Energy-State Dependent Responses of

Anopheles gambiae to an unobtainable host

by

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Abstract

Understanding how blood-seeking behavior changes with different energy levels in the malaria mosquito *Anopheles gambiae* (Diptera: Culicidae), when confronted with an unobtainable blood-host, is of interest for vector control strategies. I used a straight-tube olfactometer to mimic a domicile containing (i) a simulated blood-host (human foot smell) protected by either a plain bednet or a DEET impregnated net and (ii) a sugar source (honey scent) some distance away. I manipulated the mosquito's energy level by withholding sugar sources from females for different lengths of time. Whenever DEET was present, virtually no mosquitoes interacted with the blood-host scent at any energy level. Yet, energy levels influenced response to foot odour, probing persistence, residence time when DEET was not present. This suggests that control strategies using mosquito repellents can ignore the presence of alternative food sources (such as sugar) in the field unless the chemical efficacy is not retained over time.

Dedication

To my parents, who have never questioned my choice of higher education and pursuit of a career in science.

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Chapter 1: Introduction

Energy budgeting in insects

Energy is one of the major constraints on the life and fitness of most living organisms, including insects. Depending on their life history, food source and intake needs, insects are driven towards seeking suitable energy to fulfill both somatic and gametic functions. Often, the food item of choice is sufficiently nutritious to support different needs but in certain insects, choice is needed, as a single food item cannot be utilized for both aforementioned functions.

For example, certain parasitoid wasps are confronted with choices involving whether to oviposit in a host or to consume it. The two choices represent an immediate fitness gain versus a perceived increased future reproductive output (Heimpel and Rosenheim 1995). These types of choice are often described using dynamic state models (Collier 1995, Heimpel and Collier 1996, Ellers and Jervis 2003) in which theory predicts that choices will be geared towards augmenting lifetime fitness and serving both immediate and long-term goals.

Omnivory can involve another kind of choice-driven behaviour. In this case, the choice between eating a plant or an animal is dictated by external and internal factors, from the individual's perspective. When dealing with animals whether as hosts or prey, the foraging insect must consider a generally longer search and handling time relative to plants, as well as the potential to be killed or physically injured by the prey/host. In this case, the energy gain might outweigh the costs (see optimal foraging theory – Kamil et al. 1987). Herbivores on the other hand, require a tougher digestive apparatus, ingest lower caloric food, generally lower protein intake per unit of food relative to prey and experience physical wear from handling it (e.g. Roitberg et al. 2005).

The mosquito is a somewhat unique kind of omnivore is the mosquito. The larva feeds readily on algae and other detritus that may accumulate inside breeding pools (Merritt et al. 1992) and will also readily prey on con- or heterospecific mosquito larvae if plant nutrition does not suffice (Koenraadt et al. 2004).

In contrast, adult females can choose between feeding on sugar sources or blood from a host. Feeding on sugar alone will provide readily employable energy for flight, mating and overall metabolism (Stone et al. 2011). There is also evidence that if sugar is readily available in the environment, mosquitoes will live longer (Gary and Foster 2004) and have increased lifetime fitness (Foster 1995, Gary and Foster 2001).

However, feeding on blood remains the sole way of obtaining gametic energy (employed in oogenesis), apart for some species which can reproduce by exploiting proteins obtained as a larva (Omeara and Mook 1990). Often, in instances where larval growth conditions were sub-optimal, the energy derived from a blood meal may be directed and exploited towards structural/somatic functions (Takken et al. 1998). Furthermore, because sugar and blood are generally found in mutually exclusive locations and compete for abdominal space in the mosquito's gut, "decisions" must be made as to which resource to seek at any given time (Fernandez and Briegel 2004).

To investigate how energy issues are addressed in the insect world, I experimented with the mosquito *Anopheles gambiae*. I chose this particular species as my experimental organism because (1) it is easy to breed and has a short life cycle; (2) females are omnivores during both the larval and adult stage; (3) its fitness depends on host/food choice; (4) it has a very tight energy budget; and (5) it transmits pathogens causing life threatening diseases including malaria.

Life cycle of *Anopheles gambiae*

Gravid females of *An. gambiae* can lay 50 to 200 eggs following a full blood meal. They perform skip oviposition and assess oviposition sites using both chemical and visual cues (Chen et al. 2006). Typical oviposition sites vary and ideally are shaded, non-crowded, algae-bearing puddles and other ephemeral pools (Minakawa et al. 2004); even temporary water-filled hoof prints can be suitable (Mutuku et al. 2006). The eggs

are deposited directly on the water surface or on the dry immediate surroundings. Unhatched eggs can last up to 12 days before becoming lifeless (Jawara et al. 2008). Hatched "land larvae" are able to move the water pool by inch-worm like locomotion or by exploiting incoming rain water (Miller et al. 2007).

In non-crowded pools with abundant food larvae go through four instars within approximately 1 week before they pupate (Lyimo et al. 1992). Instars are distinguishable on the basis of size. Each instar increases approximately 120% in head capsule width (Timmermann and Briegel 1999). As noted above, larvae are opportunistic omnivores and feed on algae and bacteria but can also resort to cannibalism under adverse conditions such as crowding and lack of food (Koenraadt and Takken 2003, Koenraadt et al. 2004). Depending on conditions, the egg-to-adult cycle typically takes between 8-14 days (Jawara et al. 2008)

Freshly eclosed *An. gambiae* adults are frail and energetically deficient. Their ability to survive depends completely on accumulated teneral reserves which are quickly depleted during flight (Briegel 1990, Kaufmann and Briegel 2003). Adult males depend solely on sugar sources for energy, whereas adult females can seek sugar or blood sources. Yet, females do not readily respond to blood host cues until they are mated and after a period of at least 24 h post eclosion (Fernandes and Briegel 2004, Foster and Takken 2004).

Blood-feeding *An. gambiae* can engorge 5-10 µL of blood per meal (Fernandes and Briegel 2004). They perform pre-diuresis, expelling plasma (the less nutritious blood-fraction) from their anus for more space to retain red blood cells (Benoit and Denlinger 2010) until receptors around the gut, triggered by abdominal distension, signal to stop feeding (Klowden and Arden 1979).

Following a blood meal, a female will rest and hide from potential predators and cease responding to blood host cues until egg maturation and oviposition (Takken et al. 2001, Klowden and Briegel 1994). This can takes up to 3 days. Field mark and recapture studies showed male daily survival to be 0.79; 0.83 for females (Lehman et al. 2009). In my colony, adults lived for 2-3 weeks when provided solely with sugar *ad lib*, 4-5 weeks if blood-fed weekly (personal observations).

Malaria

Plasmodia comprise a genus of parasitic protists which are exclusively transmitted by the mosquito genus Anopheles. *Plasmodium* species are the causative agents of malaria, kill an average of 800,000 people a year (WHO 2010), and are an unquantifiable burden on the economies of countries where malaria is endemic (Gallup and Sachs 2001).

Historically, the most effective vector control method involved the heavy use of DDT and other chemical sprays (Kouznetsov 1977). These insecticides have freed much of the temperate world from malaria and are still recommended for use in tropical endemic regions by the WHO (2007). Yet, concerns grew around adverse health and environmental effects of DDT and the discovery of DDT-resistant mosquitoes (Crandell 1954, D'Alessandro and Mariani 1958, Raghavendra et al. 2011). Today, the focus of control has shifted to the use of biological control agents and insecticide-treated bednets (ITNs) bearing pyrethroids which not only protect humans with a chemical toxin but also provide a physical barrier if used correctly (Lines et al. 1987, Bockarie et al. 2002, Takken 2002, Lindblade et al. 2006, Raghavendra et al. 2011). However, there is conflicting evidence as to whether a mechanical barrier alone can lower infection incidence (Clarke et al. 2001, Mwangi et al. 2003). Other cutting edge vector control techniques which are still under scientific and economical scrutiny, involve the release of sterile, genetically engineered mosquitoes (Catteruccia et al. 2003), and habitat modifications (Bond et al. 2004, Raghavendra et al. 2011).

Of interest for this thesis is the fact that once a mosquito is infected with *Plasmodium*, its behaviour changes significantly (Hurd 2003). It becomes more strongly attracted to human scents (Lacroix et al. 2005) and exhibits more frequent and longer intradermal probing (Rossignol et al. 1983, Wekesa et al. 1992), thereby increasing its likelihood of dying by host defense mechanisms or by contacting ITNs or other chemical barriers. This may shorten its life and lower its overall fitness (Anderson et al. 2000). The increased mortality is strictly due to changes in behaviour, as field studies suggest (Chege and Beier 1990).

My questions

All else being equal, adult female *An. gambiae* are confronted with a choice of sugar, which provides solely somatic energy, and blood, which is used for both somatic and gametic functions (Briegel 1985). Yet, females may differ in size, teneral reserves, age, current energy level, previous blood host contact, parous level, perceived fitness, health and potential infection with *Plasmodium*. These internal factors are thought to modulate both a female's choice of energy source and its persistence in finding and obtaining it.

