EXPERIMENTAL ASSESSMENT OF GENETIC VARIATION IN BLOOD FEEDING DURATION AND EVALUATION OF FEEDING DYNAMICS IN THE HUMAN MALARIA VECTOR *ANOPHELES GAMBIAE* SENSU STRICTO (DIPTERA: CULICIDAE)

By

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A Thesis
Submitted to the Faculty of
Graduate Studies
In Partial Fulfillment of the
Requirements for the Degree of

Master of Science
Department of Entomology
University of Manitoba
Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

OF

MASTER OF SCIENCE

I dedicate this thesis to my two uncles, John Kleiner and Bruce Gallimore, who, although they were unable to see me complete my research, inspired me to finish nonetheless. I also dedicate this to my grandmother Muriel Derham, who has helped me in both my studies and in life, perhaps more than she knows.

ABSTRACT

EXPERIMENTAL ASSESSMENT OF GENETIC VARIATION IN BLOOD FEEDING DURATION AND EVALUATION OF FEEDING DYNAMICS IN THE HUMAN MALARIA VECTOR *ANOPHELES GAMBIAE* SENSU STRICTO (DIPTERA: CULICIDAE)

By

Scott W. H. Derham

Major Advisor: Dr. Robert A. Anderson

To preserve the effectiveness of vector control efforts aimed at minimizing the transmission of malaria parasites, such as the use of insecticide treated bed nets, an improved understanding of the feeding behaviour and dynamics of *Anopheles gambiae* sensu stricto is necessary. This study examined whether there was a genetic basis underlying variation in blood feeding duration, and the relationship between fecundity and total blood feeding duration. A total of 1530 individuals were examined, with gut-filling duration, prediuresis duration, fecundity, and wing length data recorded. Based on 34 mother-daughter pairs (iso-female lines) it was impossible to state with certainty whether a genetic basis for variation in blood feeding duration existed. A decelerating payoff curve for fecundity-feeding duration data, modeled using regression coefficients, was observed. Consequently, a significant truncation in feeding duration may be needed before a relative change in fecundity is observed.

ACKNOWLEDGEMENTS

The research that has gone into this thesis could not have been possible without the support and assistance of a great many people. I am grateful for the input and assistance of my thesis advisor, Dr. Robert A. Anderson. Dr. Anderson has generously provided financial assistance and moral support during the course of my studies. Without Dr. Anderson's assistance I would have been unable to complete this research. I am also grateful to both Dr. Gary H. Crow and Dr. Scott Forbes who helped with several of the analytical problems that arose. I also thank the input at various stages of my thesis from my committee members, Dr. Terry Galloway and Dr. Jim Hare.

I thank the staff in the Biology Department at the University of Winnipeg, in particular Terry Durham, for their assistance with my research and allowing me to conduct my trials in their facilities. I am incredibly grateful to my research assistants Mark Stepaniuk, Maria Prado and Carrie Ross, for without their help mosquito observations would have been far more tedious.

I acknowledge the financial assistance from the Natural Sciences and Engineering Research Council of Canada. Financial assistance was also provided by GDG Environment Limited. I thank Christian Back for his input, particularly his pertinent questions regarding the conclusions reached herein. Lastly, I am grateful for the moral and at times financial support of my parents Clive Derham and Patricia Cameron both of whom encouraged me to complete my studies even when the challenges seemed insurmountable.

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INTRODUCTION

Blood feeding, for a majority of mosquitoes, is essential for the acquisition of proteins for both metabolic activities and to initiate oogenesis (Jones, 1978; Briegel, 1990; Klowden, 1995; Kelley, 2001). Blood requirements facilitate the transmission of various blood-borne pathogens, such as *Plasmodium* parasites that are responsible for malaria. Mosquitoes transmit other pathogens, such as encephalitis viruses, filarial worms, yellow fever and dengue viruses that, like malaria, also cause considerable human morbidity and mortality each year (Jones, 1978; Klowden, 1995).

Mosquito behaviors have evolved that facilitate blood-host location in heterogeneous environments. They rely upon visual, chemical and physical stimuli to locate hosts (Knols, 1995). With the location of a suitable blood host, mosquitoes must engage in the risky process of blood feeding. The principal risk associated with engorgement is eliciting a host reaction, such as grooming, that may, at least, reduce blood intake, and, at worst, be fatal (Gillett, 1967; Edman and Kale, 1971; Knols, 1995; Chadee *et al.* 2002). As well, there is a risk of predation by spiders and other predators following engorgement due to increased weight from blood, and hence decreased mobility (Roitberg *et al.* 2003). Mosquitoes have evolved behavioral and physiological adaptations that reduce risk associated with blood feeding. For instance, mosquitoes salivate copiously into the wound introducing apyrase, an anti-haemostatic factor that increases blood flow to the region (Ribeiro *et al.* 1985a; Pappas *et al.* 1986). If interrupted prior to the completion of engorgement, a combination of behavioral and physiological processes determine whether or not additional blood feeding will occur to increase fecundity (Anderson and Brust, 1997; Anderson and Roitberg, 1999).

Some tropical anopheline species, including *Anopheles gambiae* Giles *sensu stricto*, have smaller mid-gut capacities than other species, a factor for which they compensate by prediuresis (Briegel, 1990). Prediuresis is a process during which damaged erythrocytes and blood plasma are actively excreted while feeding to increase the concentration of intact erythrocytes (Briegel and Rezzonico, 1985). This adaptation increases the period spent in contact with blood hosts relative to simply filling the gut and thus may increase the risk of eliciting host, anti-mosquito behavior.

