS

Apparatus

A four-choice apparatus permitting overnight sleepers in a secure arrangement was constructed at the Center for Vector Biology and Control Research (CVBCR) in Kisian, Kenya; it is a field station of the Kenya Medical Research Institute, a national biomedical research organization. It was named the Mosquito Hotel (Fig. 3.1). To

Figure 3.1. Photograph of the *Mosquito Hotel* on site, showing the main entrance to the central courtyard, and the exterior of two of four sleeping rooms.

preclude ants and other predators/scavengers, the structure was built on a platform, supported by 1 m posts resting in 10L metal cooking pots each containing ca. 7 L of used automobile engine-oil.

The floor plan is given in Fig. 3.2. Walls and rafters were 5 cm x 10 cm wood construction. Sleeping rooms were roofed by corrugated iron sheets. The exteriors of sleeping rooms were plywood, painted white. The interior walls of the sleeping rooms were covered with white, 25 mm thick Styrofoam sheets for insulation. Further, the insulation and plywood flooring were tightly lined with a removable, white polyester textile (flexibly woven, 70 g/m^2).

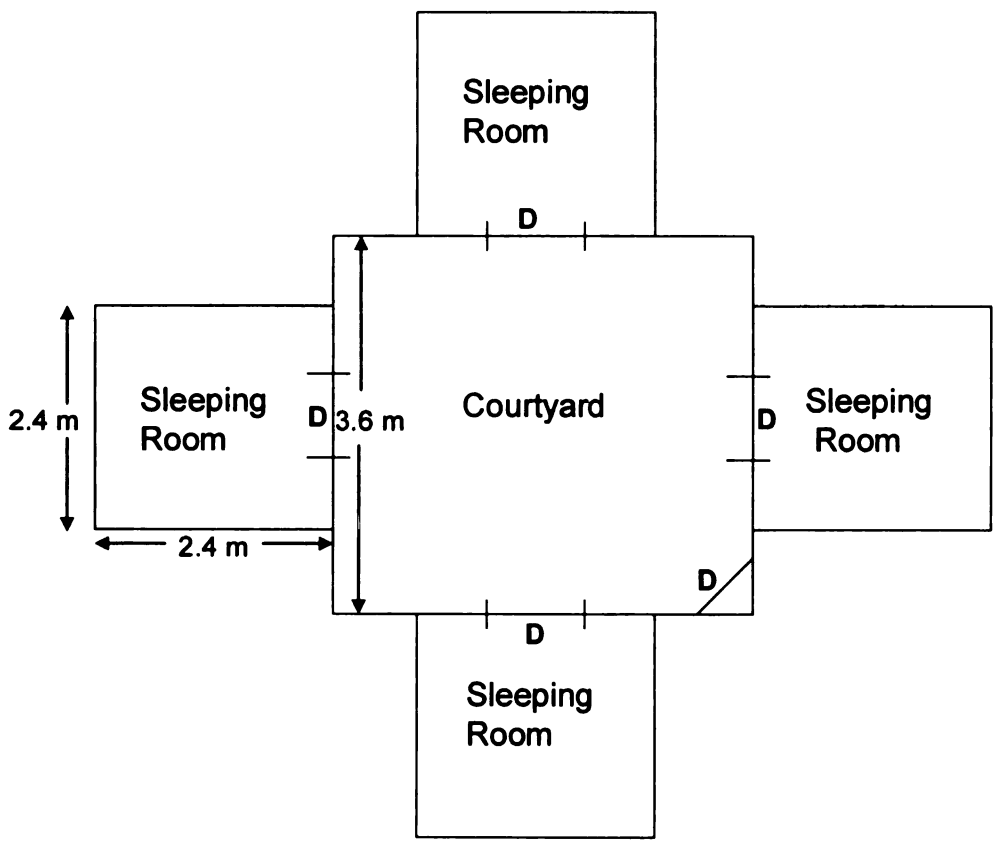


Figure 3.2. Floor plan of the *Mosquito Hotel* showing the configuration of the central courtyard and sleeping rooms. D = door.

Doors leading from the courtyard to each sleeping room measured 1.8 x 0.9 m. They were fashioned from textile split with a vertical zipper. Beds were 1.8 x 0.9 m with a foam mattress set 60 cm above the floor on a wooden frame. Rectangular nets were hung by strings anchored at the four corners where the roof joined side-walls. During tests, nets were tightly tucked under the mattress. Mosquitoes were able to access spaces under the beds. Above each door was a 20 cm high x 110 cm wide "simulated eave" opening for mosquitoes to enter or leave a sleeping room (Figure 3.3.a.). This opening was fitted with the restrictor apparatus shown in (Figure 3.3.b). The top of the restrictor consisted of netting attached to a bar that could be moved to adjust the opening from 3 to 20 cm. The screening allowed for similar levels of air exchange at any opening size.

The floor of the 3.6 x 3.6 m courtyard was also textile-covered; its ceiling was sealed with nylon mosquito netting (mesh 50 g/m^2). A 6 x 6 m green canvas completely covered the courtyard and extended over the corners of the sleeping rooms (Figure 3.1). Air exchange occurred between the canvas courtyard roof and netted ceiling. The space between sides of sleeping rooms and courtyard corners was covered with untreated mosquito netting over fabric. Between tests the fabric was unzipped to enhance ventilation of the courtyard. Night-time light intensity in sleeping rooms was 0.13 - 0.24 lux, while that in the courtyard was 0.91 – 1.30 lux; it was provided by security lamps in the vicinity of the Mosquito Hotel.

Three brands of long-lasting insecticidal net (Permanet, Olyset and Netprotect) and a net treated with alphacypermethrin (Fendona) were used in this study. Permanet, manufactured by the commercial textiles firm Vestergaard-Frandsen, is made of polyester fibers which contain ca. 55 mg deltamethrin per square meter of fabric, and is designed

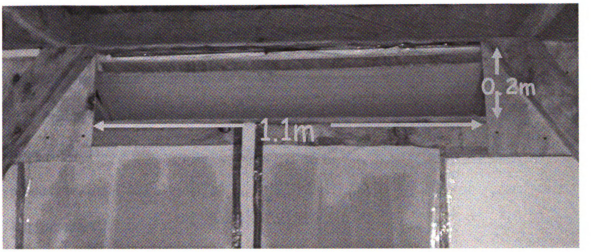


Figure 3.3.a. Simulated eave opening above the entrance of a sleeping room

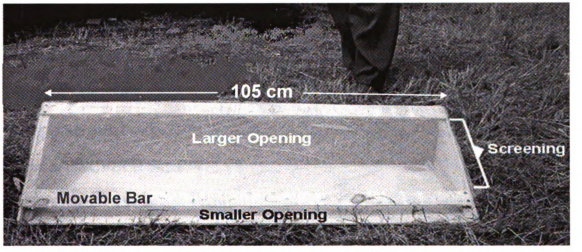


Figure 3.3.b. Entrance restrictor with screening and movable bar for adjustment of eave opening to 3, 10 or 20cm. The smaller of the two openings projected into the sleeping room.

so that the insecticide remains effective for 3-5 years. Netprotect has similar composition to Permanet; it has a polypropylene fiber. It was provided by the Intelligent Insect Control Company. The Olyset net marketed by the Sumitomo Chemical Company,

consists of ca. 1 g of permethrin incorporated per square meter of polyethylene fiber. Fendona is a recent long-lasting insecticidal net from the BASF Corporation; it uses alphacypermethrin at 25 mg per m^2 of fabric. Both Permanet and Olyset have been approved by the World Health Organization Pesticide Evaluation Scheme (WHOPES) for use in malarious areas. Netprotect and Fendona are still undergoing evaluations and efficacy testing.

Test protocol

Nets to be tested were hung outdoors in the shade for two d prior to deployment in sleeping rooms. This permitted release of any immediately volatizing active ingredient built up on surfaces while in the packaging. Preparations for a nightly test began at ca. 15:00 h when beds were prepared and nets were draped over them. Using hand-held sprayers, the floor and wall fabric linings of sleeping rooms were wetted with well water, along with the courtyard floor. Each room received 3 L and the courtyard floor received 6 L. Typically, two hundred 2-3 day-old mosquitoes from the Kisumu laboratory strain reared at the CVBCR of KEMRI, fed only 10% sugar water, were carried to the test site in 11cm diam. x 15 cm high paper cups (50 per cup) with screened tops. Mosquitoes were placed near the wet courtyard floor and allowed to acclimate. At about 21:00 h four sleepers entered the courtyard, opened the paper cups, and retired to their assigned beds for the night, making sure that their door was zipped and the eve-entrance cover was open.

At 6:30 h sleepers arose and closed the entrance apparatus; zipped all doors, and departed. Between 7:30 –8:30 h, 2-4 technicians arrived and carefully collected and counted all dead and alive mosquitoes from the courtyard and each sleeping room.

Search for mosquitoes was intensive and involved turning over and searching beneath the beds. Dead and alive mosquitoes were counted. Live mosquitoes were transferred to previously unused, 11 cm diam. x 15 cm holding cups and given access to 10% sugar solution and held in ca 75% relative humidity and subdued light in the laboratory. After 24 h, a final count was taken of the numbers of live vs. dead mosquitoes. Thus, for any given test, the number of mosquitoes occupying each room was recorded, as was the number alive and dead the morning after the experiment. The number found dead upon the first morning after release will sometimes be referred to as "immediate mortality", and the number dead after 24 h as "delayed mortality".