In addition, foraging is affected by external factors, such as the ease of obtaining a meal, the travel distance to it, and the risk of death due to predators and/or a non-compliant host (Roitberg and Roitberg 2008). Therefore, blood and nectar sources represent very different fitness potential since they differ in quality, availability and ease of access. For instance, nectar pools fluctuate during the day and flower beds may contain predators. Similarly, blood hosts may be sheltered inside well-insulated domiciles or hidden under ITNs. The focus of my thesis will be directed at understanding how mosquitoes at different energy levels react to an unobtainable (bednet-protected) blood meal given the possibility to obtain a secondary energy source (sugar).

Mathematical models addressing food choice and energy status of mosquitoes were developed by Ma and Roitberg (2008) and Roitberg and Mangel (2010) but no empirical data have been assessed so far. For example, feeding persistence models (Anderson and Roitberg 1999), predict that mosquitoes are more persistent at biting if the probability to perish and to obtain blood from a feeding attempt is low. Conversely, the models also predict that mosquitoes are less persistent at biting if the probability to perish and to obtain blood from a feeding attempt is high. In either case, there is a threshold blood meal size that determines whether mosquitoes should continue or cease feeding. This is further modulated by the relative age of the mosquito. The general predictions involve the presence of external and internal cues that once perceived and processed will trigger a reaction, which should reflect the optimal choice (risk vs. benefit) in terms of lifetime fitness.

In order to test these predictions in a controlled environment, I designed a straight-tube olfactometer and tested the insects' choice (Omrani et al. 2010, Qiu et al. 2011) between sugar and blood, in a sequential manner. The olfactometer design (see chapter 2) differed from a typical y-tube in that it simulated the presence of a mosquito inside a domicile encountering a blood host, while being aware of a sugar source some distance away.

In the second chapter of my thesis I will illustrate how mosquitoes react to an unobtainable blood-host shielded only by a physical barrier, while in the third chapter the shield is impregnated with the mosquito repellent DEET at one of two concentrations.

The focus of my research is to determine how the behaviour of a mosquito might affect its frequency of feeding and the number of hosts it encounters, and how this affects malaria transmission. To continue to improve the advancements in malaria control it is important to understand how the highly mechanized and genetically engrained behaviour of mosquitoes can be exploited. For example, understanding movements of mosquitoes in and out of domiciles and their feeding patterns (probing rate, persistence, activity levels) with respect to their energy status may help in the design of better integrated pest management strategies. If availability of sugar makes mosquitoes more likely to encounter and feed on a human host, or more likely to feed on multiple hosts, then programs aimed at eliminating nectar-bearing plants from domiciles may be initiated. My experiments take place in a small, enclosed environment but may be significant to advance understanding the dynamics at the level of an entire village where there may be multiple sources of sugar, protected or unprotected blood hosts, and predators.

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Chapter 2: Energy-State Dependent Responses of *Anopheles gambiae* (Diptera: Culicidae) to Simulated Bednet-Protected Hosts.

Abstract

In nature, *Anopheles gambiae* mosquitoes are found at various energy levels and such females must choose between seeking somatic energy from sugar sources and obtaining both somatic and gametic energy from blood hosts. I used a straight-tube olfactometer containing a simulated unobtainable blood host (human foot smell protected by a net) as well as a sugar source (honey odour). I assessed female probing rate and residence time at the net as a function of energy state (0, 24, 48, 72-h starved). In my trials, 0-h starved females showed low response to human odour, low probing rate and residence time at the human odour site. By contrast, both 48 and 72-h individuals showed high response to foot odour, longer residence time and higher probing rates. Seventy-two-hour females also flew towards the honey source less often than other groups. My findings suggest that managing sugar sources might be a viable strategy for influencing mosquito biting behaviour.

Keywords: *Anopheles gambiae*, Energy state, Malaria.

Introduction

An. gambiae is the main vector of *Plasmodium spp.*, the causative agent of malaria in Sub-Saharan Africa. The disease causes both mortality (~800,000 people yearly) and morbidity in the millions, and it is a significant social-economic burden in all endemic countries (WHO 2010). To combat the spread of malaria it is important to understand both the life history and behaviour of its vector (Chaves and Koenraadt 2010).

Upon emergence, adult *An. gambiae* have low teneral reserves and, generally, will readily feed on sugar sources (Stone et al. 2011). After two days, females begin responding to human odour stimuli (Foster and Takken 2004) and are faced with two possible feeding choices, which are often mutually exclusive due to the mosquito's size-constrained crop (Fernandes and Briegel 2004). An individual female mosquito may choose to continue feeding on readily available sugar to support somatic function, or feed from a blood host. The latter would provide energy for both somatic maintenance and reproduction but is less readily obtainable and more dangerous to acquire than nectar. Sugar availability has been linked to longer lifespan (Straif and Beier 1996, Gary and Foster 2001, Gary and Foster 2004), increased insemination rates by males (Stone et al. 2009) in that is relevant to management of malaria (Gu et al. 2011).

To maximize lifetime fitness, the choice between somatic and gametic energy sources (Stone et al. 2011) may be mediated by both external and internal factors (Ma and Roitberg 2008, Roitberg and Mangel 2010). External factors, such as the risk of death due to a defensive host, presence of potential predators and a bednet

may lower the mosquito's attack persistence when attempting to obtain a blood meal from a particular host (Walker and Edman 1985). Likewise, internal factors such as energy level, age and life expectancy of the individual may affect this persistence (Roitberg et al. 1993). Both external and internal factors may be assessed by the female prior to seeking a source of energy.

For years, insecticide treated bednets (ITNs) have been the most effective tools against *Anopheles* bites (D'Alessandro 2001). While many studies have investigated mosquito interactions with bednets (Lines et al. 1987, Takken 2002), to our knowledge, no study has tested how energy state affects these interactions, although theoretical studies have examined this relationship (Ma and Roitberg 2008, Roitberg and Mangel 2010).

Given that adults have limited ability to maintain energy at constant high levels, our interest lies in understanding the point at which a mosquito gives up on a potential blood meal and how energy state affects the probing rate and time spent with the potential host as well as the kind of interactions with an unobtainable host. Similar studies have addressed this issue (Walker and Edman 1985, Nasci 1999 with *Aedes aegypti* and Roitberg et al. 2010a with *An. gambiae*), but the mosquitoes were being actively dislocated from the host and no alternative food source was provided. In this study, we predicted that energy-deprived individuals would have increased probing rates towards an unobtainable blood host, giving up less readily than mosquitoes whose energy levels are high and thus have lower risk of starvation. In contrast, individuals with a higher energy level would have a wider range of options: they could wait for the host to become available or move on to another source of energy, seeking a more obtainable blood host or sugar source.

To test these predictions we employed a straight-tube olfactometer in which we simulated a human that is being protected by a bednet within a domicile. We tested individual female mosquitoes at various levels of starvation and gave them two potential food options: an unobtainable blood meal and a sugar source.

Materials and Methods

Colony

Anopheles gambiae s.s. was reared from a colony originating from Njagi, Tanzania, in 1997. Our laboratory culture was kept at 28 ± 2°C, 83 ± 3% RH under the L:D 12:12 h photoperiod with 1 h of twilight transition (Conviron). Eggs were collected on moist filter paper from ovipositing adults that were blood-fed by SZ, one of the authors. Eggs hatched within 72-h and first instar larvae were placed in large white plastic containers (30 cm x 45 cm x 6 cm) with 3.5 L of water. The larvae were fed fish flake food (Nutrafin - Hagen) *ad lib* until pupae were transferred in water filled containers to Perspex cages with screening on three sides (30 cm x 30 cm x 30 cm) under same RH and L:D conditions. Following eclosion, adults were provided with 5% sucrose solution *ad lib* from a medical grade cotton wick (Richmond Dental) contained in an Erlenmeyer flask. All 145 experimental females were large (wing length of 2.70-3.50 mm), 3-9 days post-eclosion, not previously blood-fed, presumed mated (mating was observed inside the cages) and without visibly swollen abdomens to increase the likelihood of female response to odour stimuli (Fernandes and Briegel 2004).

Experimental Apparatus

We used a large, custom-made glass tube (olfactometer) (Figure 1), 145 cm in length with diameter of 17 cm which allowed unconstrained flight of the mosquito. An air pump (Petcetera AP5000 double type) pushed ambient air through an activated carbon filter at 0.025 L/s and funnelled it to the tube at mid height at one end of the chamber. A cotton ball containing approximately 5 g of honey (Kidd Bros. unpasteurized alfalfa and clover honey) which was found to be attractive at full strength (SZ, pers. obs.), was placed on a metallic tray suspended midway into the tube. A semi-rigid, plastic, cylindrical, tan coloured net (10 cm in height X 2.5 cm in diameter, 1x1 mm mesh holes) which served as a simulated bednet containing a conditioned nylon sock (Secret^{TM} brand, knee high) was placed downwind from the honey, at a distance of 73 cm and was suspended from the top (Figure 1). The sock was worn by SZ for 2 consecutive days to condition it to be attractive to female mosquitoes (Braks and Takken, 1999, Smallegange et al. 2010); on the second day, SZ performed a strenuous sport. The sock was then placed in a sealed plastic bag and incubated in the experimental chamber for 1 full day prior to being used in the olfactometer. The sock was replaced every 4-5 days by a similarly-treated sock and it was randomly assigned to the different starvation treatments. A vinyl pipe was placed at the upper extremity of the simulated bednet to allow the experimenter to exhale to simulate the presence of a live blood host and stimulate host searching by the mosquitoes (Healy and Copland, 1995). At the end of the tube (60 cm from the simulated bednet), we placed a plastic release chamber with a movable metallic screen. It is important to note that the experiment was arranged such that released mosquitoes encountered the simulated blood host prior to the simulated nectar source in a linear arrangement. The experimental runs were observed under dim

red light (average of 1.79 μmol/(s*m^2)). The olfactometer was aerated and flushed daily with plain water to minimize residual odours.