Given its importance to malaria transmission, *An. gambiae* has been the subject of renewed research interest in recent years. With the sequencing of this species' genome in 2002 (Holt *et al.* 2002), research has focused upon the development and eventual introduction into the wild of transgenic mosquitoes that are refractory for *Plasmodium* species (Alphey *et al.* 2002; Tabachnick, 2003). In addition, current control efforts based on insecticide-impregnated materials and indoor residual spraying are receiving further study given their potential to alter mosquito feeding behavior in ways that may affect the epidemiology of malaria (Haridi, 1972; Charlwood *et al.* 1986; Charlwood and Graves, 1987; Lengeler *et al.* 1996; Knols and Takken, 1998; Maxwell *et al.* 1998; Mathenge *et al.* 2001; Charlwood *et al.* 2001; Braimah *et al.* 2005). Without additional research to understand these dynamics, current and future vector control efforts may be less effective, thus permitting the continued transmission of malaria.

Gillett (1967) and Chadee and Beier (1997) suggested that there may be a genetic basis underlying feeding duration in *Aedes aegypti* Linneaus. They demonstrated that feeding durations increased when individuals were given access to either restrained hosts or artificial membrane systems. It appears that, in the absence of selective pressure for

fast feeding mediated by blood-host defensive behavior, feeding duration increased. If a similar genetic basis for blood feeding behavior exists for *An. gambiae,* this may affect current and future control efforts. However, research using *Ae. aegypti* revealed that blood feeding duration may not be subject to genetic control as the differences between individuals from short and long feeding lines declined with each subsequent generation (Chadee *et al.* 2002). Blood feeding duration may be described by phenotypic plasticity. Thus, it is uncertain whether there is a genetic basis associated with variation in blood feeding duration or whether this variation is simply due to phenotypic plasticity.

Roitberg and Gordon (2005) created a fitness payoff curve for *An. gambiae* by modeling the relationship between fecundity and body weight (an indicator of blood volume), as measured in change in mass before and after feeding. Based on their research, there was a decelerating return in terms of fecundity associated with increasing body weight, regardless of body size. In addition, Anderson and Roitberg (1999) modeled feeding dynamics of mosquitoes and suggested that any benefit associated with continued blood feeding should decrease linearly over time, while the risk of eliciting host response should increase. But what is the interaction between fecundity and feeding duration? In other words, at what point does the benefit associated with increased feeding duration, in relation to fecundity, decline and how might this selection on mosquito feeding behavior affect control efforts?

Objectives of the Research

1) To determine if there is a genetic basis for variation in blood feeding parameters, such as duration of gut-filling and prediuresis, in *An. gambiae s. s.*, the most important vector of human malaria in sub-Saharan Africa.

2) To determine the relationship between fecundity and feeding duration in *An. gambiae s. s.*

3) To integrate data relating to the potential genetic control of blood feeding parameters and feeding dynamics so as to assess the potential impact that alterations to *An. gambiae* feeding behavior and feeding dynamics may have on vector control strategies aimed at minimizing the impact of malaria in sub-Saharan Africa.

THESIS ORGANIZATION

This thesis is organized into the following sections, literature review, abstract, introduction, materials and methods, results, discussion, a concluding discussion, conclusions and references. All tables and figures are numbered consecutively from the beginning of the thesis. Pertinent figures and tables are presented near where they are cited. Questions of interest and objectives are presented in detail in the introduction. Pertinent literature is considered in the literature review to provide an overview of the research material. A brief discussion is provided to summarize the conclusions.

Chapter 1

LITERATURE REVIEW

Overview:

An assessment of the epidemiology of malaria produces a discouraging picture for public health in the tropics. It is estimated that 300 to 500 million people become infected with one of four species of malaria-causing parasites (*Plasmodium falciparum* Welch, *P. vivax* Grassi and Feletti, *P. malariae* Laveran and *P. ovale* Stephens) each year (Sachs and Malaney, 2002; Knols *et al.* 2002a; Wanji *et al.* 2003). This figure should be cautiously interpreted because many affected regions lack adequate equipment and trained health professionals to diagnose non-symptomatic cases accurately, and in some instances, even symptomatic cases. As a result, current assessments may actually be underestimates of the true burden of malaria in developing nations. Further, 0.7 to 3 million people succumb to malaria annually, the vast majority of whom are children (Collins and Paskewitz, 1995; Hourgard, *et al.* 2002; Ito *et al.* 2002; Knols *et al.* 2002a).

Morbidity and mortality rates attributed to malaria infection have increased over the past two decades (Greenwood and Mutabingwa, 2002; Miller and Greenwood, 2002). For instance, in eastern and southern Africa the proportion of malaria infections that result in death has increased from 18% during the 1980s to 37% in the 1990s (Killeen *et al.* 2004). These rising rates are because of a combination of factors such as deteriorating health systems due to monetary constraints and civil disturbances, the continued evolution of insecticide and drug resistance, the negative impact of human alteration of the environment, and human migration and population displacement which is a particular problem where civil strife occurs (Hougard, *et al.* 2002; Moreira, *et al.* 2002). As a result,

the death toll attributable to malaria has been predicted to double in the coming years if no new control measures are devised and implemented (Hougard *et al.* 2002; Keiser *et al.* 2004). This is a problem of particular concern due to rapidly expanding populations of people in endemic regions already burdened by malaria.