Room equivalence tests and effect of opening height

To test for equivalent performance of rooms, untreated nets were hung in all four rooms. Four sleepers were randomly assigned to rooms the first of four nights. Sleepers then rotated clockwise on successive nights until all persons had slept in all rooms. This procedure was done with entrance openings set at 3, 10, and 20 cm.

Experimental design for testing ITNs

In most experiments reported here, two rooms were assigned control (no-insecticide) nets, and two were assigned treated nets, except in the case where three treated nets were used. Again, each of four sleepers rotated through all rooms. Data recorded each test day were: dead and alive mosquitoes in courtyard and each room, blood fed vs. non-blood fed, additional mortality after 1 d, sleeper identity, and net type in the room. A temperature and relative humidity profile were recorded by HOBO data-logger placed in each room and courtyard at a height of 1.5 m. After a round of testing,

beddings and fabric room linings were removed, soaked in quaternium detergent (A.I.S.E., Brussels) for 1 d, and flushed in clean water, and dried in the sun before reuse.

Effects of* An. gambiae *strain and age

The Kisumu laboratory strain and F1 progeny of wild-caught *An. gambiae* were reared at the CVBCR of KEMRI, and adults were fed only 10% sugar solution. The above-described test protocol was followed. To test for their survival and equivalent occupancy of rooms, untreated nets were hung in all four rooms. Four sleepers were randomly assigned to rooms the first of four nights. Sleepers then rotated clockwise on successive nights until all persons had slept in all rooms. A total of 12 replicates for each strain was performed.

Experiments on the effect of mosquito age were similarly performed but used the Kisumu laboratory strain only. The 16 day-old females were previously blood-fed, then allowed to lay eggs; then they were kept hungry and maintained on 10% sugar solution only for, 3-4 days before the test. Young mosquitoes were as described above.

Variations in site of release

To assess the extent to which mosquitoes visiting sleeping rooms were likely to exit to the courtyard, four tests were done where 50 females were released into each sleeping room and four where all 200 females were released into one room with no releases into the courtyard. All other parts of the above protocol were followed and all cloth linings of the sleeping rooms and courtyard were changed after using each of three net types: Permanet, Olyset, and Netprotect.

Data analysis

Data were analyzed by ANOVA, implemented in SAS (2000). Data were proportions derived from counts of mosquitoes, and were transformed by the arcsine of the square root. For room occupancy, the denominator in the proportionate data was the number released in the courtyard. For mortality, the denominator was the number alive and dead in the room the morning after the trial. To account for variations among rooms and sleepers, each room was considered a separate treatment in the analysis, even if the same type of net was hung in that room, as sleepers were rotated. Means were separated using Tukey's multiple comparisons test with a significance level of $P < 0.05$.

RESULTS

Temperature/RH profiles

Figure 3.4 shows selected plots of temperature and RH in the sleeping rooms over the course of some experiments. They represent the highest and lowest average night-time conditions in the test rooms and two mid temperatures. When the sleeper arrived in a room about 21:00 h, the temperature rose slightly; when the sleeper exited, the temperature fell slightly. After 6:30 h, when the rooms were evacuated and the sun appeared, the temperature in the rooms rose steadily, while the RH fell. Figure 3.5 presents selected room temperatures and RH taken at varying times of year. Higher temperatures and lower RH were evident in March than during subsequent months. Average room temperature and RH fluctuations across the seasons were modest. Room temperature fell to a minimum when sleepers exited the Mosquito Hotel, permitting some

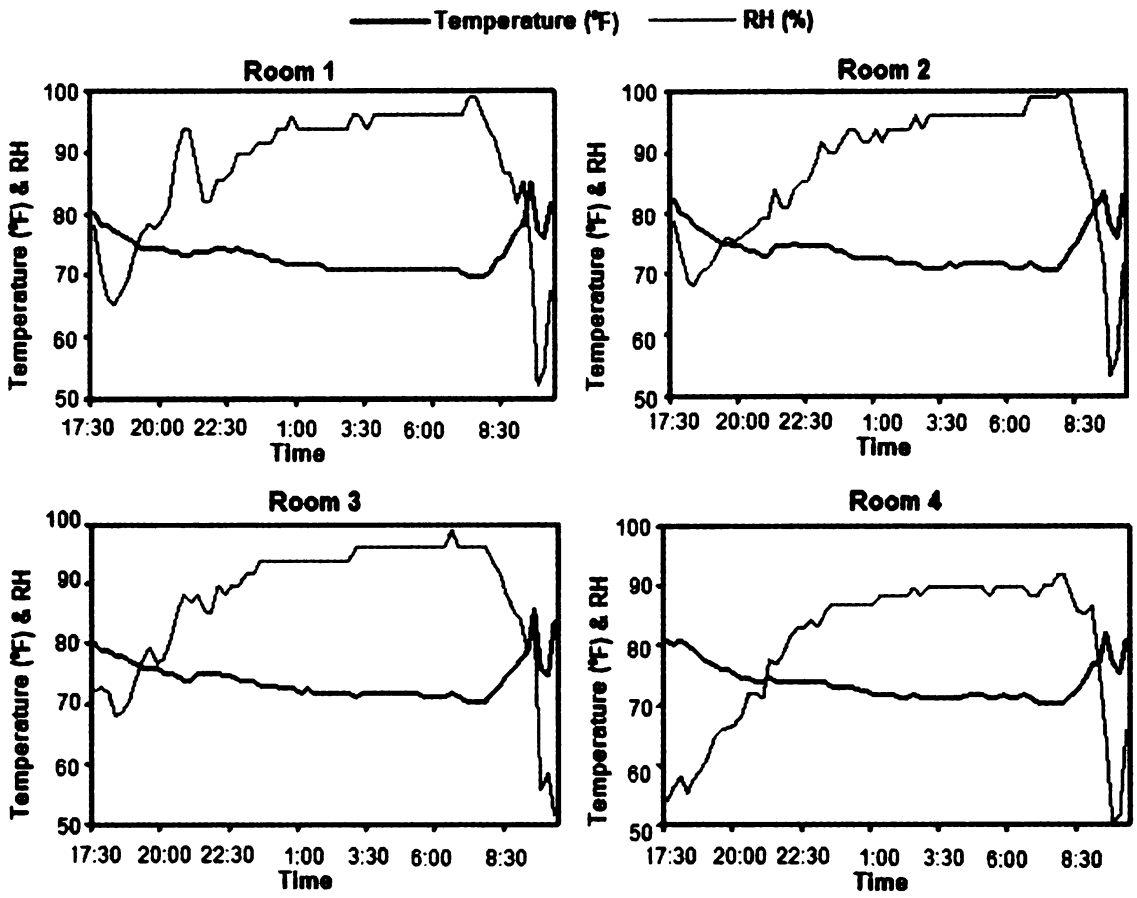


Figure 3.4. Plots of temperature and RH in the sleeping rooms on a single test day. Temperature is expressed in °F rather than °C for convenience in co-plotting %RH with Temperature.

cooler outside air to enter the apparatus. Temperature and RH across the four sleeping rooms therefore are shown to be equivalent.

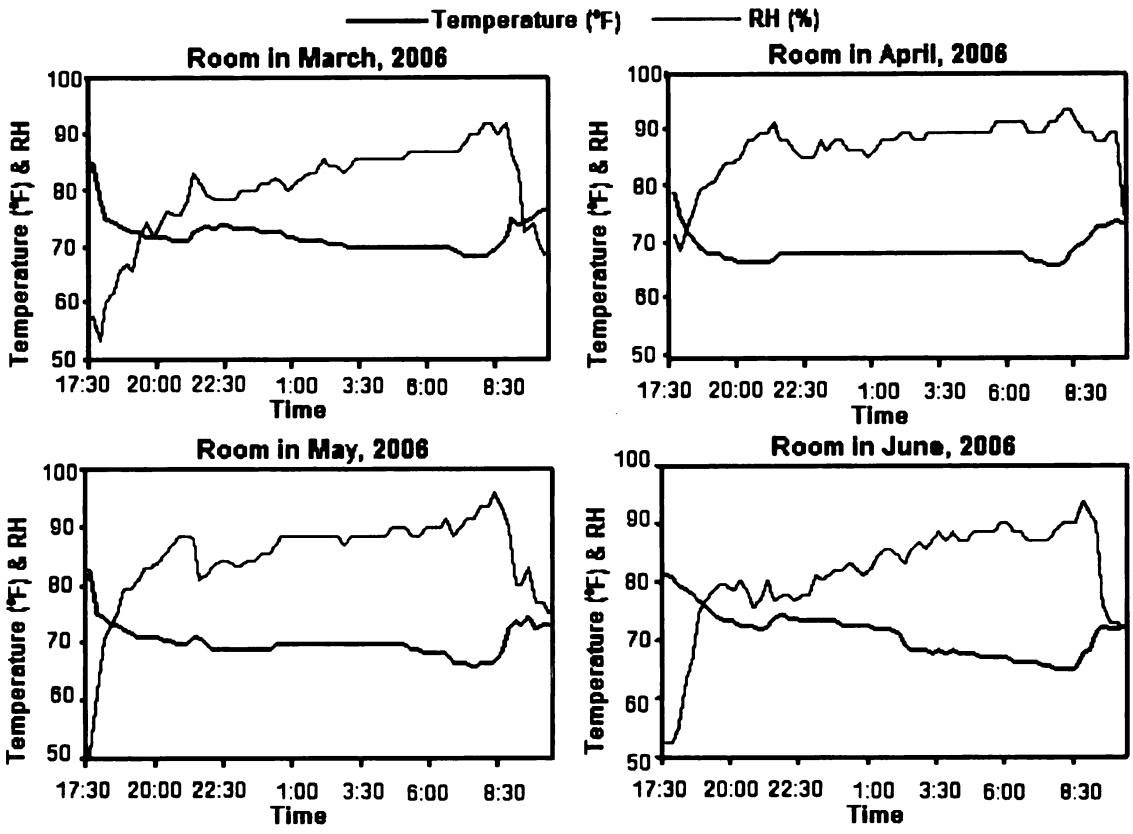


Figure 3.5. Plots of temperature and RH of sleeping rooms on different dates and test months.