Experimental Procedure

Treatments of unstarved (0-h) and 24, 48 and 72-h starvation were randomly assigned to adults aged 3 to 9 days post-pupal-eclosion to avoid any age effect. The sugar source was then removed and substituted with deionized water (Knols et al. 1994). Female mosquitoes were haphazardly selected and individually placed into a release chamber. After a 3-minute resting period, the wire screen of the release chamber was opened allowing the mosquito into the olfactometer. The experimenter then began timing the start of the run and started blowing quick pulses of air every 6-8 seconds via the vinyl tube, which were sustained until the end of the experimental run. If the mosquito was unresponsive for 3 minutes the experimenter would gently tap on the walls of the release chamber until the female exited into the olfactometer at which point the replicate started. We monitored the female's probing rate by counting the number of attempted landings/probes on the sock and net (Nasci, 1991) per unit time. We considered a landing to be completed once the female touched the net and did not fly more than 5 cm away from the net.

We also recorded the residence time at the net with sock calculated as the time spent in physical contact with the net. The experiment was terminated if the female flew upwind past a line 5 cm in front of the cotton ball containing honey or rested on the sides of the glass tube for more than 5 consecutive minutes. Once a replicate was concluded, the female was removed, dried, and its wings removed and measured using an ocular micrometer and the software "Analyzing Digital Images (Version 11-2008)."

Data Analysis

All statistics and graphs were processed using JMP 9 and GraphPad Prism 5. We used a χ^2 test to assess significance for the number of *An. gambiae* responding to the human odour stimulus as well as the number of mosquitoes that actively flew to the honey source or rested. Kruskall-Wallis test was used for analyzing significant differences in the probing rate, residence time at sock and net, as well as the average time per probing event between treatments, due to the zero inflated distribution of the values. Lastly, Log-Rank (Mantel-Cox) (i.e. survival analysis) test was used to analyze the differences in sustained activity towards obtaining a blood meal as a function of energy state. Age stratification (number of days after emergence) was included in each analysis.

Results

A total replicate size of 145 females met our criteria of evaluation described in the material and methods section. Single experimental runs lasted from a few seconds to a maximum of 27 minutes (average ~3 minutes).

Non-starved mosquitoes (0-h) responded to the human odour by probing the simulated bednet significantly fewer times than all the other treatments as only 43% of the individuals were recorded to have probed at least once during the experiment. The other groups had a higher proportion of individuals responding to the human odour at 71%, 74% and 67% for 24, 48 and 72-h respectively (χ^2 df.= 3, F= 25.91, p<0.001) (Figure 2).

The relative residence time at the sock and net (i.e. the proportion of experimental time spent probing) of non-starved females was 22±0.1% and was significantly different from the 48-h and 72-h groups (Kruskall-Wallis F= 15.45, p= 0.002). These treatments yielded to 50±0.1% and 47±0.1% relative residence time respectively while the 24-h starvation treatment averaged at 37±0.1% (Figure 3).

When considering the proportion of individuals that actively left the 'hut', by flying to the honey source at one end of the olfactometer versus the proportion that rested in the immediate surroundings of the human odour, the only significantly different group was the 72-h treatment in which 24% of the individuals moved to the honey (χ^2, df.= 3, F= 35.02, p<0.001) . The other groups scored 48%, 51% and 65% for 0, 24 and 48-h respectively (Figure 4).

The proportion of females still active in host-seeking after a given time, measured as total experimental time, yielded significant differences between the two extreme treatments: 0 and 72 h. The latter showed a much longer overall active host-seeking time length (Log-Rank Mantell-Cox F= 7.985, p=0.046) (Figure 5).

Probing rate (total probes/total experimental time) was significantly different between 0-h (0.239±0.001 bites/minute) and 48-h (0.481±0.001 bites/minute) treatments (Kruskal-Wallis, F= 9.563, p=0.023). 24-h and 72-h were recorded at 0.407±0.001 and 0.430±0.001 bites/minute respectively (Figure 6).

No significant results were obtained when comparing the average length of time per landing (~67 seconds across all levels) on the simulated bednet between energy levels (Kruskall-Wallis F= 4.232, p=0.238).

Discussion

In this experiment, mosquitoes with different energy levels were offered a choice between an unobtainable (simulated) host under a bednet and an alternative source of energy, honey, a proxy for nectar source outside of a domicile. The experiment was designed spatially such that the mosquito would encounter the blood host before the honey (Figure 1). The goal was to see if females would seek the unobtainable blood meal indefinitely or give up this potential source of energy to actively fly towards the alternative sugar source.

We found that *An. gambiae*, female mosquitoes modulate their behaviour towards trying to bite an unobtainable host based on their energy state. In nature, females are found with a broad range of energy states (Walker 2008) and our research helps elucidate how they might react to the presence of two potential energy sources with very different fitness advantages and costs for the individual female.

Mosquitoes that were granted *ad libitum* access to sugar water until the start of the experiment (0-h starved) were overall less inclined to respond to the bednet odour stimulus (Figure 2). This absence of response suggests that the high energy level, perhaps coupled with a perceived full crop, reduces meal-seeking activity (Jones and Madhukar 1976). These findings are in line with Roitberg et al. (2010a) where it was proposed that well fed females would have lower attack rates and were predicted by Ma and Roitberg (2008). In the few instances where these females probed for blood, their residence time at the sock and net was short and they tended to have lower probing rate and to quit blood-host-seeking significantly earlier than other groups (Figure 3, 4 and 5).

Here, we suggest that recently fed females may postpone the risk of attacking a blood host, or venturing away from it, until crop volume and mass drops, perhaps maximizing the size of the future engorged meal and survivorship (Roitberg and Gordon 2005, Ma and Roitberg 2008). This can be best understood using a marginal analysis wherein the marginal returns from both space-constrained blood meals and sugar meals are small for recently individuals whereas the marginal costs via mortality risk are high (see Roitberg et al. 2010b for an example from parasitic wasps). In contrast, the marginal returns for starved individuals are high in either case, but one of the two meal options might be more accessible. This statement is supported by our data. Very starved females (48 and 72-h) had longer residence time at the sock and net than other groups and 48-h females had a significantly higher probing rate. However, 72-h starved females showed no significant difference in probing rate. Perhaps females in this category are on the verge of starvation and are energetically constrained to overall low activity levels. This hypothesis is supported by the extremely high mortality in the 72-h treatment cages and the fact that this treatment yielded females that rested alongside the simulated blood-host more often than other groups; we hypothesize that the latter had sufficient energy to fly towards the sugar source in search of alternative food. However, we cannot exclude the possibility that mosquitoes that flew towards the honey source were simply flying upwind. Although some females were observed to readily feed on the cotton bearing honey, no data were collected regarding honey avidity.

The low probing rate and residence time at the sock and net by well-fed individuals suggests that the female's energy level might allow for a flight to another perhaps more readily accessible blood host (Lines et al. 1987).

Very starved individuals (48-h, 72-h treatments) not only responded more often to scent stimuli, but also showed a higher persistence rate and residence time at the sock and net. The propensity to stay in contact for a longer duration of time with the bednet could be advantageous in the field use of ITNs (Insecticide Treated Bednets); as increased contact time with the chemicals proportionally increases the likelihood of death by poisoning (Roitberg and Mangel 2010).

Removal of sugar sources from the immediate vicinity of human habitations may contribute to the overall depletion of energy in females, increasing the exposure to the chemicals to ITNs; this fact should be considered by malaria vector managers. Similar considerations are relevant for females that have undergone changes due to *Plasmodium* infection (shortened overall life expectancy and increased risky behaviour) as they may be linked to depletion of energy in the mosquito host (Ferguson and Reid 2002, Rivero and Ferguson 2003).

We expected that starved individuals would spend on average a longer time per probe but that did not occur. There are at least two explanations for this finding. First, *An. gambiae* might have evolved an innate maximum length of time allowed per unsuccessful probe (see fixed giving up times in foraging theory, Krebs and Davies 1997), which could have evolved in response to the risk of reaction by the host and therefore potential death of the mosquito. Second, by providing an additional feeding option (sugar), the energy state effect at the unobtainable host was effectively removed; *i. e.*, starved and well-fed individuals gave up at the same time but for different reasons, with starved individuals demonstrating immediate attention to energy state and well fed individuals forgoing current risky tactics for future opportunities (see Roitberg and Mangel 2010).

Our findings suggest that the management of sugar sources within a village should be a priority. In fact, as suggested by Utzinger et al. 2001, the then incidental management of vegetation within breeding grounds lead to lowering malarial occurrence in a region of Zambia. Thus, we advocate extending the management to nectar bearing plants within foraging areas in integrated pest management programs and the introduction of poisonous sugar baits which have shown to be an efficient control strategy in Israel (Müller and Schlein 2008).