Nowhere is the severity of this impact more apparent than in sub-Saharan Africa where approximately 90% of all malaria-related mortality occurs (Greenwood and Mutabingwa, 2002; Sachs and Malaney, 2002). In this geographical region, approximately 450 million people inhabit areas where malaria is endemic (Knols *et al.* 2002a). Additionally, it is further estimated that 50 million inhabitants of sub-Saharan Africa are subjected to periodic malaria epidemics.

This impact also generates serious economic problems. For example, more than 80% of the 44.7 million disability adjusted life years (D.A.L.Y.s) attributed to malaria are concentrated in sub-Saharan Africa (Keiser *et al.* 2004). Based on the global distribution of per-capita gross domestic product (GDP), a correlation exists between malaria and poverty (Sachs and Malaney, 2002; Malaney *et al.* 2004). For instance, in malaria endemic countries, the GDP in 1995 was $1,526 US compared to $8,268 US in non-endemic states (Sachs and Malaney, 2002). In endemic countries, the per capita GDP is approximately 20% that seen in non-endemic countries (Malaney *et al.* 2004). Moreover, the average growth rate for the period from 1965-1990 was 0.4% in endemic countries compared to 2.3% for non-endemic countries. It should be noted that malaria and economics may be a positive feedback cycle in the sense that malaria exacerbates poverty and economic constraints exacerbate malaria's impact, particularly given the reduction in available funds for health and control services. To alleviate this clinical and economic

burden, the World Health Organization (W.H.O.) has estimated that current control expenditures need to be increased from the US$100 million presently allotted per annum to US$2.6 billion by 2007 and eventually to US$4 billion by 2015 (Sachs and Malaney, 2002).

Mosquito Biology:

The ability of female mosquitoes to transmit pathogens is due principally to their biological requirements for blood. Most female mosquitoes require access to blood for metabolic activities and synthesis of yolk and subsequent egg development (Klowden, 1995; Kelley, 2001). Blood requirements by female mosquitoes facilitate the acquisition and transmission of blood-borne pathogens such as malaria.

Haematophagous (feeding or subsisting on blood (Pfadt, 1978)) insects such as mosquitoes, respond to chemical, visual and physical stimuli to locate potential hosts (Knols, 1995). Chemical stimuli, such as carbon dioxide, are important for mosquitoes to locate hosts over great distances (Sutcliffe, 1994). Female *Ae. aegypti* were able to detect fluctuations in ambient carbon dioxide levels of as little as 0.01%. When flying, *An. gambiae* have been observed to rely on visual cues (i.e. junction between woodland and grassland) to navigate at night (Allan *et al.* 1987). Once in the vicinity of hosts, mosquitoes orient to a combination of visual, physical (heat) and chemical cues, which may also induce landing and initiation of blood feeding (Takken and Knols, 1999). Mosquitoes are also sensitive to physical cues such as slight temperature changes. *Aedes aegypti* respond to temperature fluctuations of as little as +/- 0.2°C (Lehane, 1991).

Response to host cues is dependent on the host preference of the mosquito species in question. Depending on host availability and abundance, mosquitoes may feed on less

preferred hosts, a phenomenon closely linked to seasonal shifts in host distribution and density (Mboera and Takken, 1999). Strongly anthropophilic (those associated with human habitation and preferentially feeding upon humans (Gullan and Cranston, 2005)) species, such as *An. gambiae*, rely on a synergistic combination of carbon dioxide and human-specific kairomones (interspecific chemical messages that benefit the receiver but not the emitter (Klowden, 2002)) (Mboera *et al.* 1997; Takken *et al.* 1997). Among the cues used by *An. gambiae* to locate potential hosts are volatiles produced by bacteria, *Brevibacterium epidermidis,* from the feet, a region preferentially fed on in human hosts (De Jong and Knols, 1995).

Once hosts are located, mosquitoes engage in the risky process of blood feeding. The primary risk associated with blood feeding is the possibility of eliciting anti-mosquito behaviors, such as grooming, which may increase with increasing feeding duration (Waage and Nondo, 1982). Consequently, successful females should be those that complete blood feeding more rapidly, prior to the onset of host irritation, indicated by activities such as, grooming (Gillett, 1967; Knols, 1995; Chadee *et al.* 2002). During probing and feeding, female mosquitoes salivate copiously, causing increased blood flow to the area by dilating blood vessels and reducing haemostasis (Pappas *et al.* 1986). Haemostatic compounds in the host released by tissue injury slow blood flow causing red blood cells to clump, thus causing plasma leakage and haemoconcentration (Pappas *et al.* 1986). Salivary components play a critical role in minimizing contact time between blood hosts and mosquitoes, thus, reducing the risk of eliciting anti-mosquito behaviors (Ribeiro, 2000). Probing duration is inversely correlated with apyrase levels, a compound responsible for the breakdown of ADP and subsequent inhibition of haemostatic

pathways (Ribeiro *et al.* 1985a; Ribeiro, 2000). Blood location was significantly faster in salivating individuals compared to individuals that had had their salivary tubes ligated (Ribeiro *et al.* 1984; Ribeiro *et al.* 1985b). The average total blood feeding duration for non-salivating individuals given access to the backs and ears of guinea pigs was 420s and 223s compared to 95s and 44s for salivating individuals. Thus, salivary components, such as apyrase, permit mosquitoes to reduce their engorgement time and increase their likelihood of survival.