Room equivalence tests and effect of opening height

Tests carried out with untreated nets in rooms at a uniform eave opening height of 20 cm for all rooms, were statistically insignificant in mean percent occupancy at $P<0.05$ (Fig. 3.6). Room occupancy for certain sleepers was significantly higher than for other sleepers. However, because sleepers were always rotated through all rooms, this effect was inconsequential with respect to results and conclusions to follow.

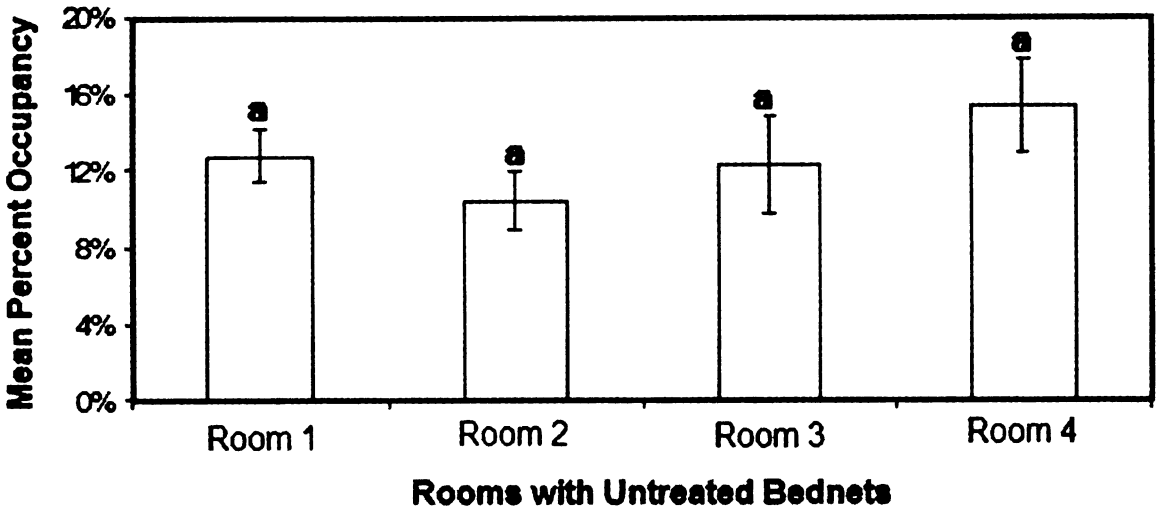


Figure 3.6. Mean percent *Anopheles gambiae* occupancy in rooms with untreated nets for simulated eave opening height of 20 cm. Two hundred mosquitoes were released on each of 12 nights of testing.

Mosquito occupancy in all four rooms with eave opening heights of 3 cm, 10 cm and 20 cm are shown in Figure 3.7a. There was no significant effect of eave opening height for entrance rate/occupancy of female *An. gambiae* s.s. ($P = 0.31$). Similarly, the mean percent mortality after 24 h was below 10% and not significantly different between the three opening heights (Fig. 3.7b).

Effects of ITN type

There was no significant difference in occupancy rate of rooms when Permanet, Olyset, and untreated nets were included in a replicated experiment with a central courtyard release of mosquitoes ($P = 0.4$) (Figure 3.8a). However, mortality at 8 and 24 h varied significantly with treatment (Figures 3.8b). Both 8 h and 24 h mortality rates were significantly higher for Permanet than Olyset or controls ($P < 0.0001$).

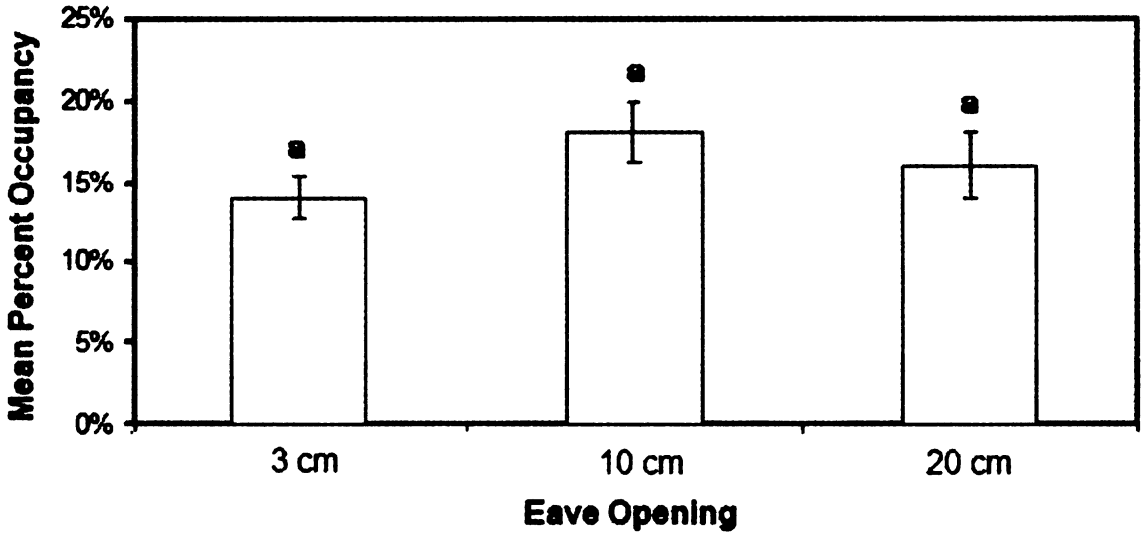


Figure 3.7.a. Mean percent *Anopheles gambiae* occupancy in rooms with untreated nets for simulated eave opening heights of 3, 10 and 20 cm. Two hundred female mosquitoes were released in the central courtyard each of 12 nights of testing.

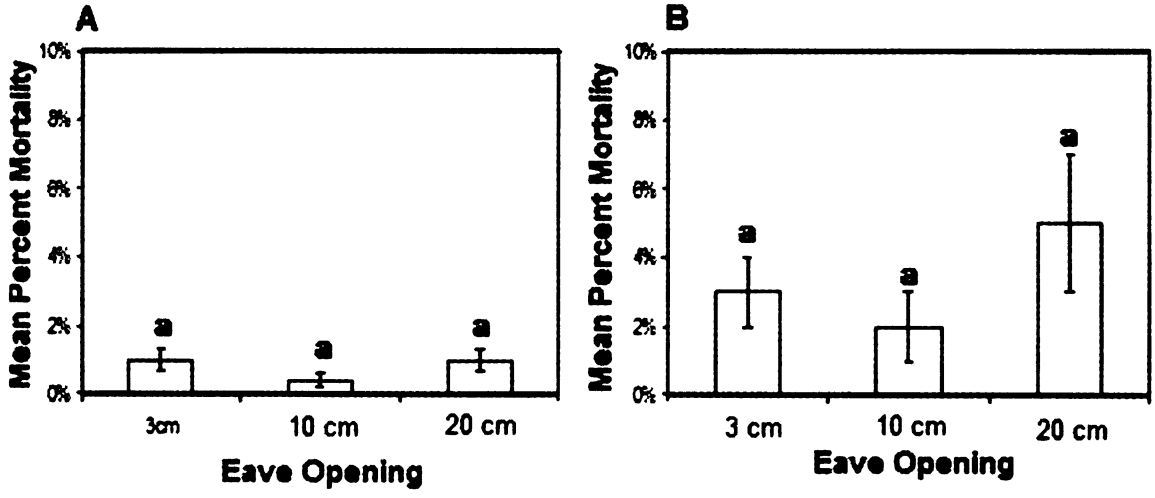


Figure 3.7.b. Mean percent *Anopheles gambiae* mortality in rooms with untreated nets for eave opening heights of 3, 10 and 20cm. A is 8 h mortality, B is 24 h mortality.