Further research is needed in this field and I am currently assessing whether *An. gambiae* behaves in the same manner in semi-field conditions.

Acknowledgements
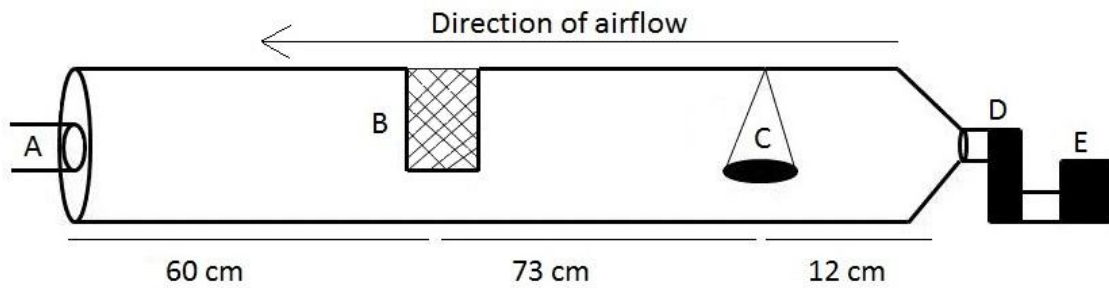
Figures

Figure 1. Experimental olfactometer setup. A. Mosquito release chamber. B. Simulated bed net containing a conditioned sock and a vinyl tube through which the experiment exhaled air. C. Position of honey attractant. D. Carbon filter. E. Air pump.
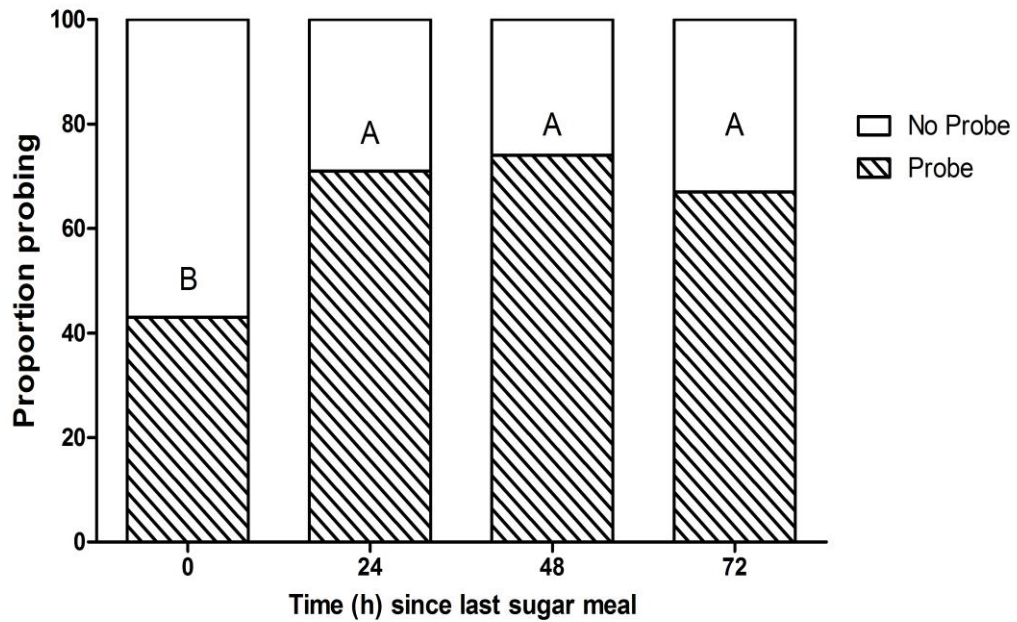
Figure 2. Proportion of *An. gambiae* mosquitoes responding to human odour by attempting to probe the simulated bednet as a function of energy state. Individuals that were not starved (0-h) showed a significantly lower propensity to respond to human foot odour (χ^2, p<0.001). Different letters indicate significant differences.

Figure 3. Mean residence time at the sock and net of female *An. gambiae* as a function of energy state. Mosquitoes that were more energy depleted (48, 72-h) spent a significantly longer portion of the experimental time probing at the bednet (Kruskall-Wallis, p=0.002). Error bars show bootstrapped 95% CI. Different letters indicate significant differences between bars.
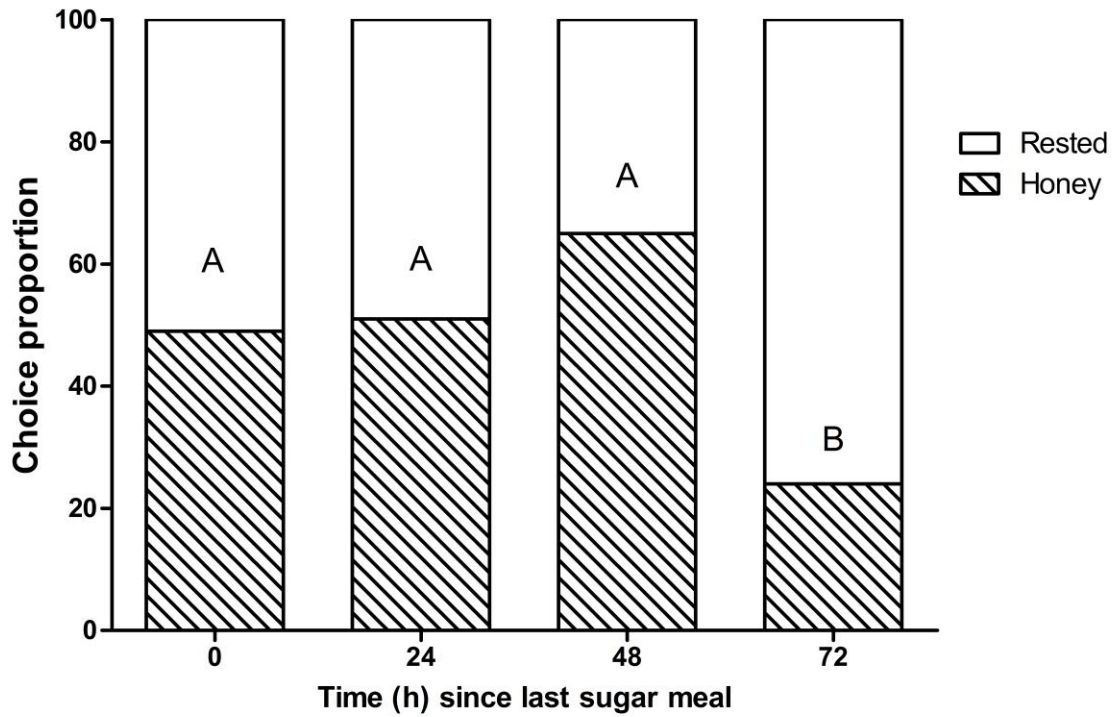
Figure 4. Proportion of *An. gambiae* that actively flew towards a honey source placed at one end of a single arm olfactometer (Figure 1) or rested for at least 5 consecutive minutes in proximity of the human foot odour source. Individuals in the 72-h food deprivation treatment flew towards the honey significantly less (χ^2, p=0.008). Different letters indicate significant differences.
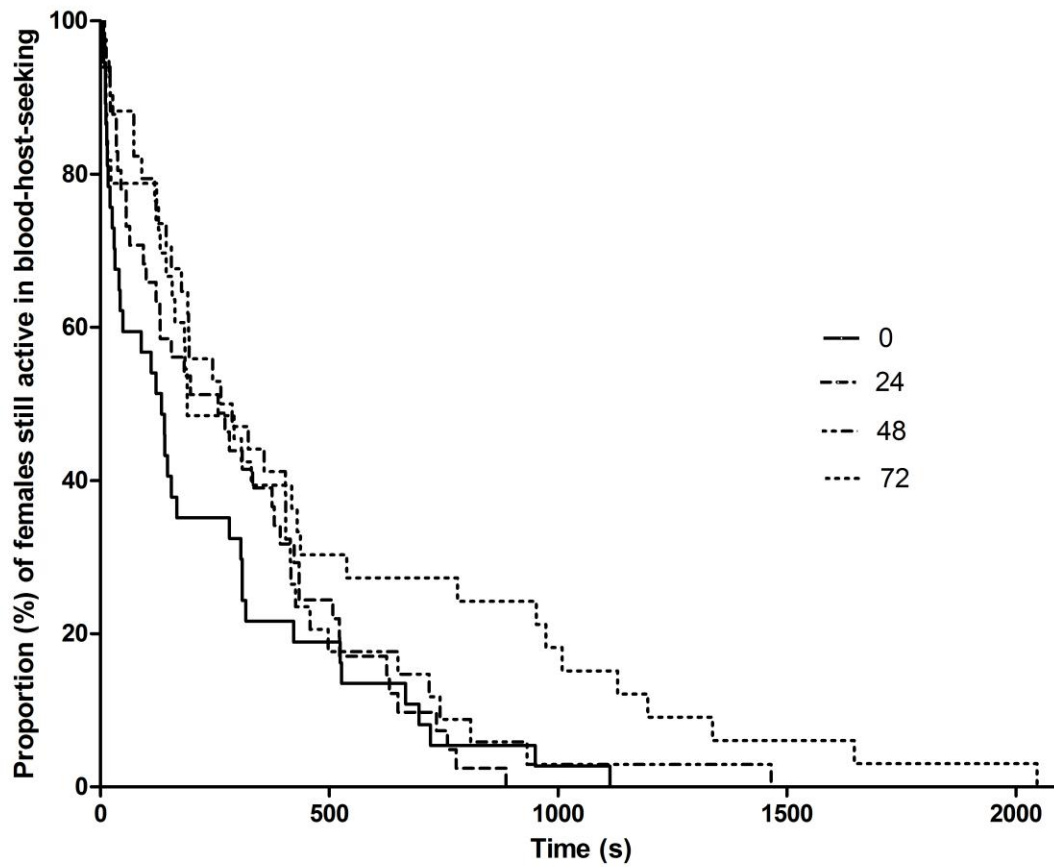
Figure 5. Kaplan-Meier survival plot of *An.gambiae* activity time (i.e. proportion of active individuals not flying towards honey scent and not resting for 5 consecutive minutes) as a function of energy state. Very starved individuals (72-h) stayed significantly more active over time around the bednet than non-starved ones (0- h) (Log-Rank Mantell-Cox, p=0.046).
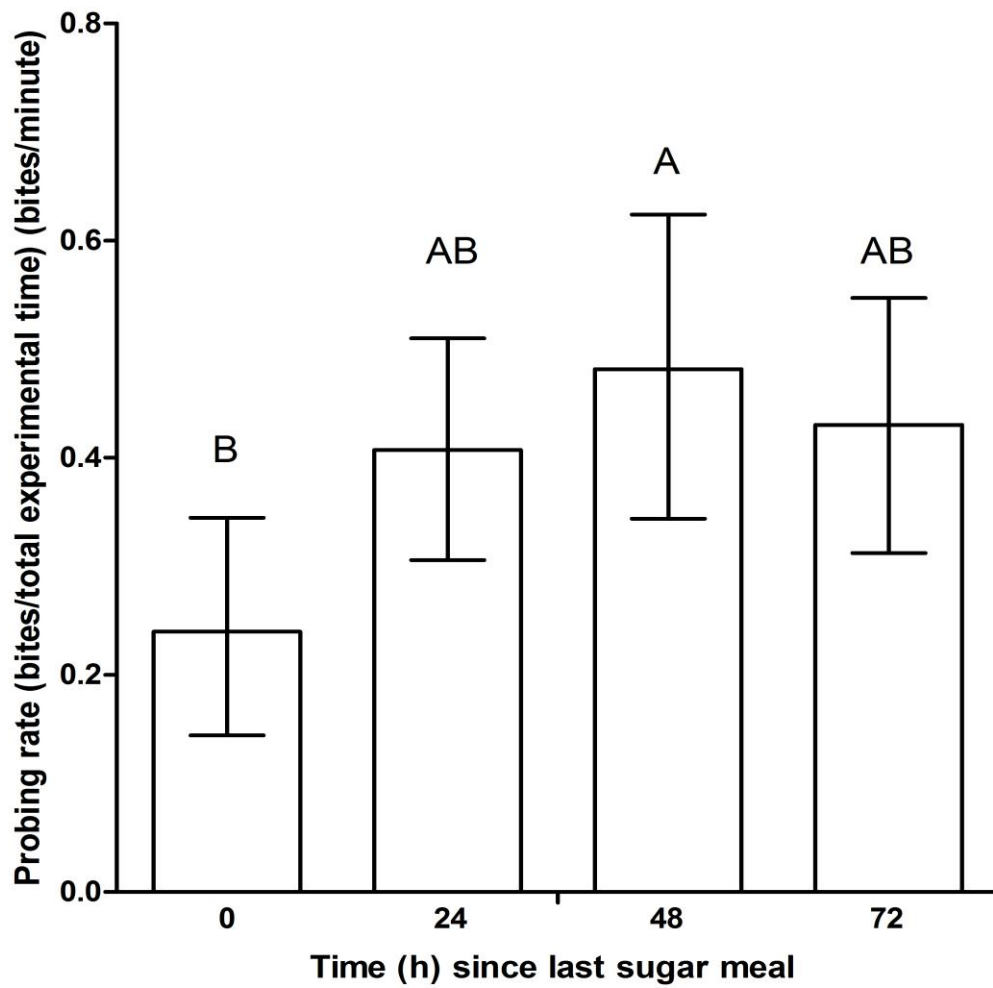
Figure 6. Mean probing rate (bites/total experimental time) of *An. gambiae* females towards the sock and net as a function of energy state. 48-h starved individuals had significantly higher probing rates compared to the 0-h treatment (Kruskall-Wallis, p=0.023). Error bars indicate bootstrapped 95% CI. Different letters show significant differences between bars.