Following oogenesis, female mosquitoes search for suitable oviposition sites. Fertilized eggs are laid either singly or in rafts dependent upon the mosquito species in question (Jones, 1978). Larvae are omnivorous and feed upon bacteria, pollen, microscopic plants and other debris. Foraging conditions during larval development may determine the success of individual development and subsequent population growth. Sub-optimal nutrition levels under crowded conditions can seriously impact adult populations (Klowden *et al.* 1988; Blackmore and Lord, 2000). When overcrowding occurs, food becomes scarce and reductions in size, teneral reserves, longevity, blood feeding success and fecundity of adult female's result (Terizan and Stahler, 1949; Haramis, 1983; Hawley, 1985; Koenraadt *et al.* 2004).

Adult body size is a determinant of fecundity among female mosquitoes (Hogg *et al.* 1996). Smaller females are less successful in reproductive terms than larger females for two principal reasons. Smaller females often require two or more blood meals to obtain sufficient protein to initiate oogenesis (Chadee and Beier, 1997; Takken *et al.* 2001). Additional host contacts may increase the probability of feeding-associated mortality (Onyabe *et al.* 1997). Consequently, smaller female mosquitoes may incur

greater survival risks due to their requirement for supplementary feeding ("*acquisition of additional blood meals after the first during a single gonotrophic cycle with the feedings being separated by intervals of several hours to a day or more*" (Clements, 1992)). Larger females emerge from pupation better nourished, often requiring only one blood meal, and as a result they are exposed to less risk than smaller individuals. For instance, Klowden *et al.* (1988) found that smaller *Ae. aegypti* were not well represented among groups of blood-fed individuals. Smaller females typically have fewer ovarioles (Hurd et al, 1995) such that, even if sufficient blood protein is obtained, their fitness may be less than larger mosquitoes. The numbers of eggs laid during each gonotrophic cycle is positively correlated with both the blood volume ingested and the number of ovarioles present, both of which are determined by body size (Klowden and Lea, 1979; Daniel and Kingsolver, 1983; Briegel, 1985).

Tropical anophelines typically emerge smaller compared to other tropical and temperate species and hence they may require additional blood meals to build up resources (Briegel, 1990). Even when food is abundant, larval anophelines are unlikely to accumulate sizable teneral reserves, a fact that may affect the behavior of emerging adults (Briegel, 1990; Takken *et al.* 2002; Briegel, 2003). This inability to accumulate sizeable nutrient reserves is due to a combination of anatomical limitations (small mid-gut volume) and typically crowded environments. Anopheline larvae lack a siphon; as a consequence, they are confined to harvesting the water surface for floating organic debris (Dahl, 1988; Briegel, 1990). Combined with the fact that they often develop in crowded, ephemeral habitats, tropical anophelines, such as *An. gambiae*, typically emerge as undernourished adults (Klowden *et al.* 1988; Clements, 1992; Takken *et al.* 1998). For

example, in a study area in the Kenyan highlands, 83.7% of larval *An. gambiae* were found in small (i.e. hoof prints, natural depressions, and holes created by human activity), potentially crowded pools (Minakawa *et al.* 2004).

Autogeny has not arisen in these species as this behavior has evolved in situations where the probability of locating suitable blood hosts is low (Clements, 1992). *An. gambiae* has evolved in close association with their preferred human hosts and as a result there has been no selective pressure to forgo blood feeding (Besansky *et al.* 2004). Consequently, tropical anophelines have evolved means other than autogeny to compensate for their inability to accumulate sizeable teneral reserves during larval development.

Tropical anophelines compensate for insufficient teneral reserves by supplementary feeding. Tropical anophelines that seek supplementary blood meals may be exposed to an increased risk associated with irritating their vertebrate host (Briegel, 2003). For instance, mosquitoes that emerge with insufficient reserves are not as likely to develop eggs during their first gonotrophic cycle because most nutrients acquired during this initial blood feed are allocated to metabolic requirements (Ramasamy *et al.* 2000). As a result most individuals require successive blood meals to supplement their teneral reserves and thus complete their first gonotrophic cycle (Klowden *et al.* 1988; Takken *et al.* 1998b; Takken *et al.* 2001).

Species, such as *An. gambiae,* may also compensate for diminished teneral reserves with an anatomical and physiological adaptation (Day and Edman, 1984; Clements, 1992; Maxwell *et al.* 1998; Takken *et al.* 2002). Prediuresis has evolved as an adaptation to compensate for limited mid-gut capacity. Prediuresis is a process by which

mosquitoes actively excrete serum or a combination of serum and some erythrocytes while feeding (Briegel and Rezzonico, 1985). This process commences shortly after the mid-gut is completely distended, the point at which other mosquitoes in other genera cease feeding. Tropical anophelines are able to concentrate critical erythrocytes thus improving potential fecundity (Briegel, 1990; Vaughan *et al.* 1991; Hogg *et al.* 1996). Prediuresis occurs as spines within the hindgut are aligned to create a sieve that allows erythrocytes to be trapped while plasma is expelled via the anus (Vaughan *et al.* 1991; Hurd, 2003). Prediuresis is defined as being distinct from diuresis as it occurs actively while feeding. Diuresis, seen in temperate and larger tropical mosquito species, involves the excretion of excess plasma to lessen the weight burden after blood feeding has been completed and usually not in the vicinity of the blood host.