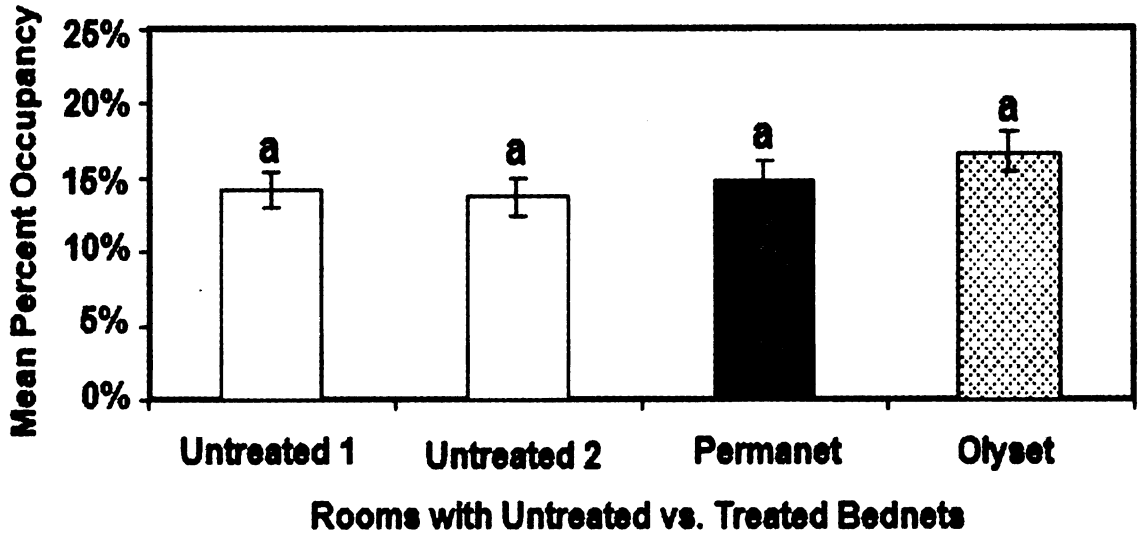


Figure 3.8.a. Mean occupancy of female *Anopheles gambiae* in sleeping rooms with Permanet and Olyset vs. untreated bednets. Two hundred mosquitoes were released in the central courtyard on each of 14 nights of testing.

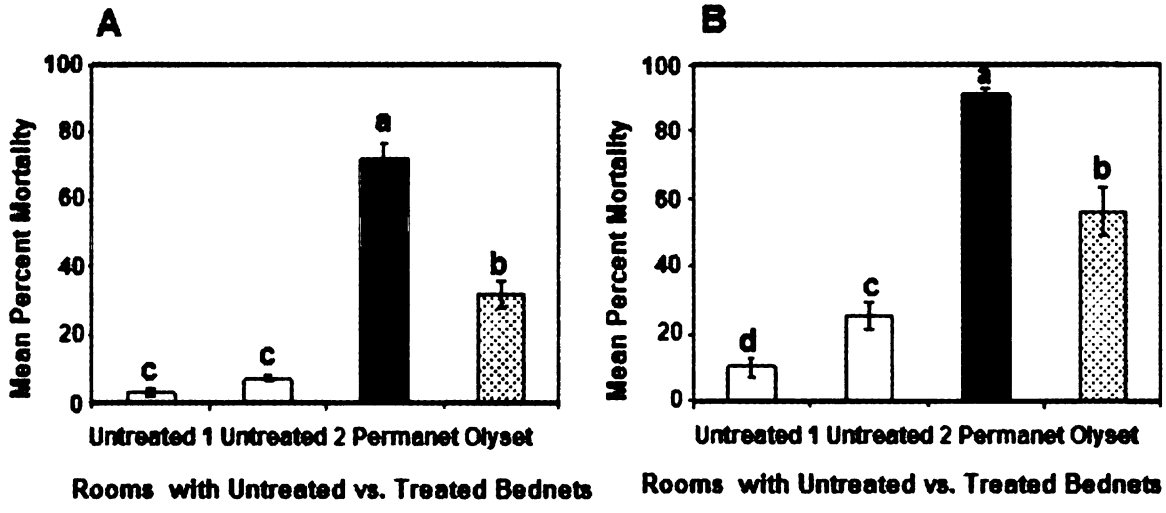


Figure 3.8.b. Eight h mortality (A) and 24 h mortality (B) of female *Anopheles gambiae* in rooms with Permanet, Olyset and untreated nets.

The mean occupancy rate for rooms fitted with Permanet, Olyset or Fendona ITNs vs. an untreated bednet was similar (P = 0.52) (Figure 3.9). Mortality at 8 h and 24 h was significantly higher for all three rooms with ITNs than for control (P < 0.0001) Figure 3.10). However, the 24 h control mortality was unusually high in this test, possibly due to mosquito handling holding problems. The overall mortality in rooms with treated nets was over 95%.

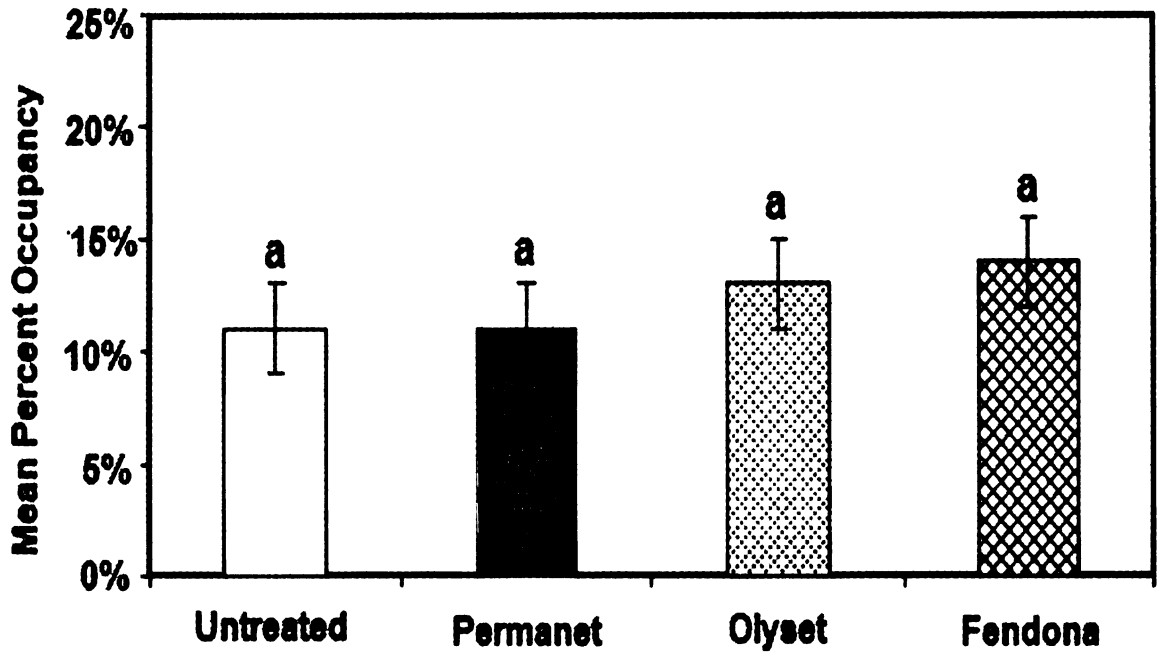


Figure 3.9. Mean occupancy of female *Anopheles gambiae* in rooms with Permanet, Olyset and Fendona vs. untreated bednets. 200 mosquitoes were released in the central courtyard on each of 12 nights of testing.

Rooms with untreated nets, and those fitted with deltamethrin chessboard nets treated with 30 x 30 cm patches of 55mg/m^2 vs. entire 30mg/m^2 nets, did not differ significantly in room occupancy (P = 0.98) (Fig. 3.11). Figure 3.12 shows 8 and 24 h

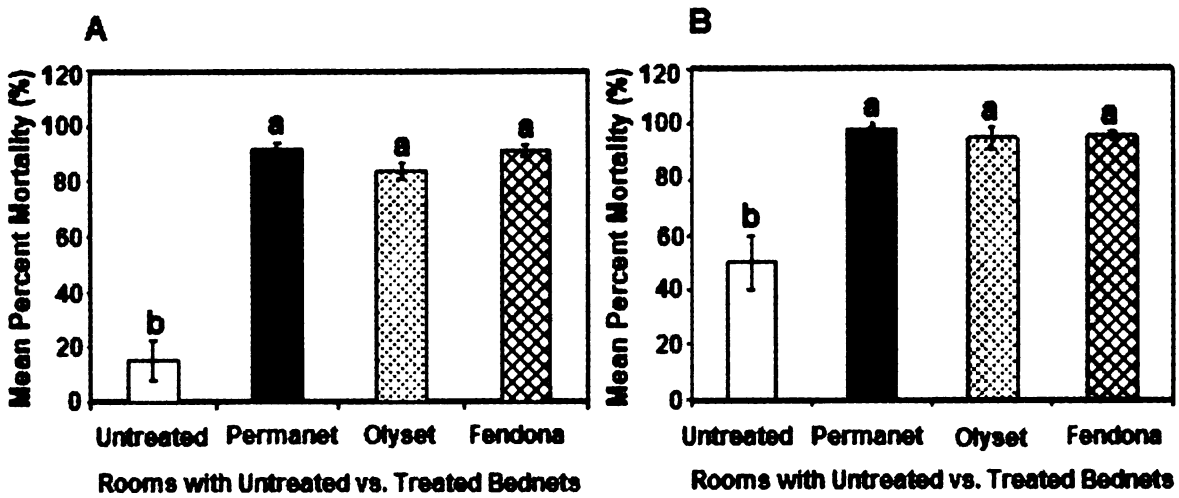


Figure 3.10. Eight h mean percent mortality (A) and 24 h mortality (B) of female *Aopheles gambiae* in rooms with Permanet, Olyset, and Fendona ITNs vs. an untreated bednet.