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Chapter 3: *Anopheles gambiae* (Diptera: Culicidae) responses to DEET protected blood-hosts under different energy levels.

Abstract

Understanding how blood seeking behavior changes with different energy levels in the malaria mosquito *Anopheles gambiae* (Diptera: Culicidae) when confronted with a known chemical repellent (N-N-diethil-meta-toluamide; DEET), is of interest for vector control strategies. I used a straight-tube olfactometer to mimic a domicile containing a simulated blood-host (human foot smell) protected by a DEET impregnated net (1% and 0.1%) and a sugar source (honey scent) 73 cm away. I manipulated the mosquito's energy level by starving females for 6, 24 and 48 h and monitored their responses. No effects on behavior were found between individual energy levels, yet significant differences in residence time at foot odour site and overall activity level were recorded when pooling groups together. In general, mosquitoes exposed to DEET responded less readily to the blood-host-scent almost never probing it. I propose that DEET is perceived by the mosquito as such a strong threat that energy levels do not affect the behavioral outcome, keeping females away from the potential blood meal. This suggests that control strategies using mosquito repellents can ignore the

presence of alternative food sources (such as sugar) in the field as long as the chemical efficacy is retained over time.

Introduction

Malaria kills ~ 800,000 people yearly worldwide and is one of the most important insect-vectored diseases in developing countries (WHO 2010). *Anopheles gambiae* is its principal vector in Sub-Saharan Africa. Its control is key to lowering the burden of malaria on human populations (Burtock et al. 1990).

Mosquito control strategies have greatly evolved over the years. They ranged from the heavy use of organochlorinated insecticides (mainly DDT) (Webb 2011), which are now almost completely banned for their deleterious environmental effects (Eskenazi et al. 2009), to the treatment of larval habitats with Bti (*Bacillus thuringiensis israelensis*) (Guillet et al. 1990, Becker 2000) or fungi (Scholte et al. 2004). The most cost-effective control method of mosquitoes is the employment of insecticide-treated bednets (ITNs) (D'Alessandro 2001), which are impregnated with pyrethroids and are beneficial for long-term protection, although resistance development is a concern (Chandre et al. 1999). ITNs are especially useful in hard-to-access communities since the pyrethroids may be effective for up to 6 years, and the nets provide a mechanical barrier which prevents mosquito feeds (Mwangi et al., Tami et al. 2004).

All these control techniques have assumed that insects are non-flexible, auto-behaving organisms with predictable, interactive patterns and choices based on pre-programmed genetic information. Yet, recent mathematical models and related experiments predict and suggest that this is not true (see chapter 2). For example, Roitberg and Mangel (2010) show that mosquito behavior is plastic and is dependent upon external and internal factors. External factors include the presence of predators and the ease of obtaining nutrition, while internal factors comprise energy level, perceived fitness and life expectancy. Additional models (Ma and Roitberg 2008) predict that mosquitoes with different energy levels choose between blood and nectar as mutually exclusive energy sources. Similarly, Blaustein et al. (2004) demonstrate flexibility in oviposition decisions related to habitat quality and the risk of predation and cannibalism. In the former case, the mosquito can choose between attaining an immediate fitness gain from gametic energy provided by blood and investing in future fitness by nectar feeding which provides somatic energy. Either choice is driven by the goal of higher fitness and is likely well considered by female mosquitoes (Stone et al. 2011). If mosquitoes face a defensive blood host, are not in dire need of food, and their likelihood of being killed is high (i.e., low return on investment), then they should leave the blood host. Similarly, if the mosquito's energy level is high, the risk of death correlated with interacting with a blood host may be outweighed by the risk of leaving to search more readily obtainable food (Ma and Roitberg 2008).

Understanding how the mosquito's behavioral plasticity affects malaria transmission may help improve future policies for integrated pest management. I build from mathematical models addressing energy state and I bioassay the response of mosquitoes with different energy levels in a straight-tube olfactometer as a domicile simulator (Ma and Roitberg 2008). I provide two attractive scents, a human-odour-conditioned sock and honey, simulating the presence of a blood host and a nectar source, respectively, while adding the well-known repellent DEET (N,N-diethyl-meta-toluamide) to the blood host bait (Paluch et al. 2010). I am interested in understanding whether energy state has any effect on female mosquitoes facing a blood-host protected by a repellent, with a secondary food source some distance away.