Prediuresis ability varies widely among anophelines. For example, *An. albimanus* Wiedemann, *An. quadrimaculatus* Say, *An. arabiensis* Paton, *An. gambiae* and *An. stephensi* Liston are capable of concentrating erythrocytes by factors of 1.9, 2.2, 1.4, 1.8 and 1.7 times respectively (Briegel and Rezzonico, 1985; Vaughan *et al.* 1991; Hogg *et al.* 1996). With an estimated maximum mid-gut capacity of approximately 2 µl, *An. stephensi* females consumed and filtered as much as 10 µl of blood. Although prediuresis alleviates the limited mid-gut capacity, it increases the total feeding duration and hence increases the risk of eliciting anti-mosquito behaviors.

When compared to other tropical anopheline species, the total blood feeding duration for *An. gambiae*, defined as the time required for probing, engorgement and prediuresis, is generally longer. For instance, when individually fed upon a human forearm, total blood feeding duration for *An. gambiae*, 231.6s +/- 79.3s (Mean +/-

Standard Deviation), was nearly one and a half minutes greater than the blood feeding duration of 143.3s +/- 41.3s seen for larger *An. albimanus* and *An. freeborni* Aitken (Chege and Beier, 1998). As a result, *An. gambiae* may be exposed to an elevated risk of being interrupted or eliciting host reaction.

Despite the increased risk, prediuresis has been documented in numerous species, such as *An. gambiae, An. stephensi, An. tessellatus* Theobald, *An. funestus* Giles, *An. culcifacies* Giles and *An. subpictus* Grassi (Mitchell and Millian, 1981; Ramasamy *et al.* 2000). When female mosquitoes are interrupted during feeding, they may either cease and lay a small batch of eggs or seek an additional blood source (Anderson and Brust, 1997; Anderson and Roitberg, 1999). There is a tradeoff between increased fecundity and decreased survival due to increased engorgement time. Consequently, any additional fitness gained, in terms of fecundity may be offset by the risk of eliciting host reaction. Thus, overall fitness is a compromise between the mosquito's immediate survival and potential lifetime fecundity.

Dynamics of malaria transmission are affected by anatomical limitations in tropical anophelines. While prediuresis enables females to achieve potentially greater reproductive fitness, the concentration of erythrocytes may potentially increase the acquisition of blood borne pathogens (Taylor and Hurd, 2001). Supplementary blood feeding may also increase the ability of *An. gambiae* to serve as a vector for *Plasmodium* parasites by creating additional transmission opportunities (Kelly and Edman, 1992). Even relatively low frequencies of multiple host contacts present additional opportunities for pathogen acquisition or transmission by female mosquitoes (Anderson and Brust, 1997).

Mosquito & Host Interaction:

As a result of the annoyance caused by feeding mosquitoes, vertebrates attempt to minimize their contact with mosquitoes. Vertebrate blood hosts exhibit a variety of motor activities that discourage, disturb and even kill haematophagous insects such as mosquitoes (Edman and Scott, 1987). Some of the more common anti-mosquito behaviors include, but are not limited to, grooming, foot stamping and foot slapping (Edman *et al.* 1974; Waage and Nondo, 1982). This irritability may serve as a selective pressure on mosquito feeding dynamics, such as feeding duration. Based on experimental models, there is a tradeoff exhibited between the length of time required to obtain an optimal blood meal to achieve maximal fecundity, and the time before mosquito probing elicits a defensive reaction from the host (Anderson and Roitberg, 1999). Host irritation, denoted by itching, is a response to mosquito salivary compounds that are injected during engorgement (Gillett, 1967). Irritation may also be caused by the mouthparts of the mosquito damaging nerve endings at the site of the bite. Thus, to engorge successfully, it may be advantageous for mosquitoes to finish feeding prior to causing host irritation.

Any benefit associated with blood feeding may be expected to decrease over time because the risk of eliciting anti-mosquito behavior increases with increased host contact (Anderson and Roitberg, 1999; Roitberg *et al.* 2003). As mosquitoes probe randomly on a non-homogenous vascular system, there are instances where blood vessels are too deep to be accessed, resulting in desistance or the cessation of probing. Regular desistance behavior benefits mosquitoes because they abandon sites where blood is too difficult to locate and thus the risk of eliciting host reaction is diminished.

Host selectivity is another means by which mosquitoes can reduce feeding-associated risks. Most blood hosts have a tolerance threshold above which they will react to nuisance mosquitoes and thus exhibit defensive behaviors (Ribeiro *et al.* 1985a; Anderson and Roitberg, 1999). Smaller vertebrates typically have the lowest biting tolerance in relative terms, and, as a result, often display the most effective defensive behaviors, possibly a function of their reduced mass that enables them to groom a greater proportion of their body (Clements, 1992).