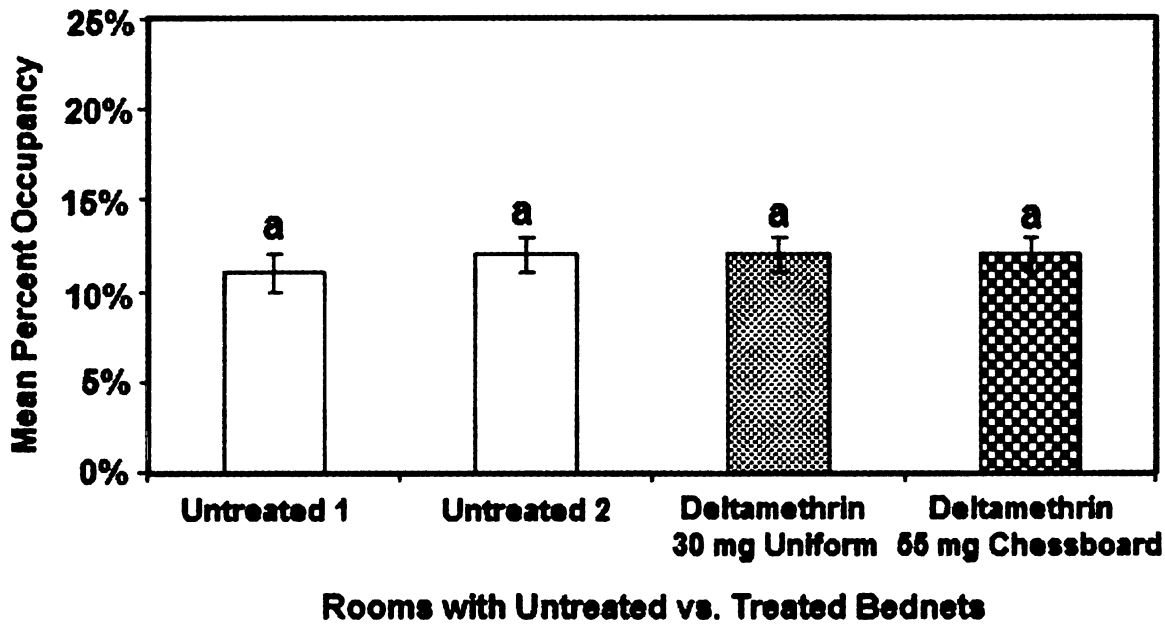


Figure 3.11. Mean occupancy of female *Anopheles gambiae* in rooms with 30 mg deltamethrin and 55mg chessboard deltamethrin vs. untreated bednets. Two hundred mosquitoes were released in the courtyard on each of 16 nights of testing.

mortality, which were significantly elevated over those for the controls ($P < 0.0001$). Mortality at 24 h was pronounced for both treated nets.

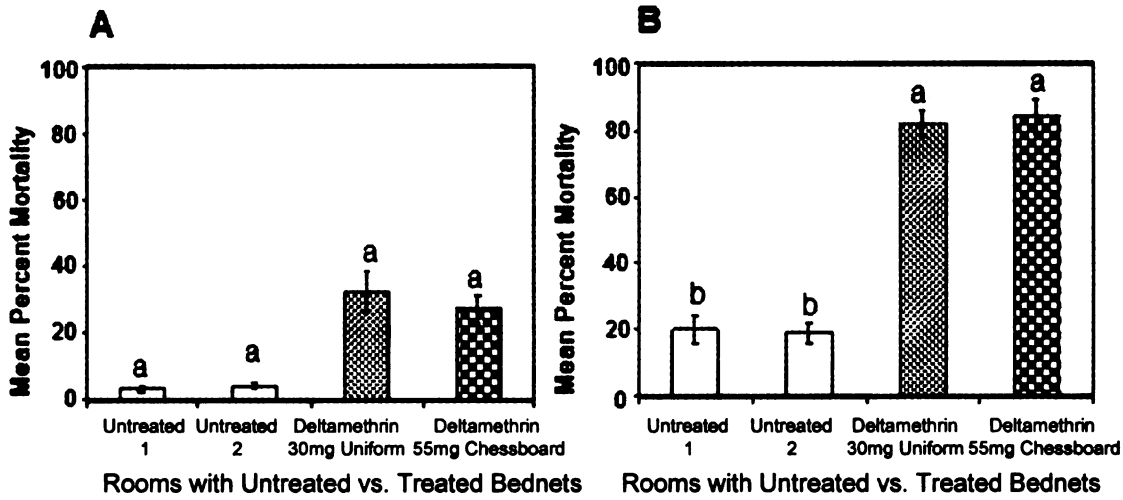


Figure 3.12. Mean 8 h percent mortality (A) and 24 h mortality (B) of female *Anopheles gambiae* in rooms with 30 mg deltamethrin and 55mg chessboard deltamethrin ITNs.

Effect of An. gambiae *Strain and Age*

No significant difference was found for occupancy ($P = 0.49$) or mortality rates ($P = 0.75$) when F1 progeny of wild-caught *An. gambiae* were tested against the Kisumu laboratory strain of mosquitoes (Fig 3.13).

The mean occupancy rate for 16-day old females was slightly but significantly higher than that for 4-day old females ($P < 0.032$). However, the mean mortality rate after 24 h was not significantly different for the two age groups (Figure 3.14).

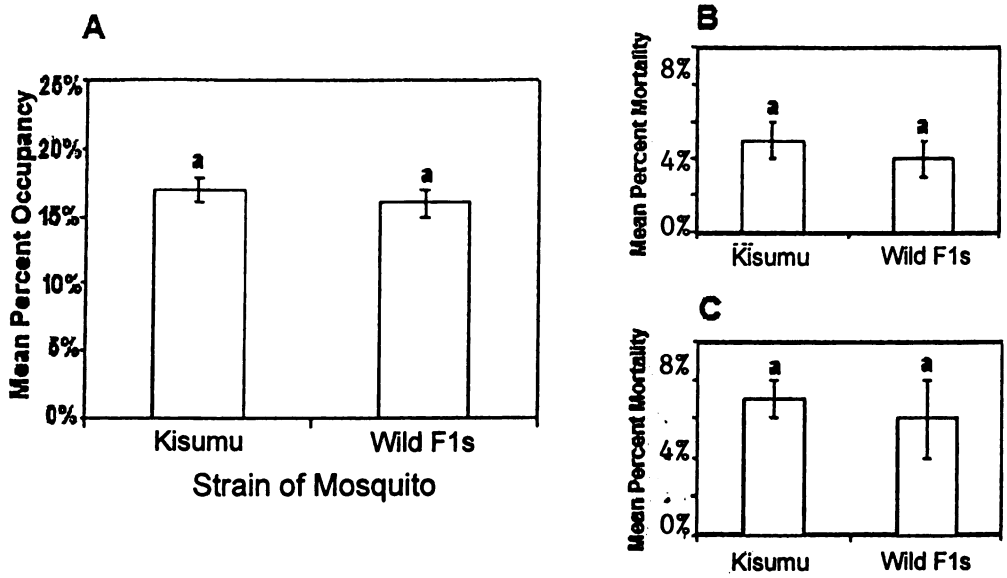


Figure 3.13. Mean occupancy and mortality of the Kisumu laboratory strain vs. F1 progeny of wild-caught *Anopheles gambiae* females in rooms with untreated bednets. (A) Mean percent occupancy, (B) 8 h mortality, (C) 24 h mortality. Two hundred mosquitoes were released in the courtyard on each of 16 nights.

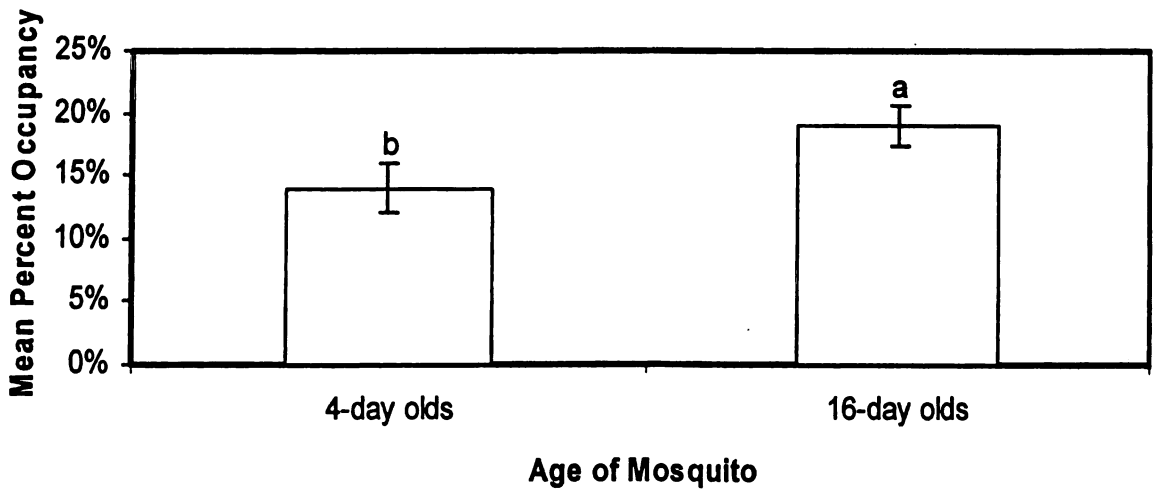


Figure 3.14. Mean percent occupancy for 4 day vs. 16 day-old female *Anopheles gambiae*. Two hundred mosquitoes were released in the courtyard on each of 12 nights.

Variation in release site

On average, more than 95% of the *An. gambiae* females released into sleeping rooms were found there and no significant differences were found between Permanet and Olyset ITNs vs. untreated bednets (Figure 3.15). Mortality at 8 h was significantly higher for Permanet vs. control rooms ($P < 0.0001$) and for Olyset vs. control rooms ($P < 0.0001$), although mortality was higher with Permanet than Olyset. The 24 h mortality was not significant different in either case from controls, possibly indicating problems with handling or holding mosquitoes during this experiment (Figure 3.16).