From this premise, I construct a factorial design which allowed me to detect interactions between energy state and DEET concentration. If DEET is perceived as dangerous, then starved mosquitoes should try to blood feed more frequently in "desperate" attempts to obtain energy than well-fed ones which are not under the immediate threat of starvation. This trend should be retained at different DEET concentrations as the trade-off between the benefits of a blood meal and danger is re-evaluated. At low concentrations, I expect many well-fed females to respond to blood host scent while at high concentration I expect only starved females to respond. Yet, as DEET is clearly perceived by olfactory cells and avoided by mosquitoes (Syed and Leal 2008), I alternatively could also expect no interaction between energy state and DEET concentration, as

mosquitoes would tend to avoid the blood host altogether in any of the combinations tested under the factorial design.

Materials and Methods

Colony

I used a colony of *An. gambiae s.s.* (Diptera: Culicidae) which originated from Tanzania (Njagi) in 1997. The colony was mantained in an environmental chamber (Conviron) at 28 ± 2°C, 83 ± 3% RH, a 12:12 h (L:D) photoperiod with 1 h of dusk/dawn transition obtained with automatic light dimmers. Females were allowed to blood-feed on my arm for 8-10 min to provide nutrition to mature eggs which they then oviposited on moist filter paper (90mm Whatman, UK) approximately 3 days after bloodfeeding. Hatched larvae were placed in large white plastic trays (30 X 45 X 6 cm) filled with 3.5 L of water. Larvae were then provided with fish flake food (Nutrafin Basix – Hagen, Taiwan) *ad libitum* until pupation, after which they were transferred in water filled glass containers and placed in large PerspexTM screen cages with screening on three sides (30 X 30 X 30 cm). Emerged adults were allowed to feed on a 5% sugar water solution *ad lib* from Erlenmeyer flasks containing a medical grade cotton wick (Richmond Dental, USA).

Experimental Apparatus

I used a total of 152 females with wing lengths ranging from 2.72-3.31 mm. Females were 4-7 days post eclosion, not previously blood-fed, presumed

mated based on observed copulations, and they did not exhibit swollen

abdomens to increase response to odour stimuli (Fernandes and Briegel 2004).

Females were introduced singly into a custom-made glass tube olfactometer

(145 x 17 cm, Figure 1) within which they could readily fly. Filtered air was drawn

into the tube by a pump (Petcetera AP5000 double type) at a constant flow of

0.025 L/s. Sixty centimeters upwind from a release chamber was a cylindrical

semi-rigid, tan coloured, plastic piece of netting (10 X 2.5 cm, 1 X 1 mm holes)

impregnated with 1% or 0.1% DEET in hexane (treatment) or hexane (control).

Hexane was allowed to evaporate by placing the net in a fume hood for 10 min.

Inside the net was a preconditioned nylon sock (Secret[TM] brand, knee high),

which had been worn for two consecutive days by me and incubated at 28 ± 2°C

for 24 h, to simulate human presence (Smallegange et al., 2010). The sock was

changed every 4-5 days to prevent mold growth. Seventy-three centimeters

upwind from the net was suspended a metallic tray carrying a honey (5 g) soaked

cotton ball (Kidd Bros. unpasteurized alfalfa and clover, Canada; full strength) to

simulate the presence of a nectar source some distance away. All experiments

were run under dim red light averaging 1.79 μmol/(s*m2).

Experimental Procedure

I conducted a 4 X 3 factorial-design experiment testing for the effects of

post-pupal-eclosion age and starvation level. All bioassay insects were 4, 5, 6

and 7 days post eclosion and had been starved for 6, 24 or 48 h by substituting

5% sugar water with deionized water. Starvation periods were assigned

randomly to adults following eclosion. Females were randomly selected and placed singly in the release chamber of the olfactometer. After a 3-min rest period, the metal screen gate of the release chamber was tilted, allowing the female to enter the olfactometer. If she failed to do so within 3 min, she was stimulated by light tapping. Following her entry into the olfactometer, the bioassay started, and the experimenter began to exhale every 4-5 s into a vinyl tube leading to the sock (Figure 1) to stimulate searching behavior by the female. Her movement was monitored and recorded as (*i*) the total number of biting attempts, (*ii*) the total time spent probing, and (*iii*) flight past the sock. The bioassay was terminated when the mosquito reached the honey source or rested for 5 consecutive minutes. Females were then sacrificed and dried for a minimum of 3 days following which their wings were measured using the software "Analyzing Digital Images (Version 11-2008)."

Data Analysis

I used GraphPad Prism 5 for all statistical analyses and graphs. I used non-parametric Kruskal-Wallis test to assess significant differences in the *An. gambiae* residence time at sock and net and Log-rank (Mantel-Cox) (i.e. survival analysis) to test differences in the length of time actively spent seeking the blood-host between treatment groups. Finally, χ^2 tests were employed to analyze the number of mosquitoes that passed by the sock, the number of non-responders, and the number of mosquitoes that rested or reached the honey.

Results

I found no effect of energy level on any of the following recorded variables: proportion of non-responders, defined as the proportion of individuals that failed to probe the sock-containing net (x^2, df.= 2, F= 1.33, p= 0.514), proportion of total experimental time spent probing (Kruskall-Wallis test, df.= 2, F= 1.84, p= 0.400), proportion flying past the sock bait towards the honey (x^2, df.= 2, F= 1.044, p= 0.593), and persistence, defined as, proportion of mosquitoes still active in the olfactometer over time (Log-Rank Mantell-Cox test, df.= 2, F= 2.507, p= 0.285).

Yet, when mosquitoes of different energy levels were combined in the three respective groups that exposed to the nets treated with hexane, 1% DEET or 0.1% DEET, statistically significant differences were detected between groups. In the presence of 1% and 0.1% DEET on nets, 4% and 13%, respectively, of mosquitoes, probed the net with the blood-host odour compared to 58% of females that did so when nets were treated with hexane only (Figure 2, x^2, df.= 2, F= 89.28, p<0.001). Similarly, 88% of females flew past hexane-treated nets compared to 70% and 59% of mosquitoes, respectively, that flew past nets treated with 1% or 0.1% DEET (Figure 3, x^2, df.= 2, F= 21.42, p<0.001).

I detected significant differences also in residence time at sock and net. When nets were treated with 1% and 0.1% DEET, female spent respectively 1.3% (±0.04) and 3.1% (±0.02) of time at the sock and net, while females spent 27% (±0.09) of time at nets treated with hexane (Figure 4, Kruskal-Wallis test, F= 48.88, p<0.001). The presence of DEET also significantly reduced the length of

sustained mosquito activities such as investigating the blood host odour bait (Figure 5, Log-Rank Mantell-Cox test on a Kaplan-Meier survival plot, df.= 2, F= 12.39, p=0.002).

Mean length of time per landing on the net (Kruskall-Wallis F= 3.126, p=0.210) and final outcome (wether the mosquito rested or flew to the honey at the end of the experiment) (χ^2 df.= 2, F= 5.11, p= 0.077) did not differ between treatment groups, even after combining mosquitoes of the three energy levels.

Discussion

My goal was to test whether female *An. gambiae* with different energy levels behave differently when facing an unobtainable host shielded by a repellent (DEET). The fact that females of different energy level did not differ in their response to host cues when facing barriers with high or low concentrations of DEET suggests that the repellent effect of DEET is very strong even at low concentrations. Mosquitoes exposed to DEET-treated nets were significantly less active and less likely to probe the host-scented sock than the mosquitoes exposed to hexane-treated control nets. In the very few instances where the mosquito probed, the residence time at sock and net was low compared to the control group.

Anopheles gambiae females exposed to DEET were also less likely to fly past the DEET-treated net. These findings confirm that DEET is easily perceived by the mosquito's olfactory sensory system (Syed and Leal 2008). Signals from

sensillae, after reaching the brain, are interpreted as such a strong threat that not even the risk of starvation triggers a positive taxis towards the blood-host scent making the mosquito unable to exploit the potential blood meal.

The argued notion that DEET obfuscates the blood host smell (especially lactic acid), making host detection more challenging if not impossible (Dogan et al. 1999) finds no support in the presented data. Further, other studies on similar chemoreceptors in Drosophila (Ditzen et al. 2008 and Lee et al. 2010), and in mosquitoes (Syed and Leal 2008), show that mosquitoes can smell and actively avoid DEET. The present study elucidates our understanding of the matter. By providing a stream of human breath in the immediate vicinity of the sock I funneled into the olfactometer not only the bi-products of foot smell but also chemicals present in the breath. If the sensillae perception of lactic acid is inhibited by DEET (Dogan et al. 1999), then the other chemicals should have elicited to at least an "investigational" response from the female which was not noticed. Furthermore, human breath and human sweat scent contain an array of chemicals so different from one another (Qiu et al. 2011) that different cells sensillae should detect them. The fact that females confronted with DEET crossed to the opposite end of the olfactometer less often than control individuals also suggests that an active repellent effect is indeed present.

Of course, it is also possible that in a dilution series, that at some very low level of DEET, one would begin to detect an effect on energy state. Even if this were true, however, such low levels would almost certainly not be employed in

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management programs thus my conclusions hold for management-level concentrations.

I found that energy levels of individual *An. gambiae* did not mitigate effects of DEET; mosquitoes simply avoided it. Further studies should be undertaken to determine whether energetic conditions influence the effects of other compounds commonly used to treat bednets, such as pyrethroids. If my findings hold true for these other compounds, then the presence of alternative food sources such as nectar in the vicinity of domiciles (see chapter 2) should not affect biting rate in the presence of treated bednets; it should drop regardless of energy level when a chemical is present.