With a few exceptions, mosquitoes do not often engorge successfully on some rodent species and hence they tend not blood feed on these small mammals, a factor believed to be attributable to their effective anti-mosquito behaviors (Edman and Kale, 1971; Walker and Edman, 1985). In laboratory trials, mosquitoes were effectively prevented from engorging on unrestrained rodents such as *Peromyscus gossypinus* (cotton mouse), *Sigmodon hispidus* (cotton rat) and *Sciurus carolinensis* (gray squirrel) (Edman and Kale, 1971). Mosquito engorgement may be facilitated on larger hosts if they are available, particularly as their tolerance threshold is expected to be greater, a significant determinant of feeding success in species such as *Culex nigripalpus* Theobold (Edman *et al.* 1974). The difficulty associated with feeding on small blood hosts, such as birds and mammals, may also be due to mosquito density which is inversely correlated with blood feeding success (Day and Edman, 1984; Clements, 1992). Studies have shown that host defensive behavior is strongly correlated with mosquito density (Edman and Scott, 1987). For example, the engorgement success of *Cx. nigripalpus* decreased with increasing density when exposed to unrestrained avian chicks (*Nycticorax nycticorax, Butorides virescens, Eudocimus albus* and *Bubulcus ibis*) (Edman *et al.* 1972). Similar

observations were seen for *Ae. triseriatus* Say and *Lutzomyia longipalpis* Lutz and Nevia, when exposed to small mammals (Klowden and Lea, 1979; Coleman and Edman, 1987).

Host health and age may also affect successful engorgement of mosquitoes. Young hosts often lack structural and behavioral defensive abilities, and as such, are more susceptible to mosquitoes than adults unless protected by their parents (Edman and Scott, 1987; Clements, 1992). Sick hosts often possess defensive skills required to reduce engorgement by mosquitoes however; their defensive abilities may be compromised. When presented with a choice of healthy or malaria-infected mice, mosquitoes fed predominantly on sickened mice as they offered the least resistance (Day *et al.* 1983). For example, when placed in a cage with one infected and one uninfected mouse, 50% of mosquitoes fed. Yet, when placed in a cage with two infected mice, 80% of mosquitoes successfully engorged, thereby illustrating an ease of engorgement on less defensive hosts.

A further impact of host-illness relates to parasite-induced anaemia. Mosquitoes are often interrupted while blood feeding, particularly on healthy hosts, before they can successfully acquire a sufficient blood volume to initiate oogenesis. Engorgement on anaemic hosts, however, proceeds more rapidly due to decreased blood viscosity. As a result, the threshold required to initiate oogenesis can be attained faster, albeit with reduced fecundity due to lowered erythrocyte concentration (Shieh and Rossignol, 1992). This ability to acquire blood rapidly from anaemic individuals could significantly alter malaria transmission dynamics (Rossignol *et al.* 1985). Optimal haematocrit levels for engorgement are often below those commonly found in healthy hosts, but not as low as in severely affected hosts with advanced malaria.

Mosquitoes that can engorge rapidly should be more likely to escape host defensive responses (Gillett, 1967). These individuals may be able to complete a greater number of gonotrophic cycles, and have a higher overall lifetime fecundity (Chadee *et al.* 2002). When individual *Ae. aegypti* were exposed to restrained hosts, or artificial systems, blood feeding duration lengthened (Chadee and Beier, 1997). The increase in blood feeding duration may be due to the absence of the selective pressure posed by host defensive behaviors. Thus, host reaction may be a selective force influencing feeding duration in mosquitoes.

Genetic Basis:

Has the risk of eliciting host defensive responses influenced the feeding behavior of *An. gambiae*? Any trait that has a genetic basis should, theoretically, respond to selective pressures such as blood-host defensive behavior. The possible existence of heritable traits such as blood feeding duration of *An. gambiae* may affect current and future vector control efforts. Whereas the presence of a genetic basis for variation in blood feeding duration in *An. gambiae* is uncertain, the genetic modulation of other behavioral parameters has been demonstrated experimentally.

In one of the first studies examining the genetic basis of behavior, E. C. Tolman (1924) attempted to determine whether traits such as intelligence were genetically modulated. Two independent strains of rats, one "dull" and one "bright", were established by selective breeding. Intelligence was selected based on two parameters; time to complete a maze and the number of errors committed along the way. This initial population was reduced through the creation of a distribution curve from which equal numbers of males and females were selected to form 'bright' and 'dull' parental colonies.

Bright parental lines produced similarly intelligent offspring, particularly when compared to the offspring from dull lines. Yet, continued selection through the F2 and subsequent generations failed to reveal an increasing intelligence. Tolman postulated that these reduced effects might have resulted from excessive inbreeding that could have uncovered a debilitating recessive trait such as nervousness. In addition, the lack of heritability may also have been due to an age restriction that may have excluded individuals of optimal ages from the trials.

W. C. Rothenbuhler (1964) examined the behavioral genetics of nest cleaning in honey bees. Brood rearing in honey bees requires the repetitive use of waxen cells in the combs of their nests. These cells are cleaned after each brood has been reared; however, when larvae or pupae die within cells, they are left to accumulate. In some colonies, particularly those infected with *Bacillus larvae* White (causative organism of American foulbrood, AFB), dead individuals are left to accumulate progressively within the nest. Not all honey bees are capable or willing to clean these cells. Some honey bees have demonstrated a 'hygienic behavior' in response to larval and pupal mortality by removing their dead young. Rothenbuhler selected four lines of honey bees, two that were resistant to AFB and displayed 'hygienic behavior' and two that were susceptible to AFB. Resistant strains removed most of their brood killed by AFB, whereas susceptible lines permitted AFB victims to accumulate. Through inbreeding and environmental manipulation (confining the queen to a given colony), Rothenbuhler was able to select for AFB resistance among honey bees. Given Rothenbuhler's results, it was illustrated that 'hygienic' behavior was genetically modulated because artificial selection permitted the establishment of AFB-resistant lines.