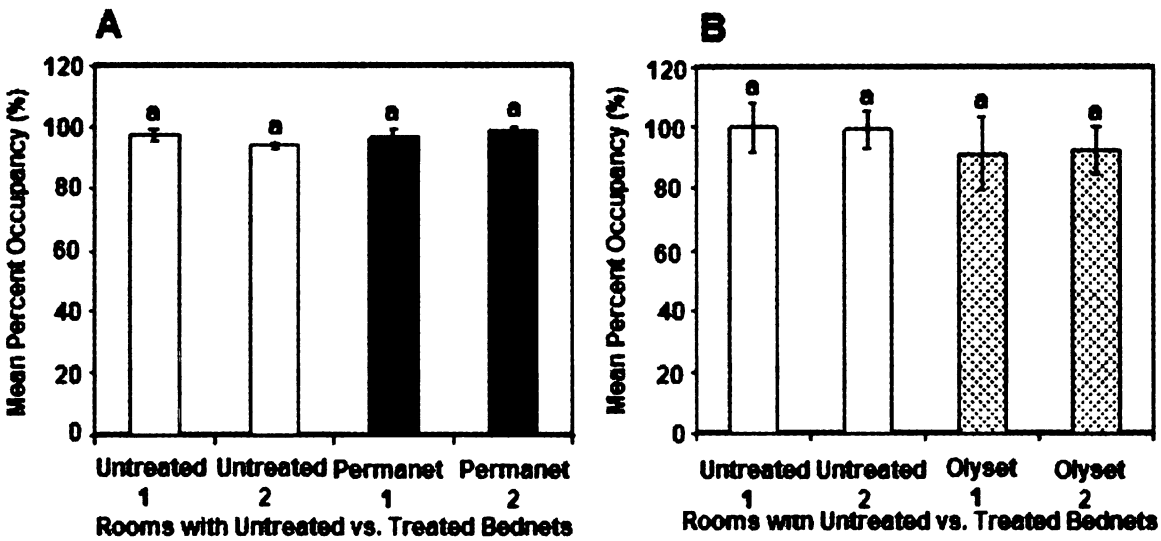


Figure 3.15. Mean percent occupancy of female *Anopheles gambiae* in rooms with Permanet vs. untreated nets (A) and with Olyset vs. untreated nets (B) when 50 mosquitoes were released into each room on each of 8 test nights.

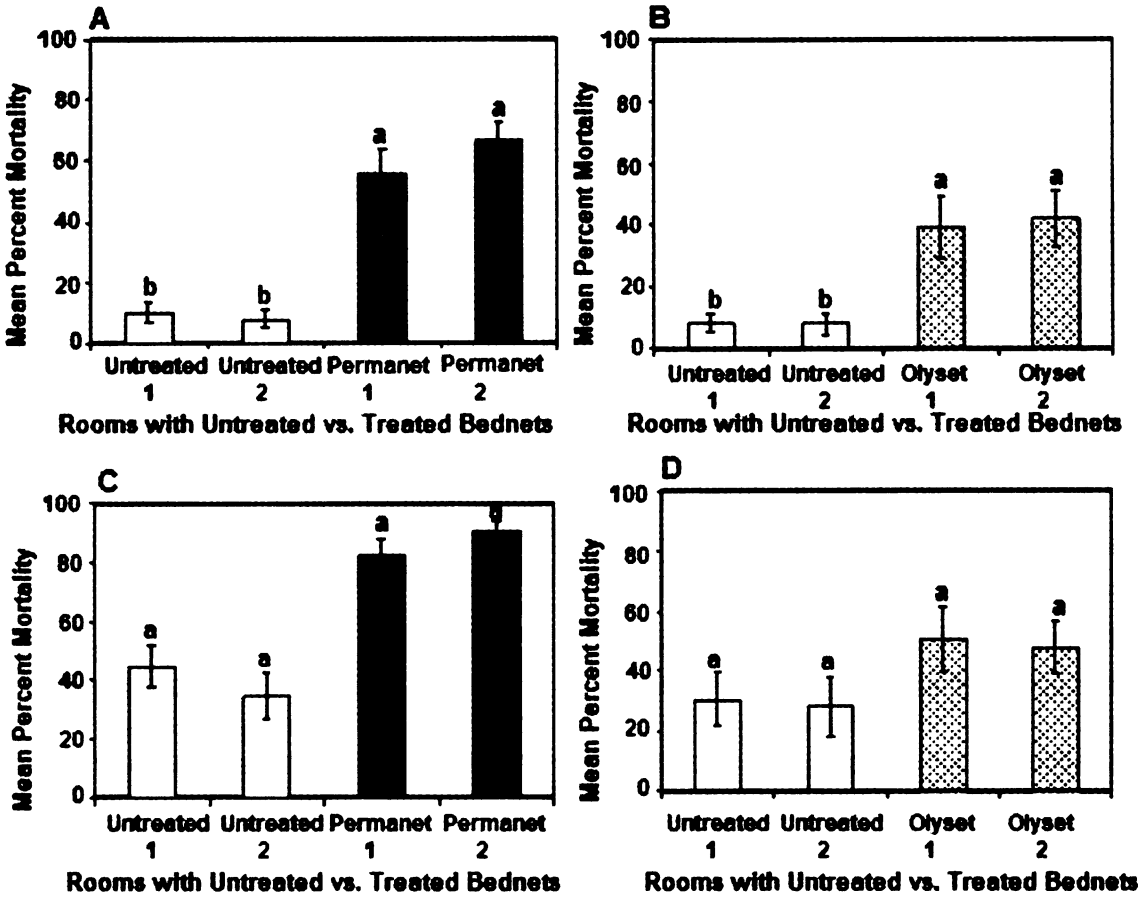


Figure 3.16. Mean percent mortality of female *Anopheles gambiae* in rooms with treated vs. untreated bednets. (A and B) = 8 h mortality; (C and D) = 24 h mortality, respectively.

There was no difference in occupancy, 8 h, or 24 hr mortality when eaves were open or closed and mosquitoes were released inside the sleeping rooms (Fig. 3.17).

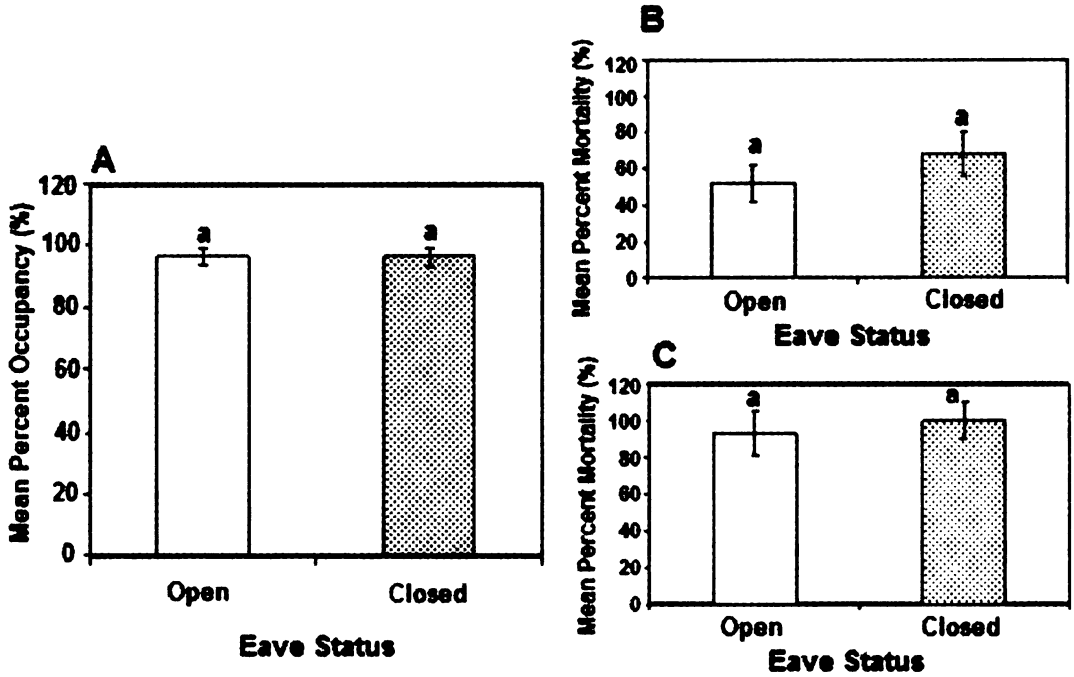


Figure 3.17. Occupancy and mortality of *Anopheles gambiae* females in rooms with eaves open or closed. A = mean percent occupancy, B = 8 h mortality, C = 24 h mortality.

When 200 females were released in one of the four sleeping rooms and their appearance in the other sleeping rooms and the courtyard was assessed after 8 h, the mean occupancy rate for the release room was 78%. A total of 7% of the emigrating mosquitoes was equally distributed in the other sleeping rooms (Fig. 3.18), and the remainder were recovered in the courtyard.