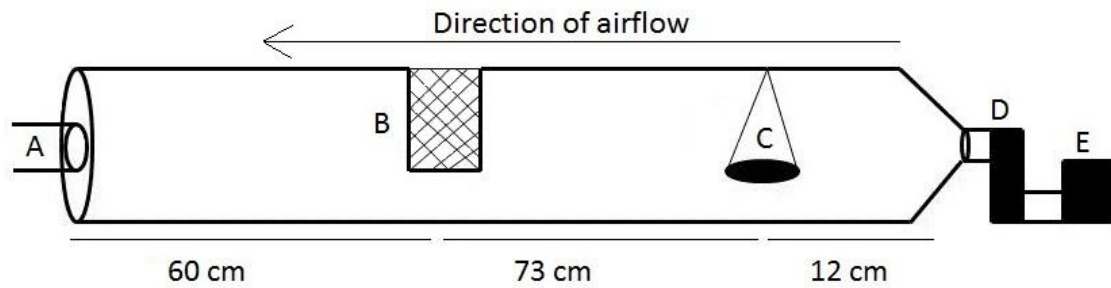## Acknowledgements

Figures

Figure 1. Experimental olfactometer setup. A. Mosquito release chamber. B. Simulated bed net containing a conditioned sock and a vinyl tube through which the experiment exhaled air. C. Position of honey attractant. D. Carbon filter. E. Air pump.
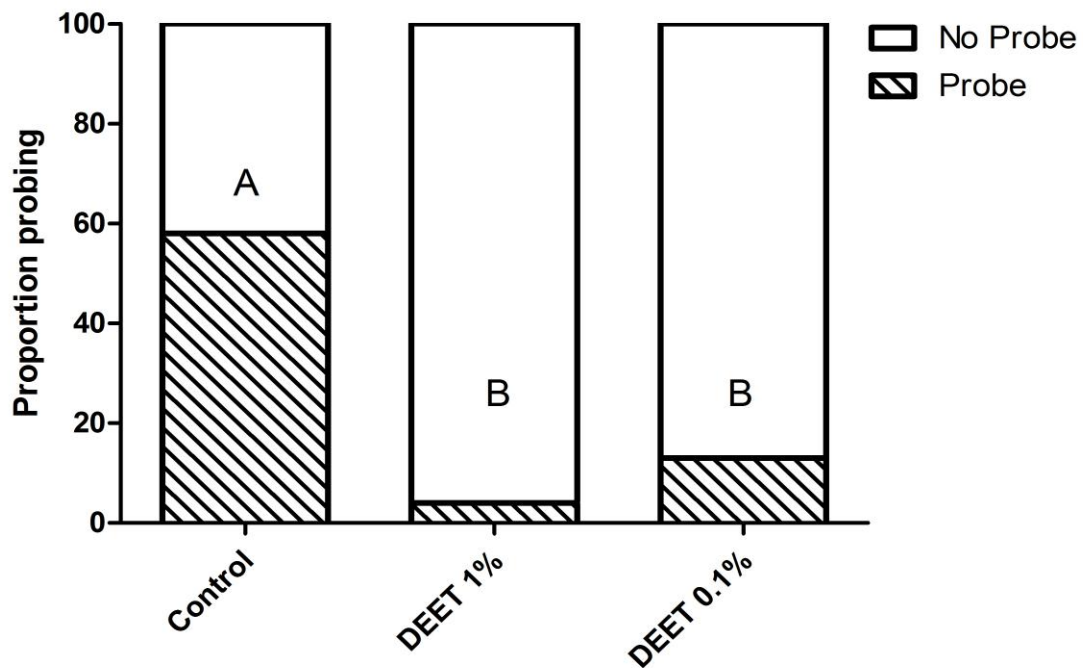
Figure 2. Proportion of *An. gambiae* probing the sock containing net as a function of DEET concentration (all ages and energy levels compounded). Any concentration of DEET yielded to significantly low number of responding females compared to controls (χ^2, $p<0.001$). Different letter indicate significant differences.
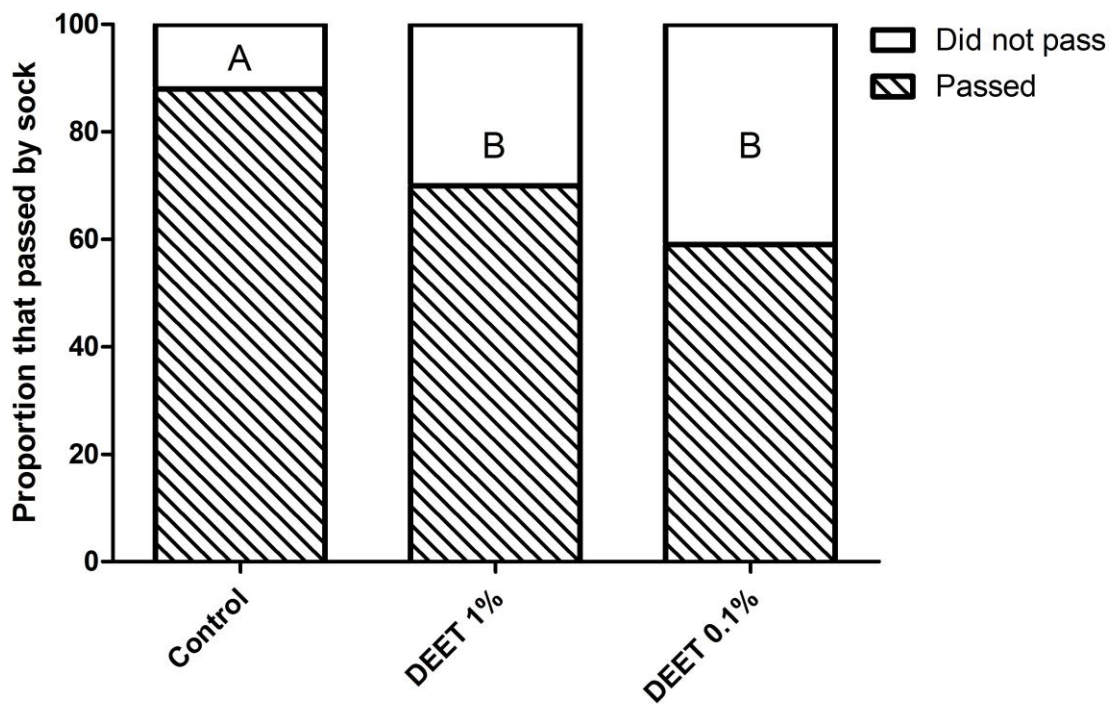
Figure 3. Proportion of *An. gambiae* that flew by the sock in the olfactometer (all ages and energy levels compounded). DEET treatments yielded to lower amount of mosquitoes passing by the impregnated plastic net compared to control (χ^2, $p<0.001$). Different letter indicate significant differences.
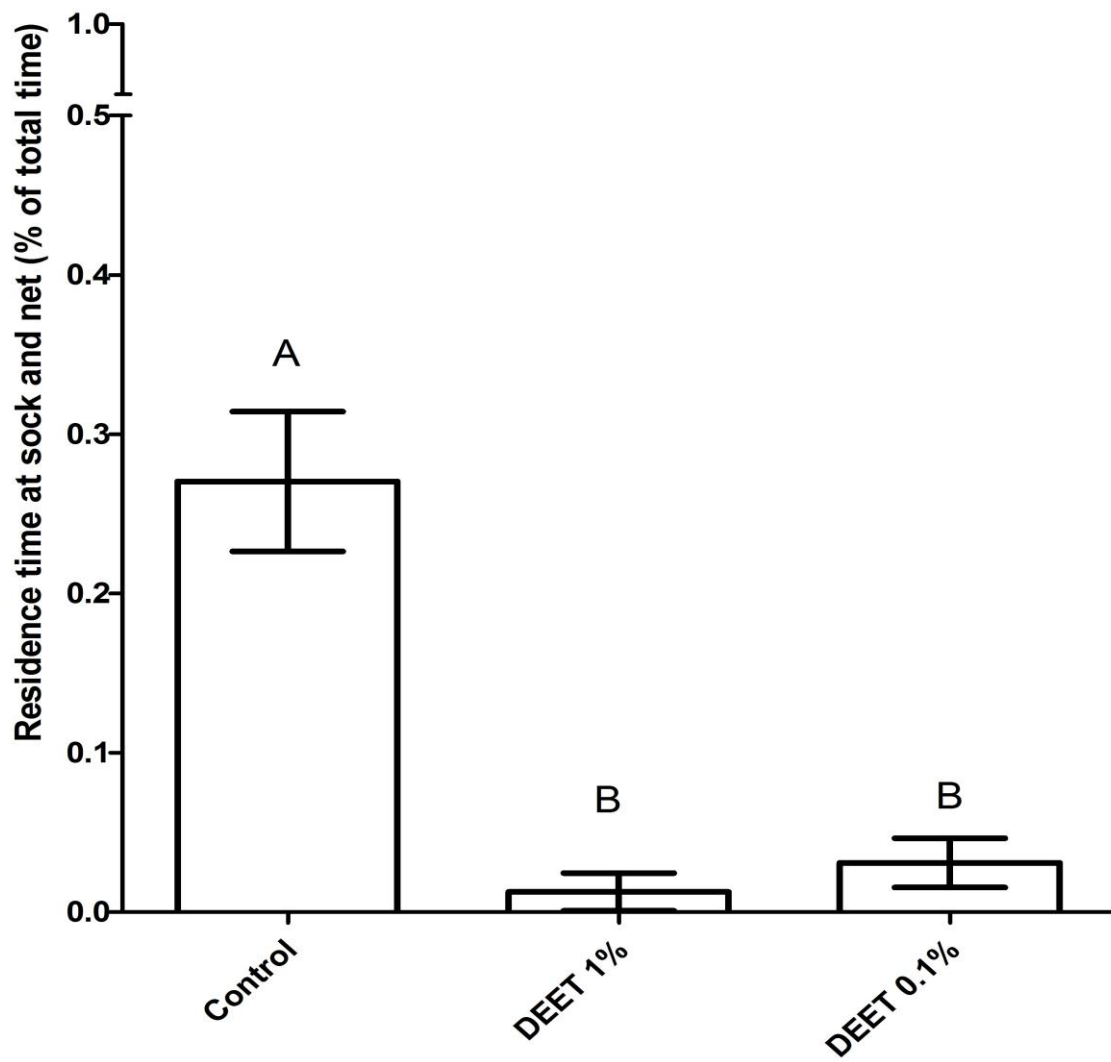
Figure 4. Mean proportion of time spent probing the sock by *An. gambiae* as a function of DEET concentration (all ages and starvation levels included). Treatments with DEET impregnated nets yielded to significantly fewer probing events compared with control (Kruskal-Wallis test, p<0.001). Different letters indicate significant differences.