Researchers examined the genetic control of song patterns in two genera of crickets, *Teleogryllus* and *Gryllus (T. commodus, T. oceanicus, G. armatus, G. rubens and G. campestris)* (Bentley and Hoy, 1972). The genetic component of song pattern in crickets was demonstrated along three principle lines of evidence. Songs of individuals possessing the same genotype were very similar when measured, and this homogeneity was observed even when rearing conditions differed between individuals. Homogeneity was also maintained when alterations were made to parameters such as diet, light cycles, temperature fluctuations and seasonal changes. Songs of individuals with differing genotypes remained distinct, even when environmental conditions were identical. A sex-linked control of song patterns was eventually demonstrated. When hybridized with non chirping males, females produced offspring that chirped unlike their fathers. The authors concluded that cricket song patterns were controlled by a complex polygenic system, and hence these traits were heritable.

Selection experiments with mosquitoes began in the 1960s. Gillies (1964) attempted to select artificially for feeding preference among wild *An. gambiae* strains to determine if this trait was genetically based. Selection experiments were conducted within a screened and divided hut with cattle bait on one side and human bait on the other. The parental generation was released into a room separating the two baits, given equal access to each, and allowed to feed uninterrupted. Those that had engorged on cattle formed the parental generation for cattle preference, while those feeding on humans formed the parental generation for human preference. The F1 and subsequent generations were released into the dividing room and permitted to feed uninterrupted. A well-defined host-preference difference was observed between the two groups by the F2 generation.

The cattle strain was carried through for five generations, and all of these showed a higher preference for cattle than their parents. Similarly, the human strain was reared through seven generations, and significant differences were observed between the F4 and F5 generations and the parental generation. Thus, Gillies demonstrated that it was possible to select for feeding preference within a few generations. Further, natural populations of *An. gambiae* exhibited a degree of polymorphism for host preference and this may provide the basis upon which selection for behavioral changes could operate (Gillies, 1964).

Mukwaya (1977) sought to determine the importance of genetic and environmental effects on feeding preference in *Aedes simpsoni* Theobald and *Ae. aegypti* in the wild. Mukwaya hypothesized that, if feeding preference is genetic in origin, this may be due to uneven distribution of gene frequencies maintained by partial or complete genetic, geographical or behavioral barriers. Biting preferences persisted regardless of environmental influences, and differences remained even when temperatures were identical. Thus, host preferences were not affected by environmental factors, suggesting that this trait may have a genetic component.

Feeding preference selection in the weakly anthropophilic *Ae. aegypti* Kampala strain was successful after a few generations (Mukwaya, 1977). Moreover, confirmation of the existence of genetic control for feeding preferences was illustrated by the creation of inter-strain hybrids (weak and strongly anthropophilic individuals) that exhibited intermediate host preferences when compared to their respective parents. Further, given that only a few generations were required to successfully alter feeding preferences, it was postulated that few genes might regulate this trait.

Vertebrate host defensive behavior may serve as a selective mechanism that constrains feeding duration (Gillett, 1967). Gillett (1967) defined the period during which successful engorgement should occur as the 'safety period'. The 'safety period' is defined as the point before which host irritation is elicited, and as such, successful mosquitoes are those that are able to complete blood feeding, including prediuresis, during this period. Host irritation/ inflammation in response to probing/ blood feeding by mosquitoes may arise in two ways. Initially, there exists a high probability that mosquitoes may cut through a nerve receptor while probing or feeding (Gillett, 1967; Clements, 1992). The probability of cutting a nerve receptor declines markedly after the probing period, as long as the mosquito does not need to re-position their proboscis to access a better engorgement site. Inflammation/ irritation occurs as a response to the salivary compounds injected by the mosquito to facilitate blood feeding, and the degree to which hosts exhibit this is dependent on their genetic makeup and prior exposure (Gillett, 1967). Desensitization may be extremely localized in its distribution even within hosts that have been exposed to large numbers of mosquitoes. Although responses to blood feeding varies between hosts, at the population level, selection for the degree of irritation acts on average response, as such, it is this average that influences feeding behavior of mosquito populations. Therefore, if blood feeding duration has a heritable component, two assumptions can be made. Fast feeders should have a greater probability of completing engorgement prior to eliciting host reaction and therefore, their survival rate and fecundity may be greater. Slower feeding mosquitoes should have reduced fitness due to smaller blood meals and an increased risk of eliciting anti-mosquito behaviors. Yet, if longer feeding duration coupled with prediuresis, can compensate for small mid-gut

volume, then, in laboratory situations individual mosquitoes should be seen to engorge for a longer period after the removal of vertebrate host pressure (Gillett, 1967; Chadee and Beier, 1997).

To test this hypothesis, Gillett (1967) compared feeding speed between wild-caught *Ae. africanus* Theobold, and colonized *Ae. aegypti* at the population level. Although the 'safety period' was found to be similar for both species, overall feeding duration differed considerably. Colonized *Ae. aegypti* that had not been exposed to selective pressures, such as host irritability, had a feeding period that was approximately 80% longer compared to wild *Ae. africanus*. Faster feeding durations observed for wild female *Ae. africanus* were attributed to the impact of defensive behaviors exhibited among sensitized blood hosts. Despite the support for this hypothesis, Gillett's conclusions must be interpreted with caution because two different species of mosquitoes were compared.