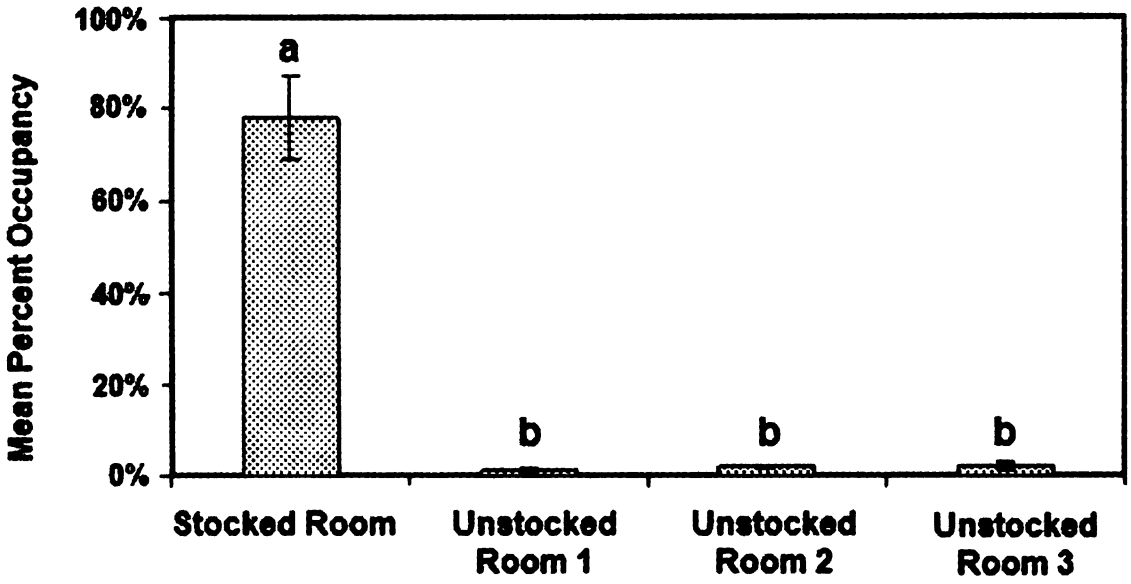


Figure 3.18. Distribution of *Anopheles gambiae* females in rooms with 20 cm eave openings when all 200 mosquitoes were released in only 1 of 4 sleeping rooms.

Netprotect

No significant differences were evident in occupancy rate of rooms by *An. gambiae* females when Permanet and Netprotect ITNs were paired against untreated bednets and mosquitoes were released into the courtyard (Figure 3.19). Mortality at 8 h was significantly elevated for ITNs over the controls ($P < 0.0007$). At 24 h, mortality was likewise significantly elevated ($P < 0.028$). Mortality rates for Permanet and Netprotect were not significantly different from each other. However, 24 h mortality for Netprotect tended to be greatest. For both Permanet and Netprotect, there was no evidence of avoidance.

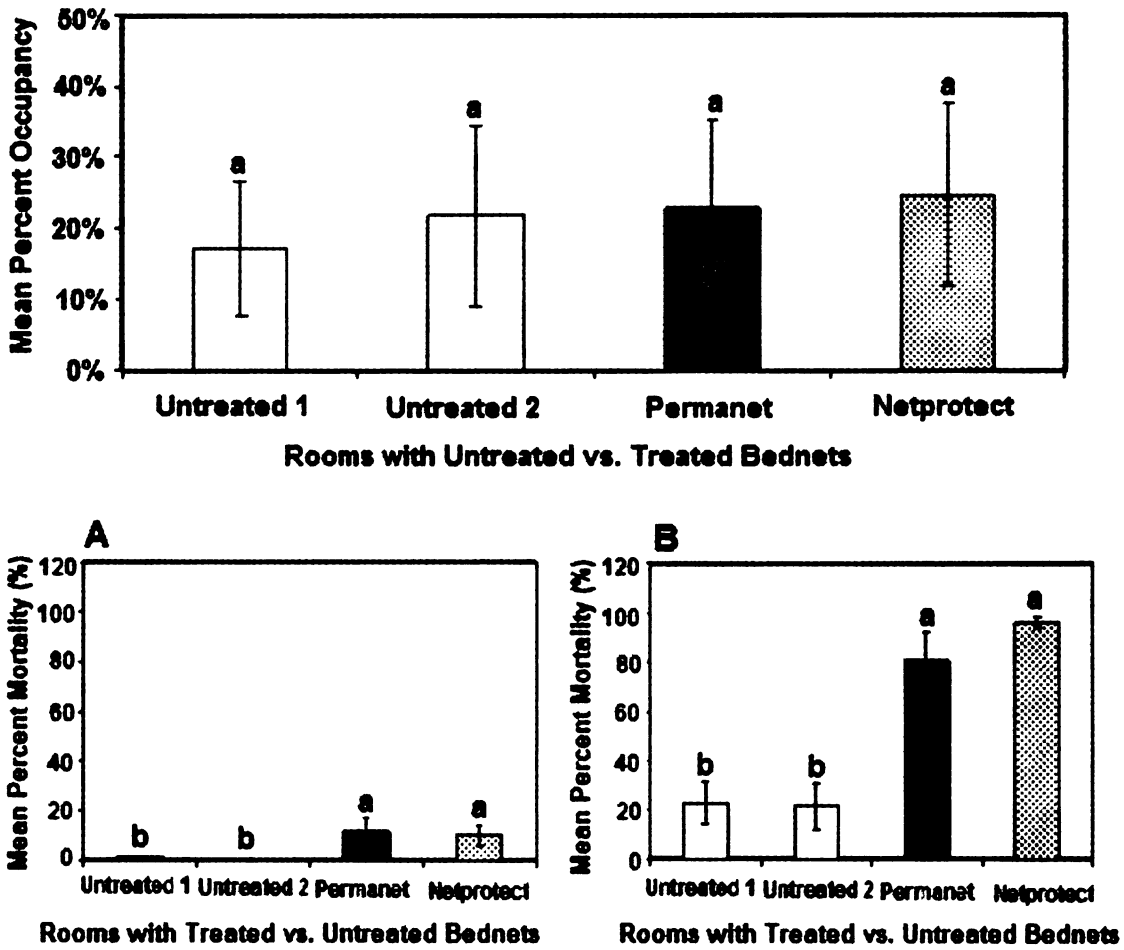


Figure 3.19. Occupancy and mortality of *Anopheles gambiae* females in rooms treated with Permanet and Netprotect vs. untreated nets. A = 8 h mortality, B = 24 h mortality. Two hundred mosquitoes were released in the courtyard on each of 16 nights of testing.

When only Netprotect was deployed in two rooms vs. untreated bednets in the remaining two rooms, the occupancy of *An. gambiae* females was slightly but not significantly higher in rooms with Netprotect than in rooms with control ($P = 0.13$) (Fig. 3. 20). Both 8 h and 24 h mortality rates were highly significant relative to the control bednets ($P < 0.0001$).

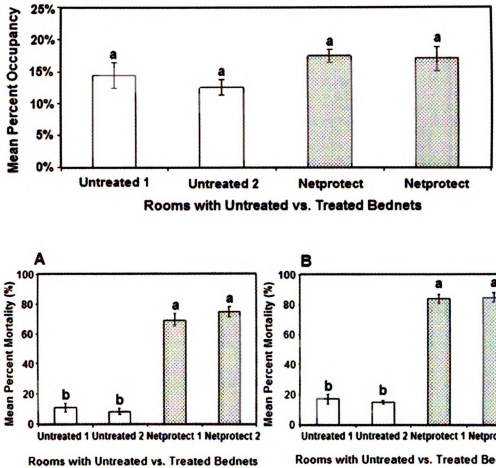


Figure 3.20. Occupancy and mortality of *Anopheles gambiae* females in rooms treated with Netprotect vs. untreated nets. A = 8 h mortality, b = 24 h mortality. Two hundred mosquitoes were released into the central courtyard on each of 19 nights of testing.

The mean occupancy rate in rooms treated with Netprotect ITNs vs. untreated bednets before washing and after one and two washes did not differ (Figure 3.21). There was no significant difference in mortality at 8 h and 24 h for Netprotect before and after washing. However, there was a significant difference between Netprotect and untreated controls for all three treatments ($P < 0.0001$).