Figure 5. Kaplan-Meier survival plot of *An. gambiae* persistence time (i.e. proportion of still active individuals; not flying towards honey scent and not resting for 5 consecutive minutes) as a function of DEET concentration. Individuals exposed to DEET impregnated nets decreased their activity significantly sooner than control treatments (Log-Rank Mantell-Cox, p=0.002).

List of References

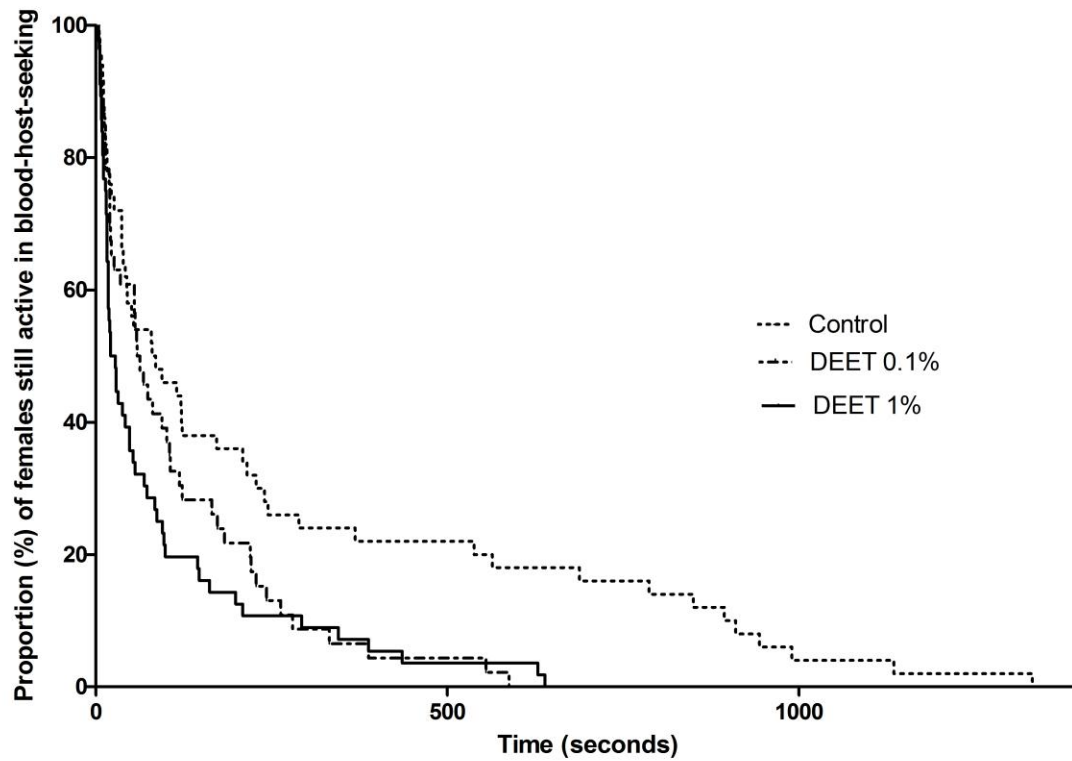Becker, N. 2000. Bacterial control of vector-mosquitoes and black flies. In Charles, J., Delécluse, A., Nielsen-LeRoux, C. (Eds) *Entomopathogenic Bacteria: From Laboratory to Field Application,* Kluwer Academic Publishers, Dordrecht, p. 383-396.

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Chapter 4: Conclusion

The goal of this thesis was to understand how differences in energy levels influence the behavior of mosquitoes towards blood-hosts protected by a physical-chemical barrier. Further, I wanted to understand if managing sugar sources in a typical African village would be a viable strategy to lower the incidence of malaria.

My results showed that energy levels indeed change the persistence of mosquitoes that attempt to obtain a bloodmeal, provided no repellent is present. Energy-depleted mosquitoes reacted by biting more often and for longer periods of time while non-starved ones responded less readily to blood-host smell. In the instances where DEET was applied as a chemical barrier, even at very low concentrations, the mosquito's reaction was an all-or-none decision. DEET repelled mosquitoes of all energy levels tested very efficiently, by shortening their activity time, probing time and overall kept them to one side of the olfactometer. This shows that in instances where chemical repellents cannot be applied to bednets, or have lost their efficacy, managing sugar sources could be an interesting strategy for mosquito control. Eliminating sugar sources would mean frailer, more starved mosquitoes which would also be more likely to die. Induced starvation would be beneficial for control strategies as it would increase risky behavior in "desperate" hungry individuals that try to obtain bloodmeals. Yet, while in this state, mosquitoes might be more successful at obtain a blood-meal through an untreated net.

Adding a degree of complexity to this already complex system is the fact that in a village setting, we find a mosaic of huts containing no nets, treated and untreated nets and even within huts, different people might use their nets differently. For example, firmly tucking the net underneath the sleeping surface may protect the person more efficiently than just loosely placing the net. Furthermore, in some domiciles, nets are used only for protecting children, while parents sleep without any protection. In these scenarios,

mosquitoes may be able to exploit and interact with more cues, which may influence their behavior.

 "Desperation" can be seen as both a benefit (increased death rate) and a disadvantage (higher success) but the persistence towards obtaining a potential blood-meal may be determined by a series of other factors that may work in synergy. To try to elucidate these points it would be important to conduct a few more experiments:

First, I would suggest a laboratory study in which adult size is manipulated in addition to energy state. Freshly emerged *An. gambiae*, as described in chapter 1, are frail and energy starved teneral adults looking to improve their body structure with an influx of proteins. This introduces the supposition that the reason to seek a first blood-meal for different size adults might go from mostly gametic (large females) to mostly structural (small females). Perhaps this change would also change the behavior towards the unobtainable blood-host as I expect that the small female might behave in a more "desperate" way than its larger counterpart.

Second, it is necessary to assess if the observed results are replicable in a large scale field experiment which can mimic the size of a small village more reliably. The olfactometer can be viewed as a tiny hut, where everything is scaled down (sugar source, blood-host, bednet, distances, etc…) while the mosquito is left at "actual size". In reality, the proportional differences between blood-host and mosquito are exponentially magnified and so are the distances between the inside and outside of the hut. In order to confirm my findings this work needs to be repeated in a field setting where proportions and distances are more realistic, I am currently planning a series of experiments to be carried in the small village of Mbita, Kenya. I will place four mud huts in a green house, equidistant from eachother and from a sugar source and equipped with either treated or untreated bednets and run experiments in which mosquitoes of different size and energy level releasing at dusk and recapturing them at dawn. Movements between huts will be recorded and possibly the contents of the mosquito's guts analyzed. This should shed a light on how mosquitoes behave when in a larger setting and during a longer exposure to the potential protected blood-host.

Third, in my design and interpretation of the results, I implicitly assumed that mosquitoes are unable to learn anything about host availability and likelihood of blood-feeding success. I also would suggest an experiment in which the same individuals are exposed to an unobtainable host repeated times to see whether the mosquito's persistence at biting the blood-host decreases with the now gained "knowledge" of its unobtainable nature. Would mosquitoes that "learned" then be less persistent in subsequent exposure to the host smell? Would they be less reactive?

In conclusion, from this thesis, I discovered that energy state does modulate behavior in *An. gambiae* when confronted with a physical barrier (bednet) protecting a blood-host. Yet, energy state is irrelevant if the blood-host is protected by a chemical repellent (DEET) as the chemical threat seems to overrule the urge to feed.