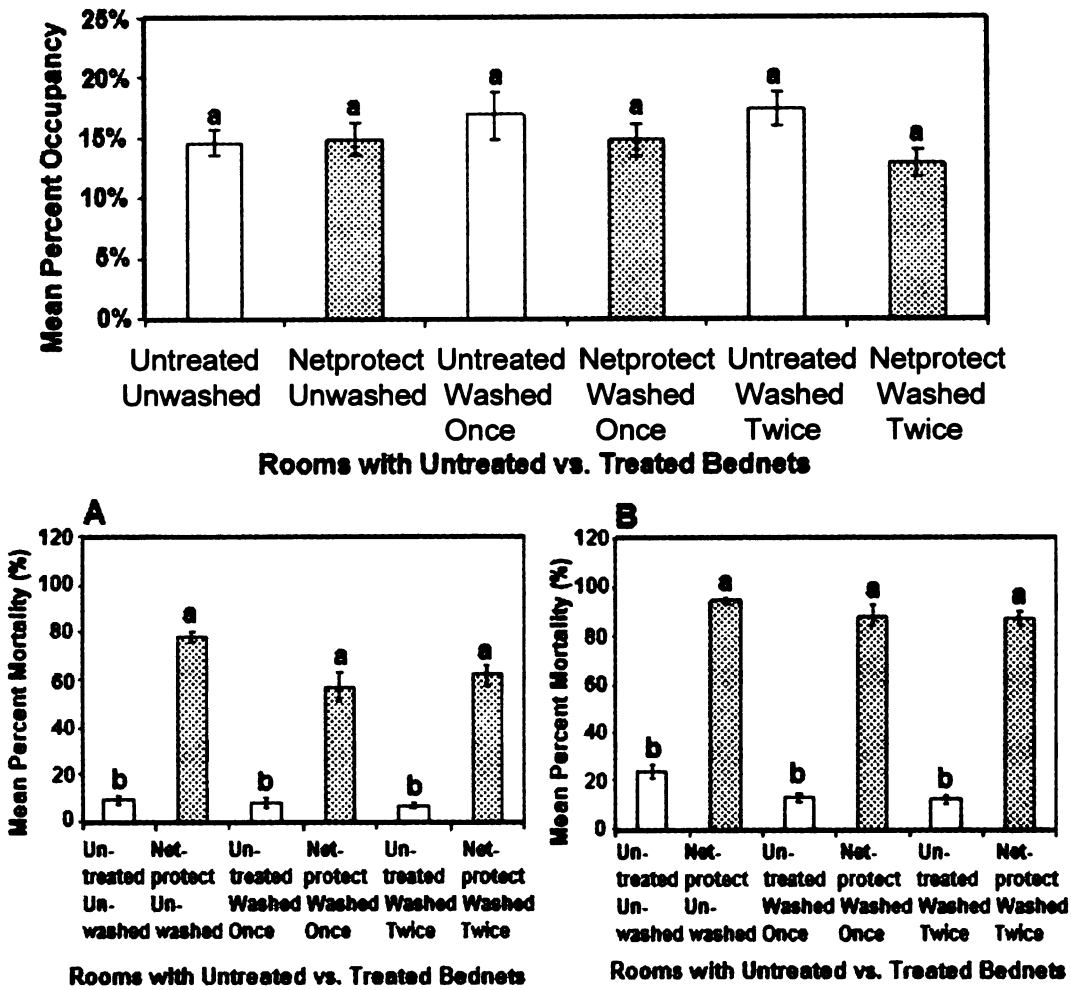


Figure 3.21. Occupancy and mortality of *Anopheles gambiae* females in rooms treated with Netprotect vs. untreated nets before and after washing. A = 8 h mortality, B = 24 h mortality. Two hundred mosquitoes were released in the central courtyard on each of 16 nights of testing.

Mortality after 24 h for Netprotect was remarkably high. No evidence for avoidance was found for any treatment.

DISCUSSION

Performance of the test apparatus

The "Mosquito Hotel" proved effective as a tool for gathering data helpful in elucidating the mode of action of ITNs at the house-scale. Arranging four equivalent sleeping rooms around a common courtyard into which a common pool of mosquitoes was released and having a common set of four sleepers always rotate through each of the four rooms provided unusually high statistical power for this type of study. Across all tests where mosquitoes were released only in the courtyard, no significant differences were ever found in entrance/occupancy rates for sleeping rooms, irrespective of whether their bednets were treated or untreated with insecticides. In a few cases, however, mortality rates were variable across sleeping rooms thought to present equivalent conditions, e.g., Figure 3.8bB. The most likely explanation for this outcome is that we may have failed to train some of the research assistants to be sufficiently gentle when they collected and transferred mosquitoes using mouth aspirators. In retrospect, we also erred in permitting certain assistants to collect mosquitoes from the same room on successive days, rather than having assistants rotate to successive rooms each day so the variation each imparted to mosquito mortality was equally distributed across treatments. These deficiencies were corrected after 24 h mortalities exceeding the 20% acceptable under WHO (2002) guidelines were obtained in several tests (Figure 3.8bB; Figure 3.10B). Further experiments proved the source of this variability in mortality was not attributable to the choice-test apparatus.

Eave openings functioned strongly as inlets, and weakly as outlets

An. gambiae females readily entered sleeping rooms through the 20 cm high x 110 cm wide simulated eave openings at the top of the courtyard walls. Cues emanating from the sleepers were necessary for high entrance/occupancy rates, as rates of entry/occupancy for rooms without sleepers were trivial in pilot tests (data not shown). No significant effect was found when the eave openings were fitted with a restrictor (Figure 3.6 a and b) whose opening into the sleeping room could be regulated from 20 to 3 cm. Perhaps this is not surprising, given that this apparatus acted as a funnel whose wide end remained unaltered. We elected to conduct the preponderance of tests using the 20 cm eave opening, because Luo houses always present an eave opening at least this large unlike in West African studies where entrance is at window level (Asidi et al., 2004). Ready entrance through eave openings in the Mosquito Hotel is consistent with the report of Snow et al. (2007) that *An. gambiae* encountering a house wall fly upward against the wall and readily find gaps at its top.

Surprisingly few mosquitoes released into a room housing a sleeper under an untreated bednet exited when the only portal was the 20 cm eave opening into the courtyard. This suggests that hungry females are disinclined to fly down a concentration gradient of host cues even after being repeatedly thwarted from feeding by the physical barrier of the netting. Host cues appear to be a powerful and long-lasting arrestant. In retrospect, it would have been informative to have released mosquitoes into a room with no sleeper to determine whether exit rate was higher without than with host cues. Perhaps the probability of obtaining a bloodmeal is greater when mosquitoes remain in an occupied bedroom and wait for the sleeper to exit the bednet than when mosquitoes exit

that house and search for another domicile. Likewise, few mosquitoes released into a room housing a sleeper under a treated bednet exited. This suggests that insecticides present in that room did not drive mosquitoes out through the eave opening.

ITN mode of action: avoidance vs. lethality

The current data overwhelmingly support lethality as the mode of action of the ITNs tested and seem to falsify the avoidance hypothesis at the spatial scale of room entry and exit. No evidence whatsoever was found for reduced entrance rates into rooms with bednets treated with insecticides vs. rooms with untreated bednets. This outcome contradicts the conclusions from various hut studies conducted in Western Africa. For example, Lindsay et al. (1991) reported that Permethrin-impregnated bednets deterred mosquitoes from entering the huts with a degree of deterrency proportional to the dosage of Permethrin. Miller et al., (1991) reported that unwashed Permethrin-treated bednet reduced the number of *An. gambiae* s.l entering huts by 60%. Difference in methodology and test conditions may partially explain these discrepancies. West African houses do not offer the large eave openings for mosquito entry typical of East African houses. Rather, mosquitoes are reported to enter through a series of cracks and slits distributed at various heights along the walls. Mosquitoes would need to crawl rather than fly through such small openings. Perhaps, when walking, mosquitoes taste insecticide deposits that function as repellents or deterrents. On the other hand, mosquitoes flying into a room with an ITN might not be able to smell pyrethroid insecticides of high molecular weights and thus very low vapor pressures. Differences in the height of portals used as entrance or exit traps could strongly influence measures of exophily. Since we wished to understand the behavior of mosquitoes visiting humans in Luo-type housing, openings for mosquito

passage were at wall-top level in all our tests. Additionally, mosquito age and experience might have some bearing on outcomes of house-entry tests. In our study, all mosquitoes were naïve and foraging for their first bloodmeal. In studies using feral females, the argument can be made that females surviving encounters with ITNs might avoid returning to those houses or entering rooms presenting cues now associated with a negative experience. Finally, some studies of mosquito entry and exit rates using normal housing conditions may have suffered from problems with accuracy in assessing avoidance vs. lethality due to difficulties in accurately counting dead mosquitoes. In a typical African house with dirt floors and the clutter of necessary house contents, it is very difficult to find dead mosquitoes. Moreover, predators and scavengers like spiders and ants can be abundant. One of the many advantages of the Mosquito Hotel was that all floor and wall surfaces were covered by white cloth that minimized the difficulty in finding mosquitoes, be they dead or alive. Effective anti-predator and anti-scavenger measures were always in place in our tests. Even so, we typically could not account for ca. 10 % of the mosquitoes released on any given night, suspecting that they escaped through tiny cracks and crevices that are difficult to eliminate entirely from any housing structure. Within the protocol limits specified, we consider that these Mosquito Hotel test results are the most robust and reliable data produced to date for directly addressing the question of the relative contribution of avoidance vs. lethality to ITN function for a house containing human sleepers. Lethality is overwhelmingly supported for all types of ITNs tested here. This was true irrespective of *An. gambiae* strain (Kisumu laboratory strain vs. feral house-caught mosquitoes) or age (4-day and 16-day old female mosquitoes).

Slight differences with ITN brand

Where mortality for control rooms fell below or near the 20% WHO (2000) guidelines, Permanet and Netprotect ITNs yielded higher nightly kill of *An. gambiae* females than did Olyset bednets (see Figures 3.8b; 3.16). This outcome is consistent with the finding of Chapter 2 that mosquitoes alighting on Olyset departed sooner than those landing on Permanet. Thus, less toxicant might be absorbed per visit on Olyset, given that the dosages of insecticide loaded into each net type are similar.

Recommendation

Under the housing conditions of Western Kenya, all available data indicate that ITNs operate as lethal traps. We recommend Netprotect and Permanet ITNs over Olyset, because of more consistent and higher kill. As mortality from deltamethrin was dosage dependent (Figures 3.12 and 3.19), we support the high-dosage tactic (Guillet et al., 2001; Corbel et al. 2004), where an overwhelming dose of deltamethrin is delivered to visiting mosquitoes so that there are very few to no survivors left in a room presenting a bednet. Under this approach, ITNs are likely to make huge contributions in reducing human deaths and suffering due to malaria. However, vector managers will need to be vigilant and be ready to adjust to counter-adaptations by *An. gambiae*; experience teaches us that no pest-control tactic is forever fail-proof.

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