Day and Edman (1984) also speculated that feeding patterns in mosquitoes might be genetically based and hence predetermined. Smaller hosts are subject to fewer attacks by mosquitoes; this situation may have been selected to avoid small rodent hosts because of their elevated defensive behavior. Wild mosquitoes exhibited reduced host seeking and probing durations compared to mosquitoes from laboratory colonies that were not usually exposed to defensive blood hosts. Moreover, wild individuals had lower mortality rates, a factor that may be attributed to rapid feeding speed. Therefore, mosquito blood feeding preference should be biased toward those hosts that display reduced or less effective defensive reactions, thus facilitating engorgement while increasing individual survival.

However, consider the results of experiments carried out in Trinidad, West Indies regarding mosquito blood feeding dynamics at an individual level among wild and laboratory colonies of *Ae. aegypti* (Chadee and Beier, 1997; Chadee *et al.* 2002). Chadee and Beier (1997) hypothesized that, if blood feeding duration in *Ae. aegypti* was genetically determined, fast-feeding individuals should be selected against and subsequently lost in laboratory colonies. In the absence of vertebrate host selective pressure, fast feeding individuals should have a compromised fecundity compared to slow feeding individuals who are better able to accumulate more blood proteins, and as a result, over time, this fast feeding trait will be lost (Gillett, 1967; Chadee *et al.* 2002). While more than 71% of female mosquitoes in the parental generation were fast feeders, this trait was not maintained through the F1 and F2 generations. Extended feeding times observed in natural and laboratory settings have been hypothesized to evolve in response to reduced host reactions (Edman and Scott, 1987; Vaughan *et al.* 1991).

Chadee *et al.* (2002) challenged the genetic hypothesis when they revealed that colonized female *Ae. aegypti* did not maintain fast and slow blood feeding traits through F1, F2, F3 and F4 generations. Slow feeders generally fed faster in later generations, whereas fast feeders subsequently took longer to feed in later generations. Additionally, there was little difference between the numbers of eggs laid by slow and fast feeders throughout the trials, suggesting that blood feeding duration may not compromise fecundity. Consequently, female *Ae. aegypti* may retain the ability to regulate their feeding duration dependant upon environmental conditions. Blood-feeding duration in *Ae. aegypti* therefore, may not have a genetic basis associated with it, but rather may be an example of phenotypic plasticity.

Feeding Dynamics & Control Efforts:

Malaria intervention efforts, including molecular and field-based techniques, are subject to numerous uncertainties. To improve current and future strategies, additional knowledge is needed regarding the ecological and evolutionary aspects of malaria transmission as well as vector biology.

One approach that has seen significant development in recent years is the genetic engineering of mosquitoes to be refractory to infection with human malaria parasites. The World Health Organization identified three primary goals that needed to be addressed prior to the release of any transgenic mosquitoes. The first step involved the identification of potentially refractory genes. Two principal genes have been identified that inhibit *Plasmodium* development by encapsulation of ookinetes in melanin or lysis of ookinetes shortly after they penetrate the mid-gut epithelium (Collins *et al.* 1986; Collins and Besansky, 1994; Tabachnick, 2003). Second, the appropriate technology was required to facilitate the insertion of these refractory genes into the targeted mosquito genome. The final goal is to introduce these genetically modified mosquitoes into natural populations.

With the completion of genome sequencing for *An. gambiae* (Holt *et al.* 2002), molecular research has shifted to the development of methods to drive *Plasmodium*-refractory genes to fixation in natural populations. However, there are a series of biological and ecological questions regarding the successful introduction of these transgenic mosquitoes into the wild (Knols *et al.* 2002b; Ito *et al.* 2002; Scott *et al.* 2002; Nirmala and James, 2003). For instance, Tabachnick (2003) highlighted the uncertainty surrounding both the competency and viability of laboratory modified vectors in field situations. How will these genetically altered mosquitoes react to the broad range of

environments and genetic variability of conspecifics encountered in natural settings? More concerning is the question of how will the introduced genes affect the fitness of target species (Enserink, 2002). Will transgenic mosquitoes be capable of competing with wild mosquitoes for mates and thereby propagate their genetic information? Further, how efficient will transgenic mosquitoes be in terms of feeding on intolerant hosts, a significant component of overall fitness. Given these uncertainties, the release of genetically modified vectors into natural settings to control malaria transmission remains many years away (Alphey *et al.* 2002; Killeen *et al.* 2002).

Insecticide treated bed nets (ITBN) (mosquito nets impregnated with synthetic pyrethroids) have been adopted by the Roll Back Malaria initiative to combat malaria (Enayati and Hemingway, 2006). Although programs that rely on insecticide-impregnated materials have shown promise, there are uncertainties associated with them. One key concern relates to the impact that their prolonged use may exert upon blood-feeding dynamics, in particular blood-feeding duration, of *An. gambiae* (Lengeler *et al.* 1996).

ITBN impede mosquito feeding success and hence malaria transmission in three ways. First, ITBN act by killing individual mosquitoes, during or after engorgement, by exposing them to a lethal insecticide dose (Knols and Takken, 1998; Takken, 2002). Even if mosquitoes feed successfully, they may not survive long enough for the *Plasmodium* parasites to develop to their infective sporozoite stage. Second, commonly-used insecticides exert an excito-repellent effect and thus mosquitoes may only rest briefly on the net fabric before leaving the house (Knols and Takken, 1998; Corbel *et al.* 2004). Third, some insecticide formulations deter mosquitoes from entering treated houses all

together. For example, marked exophily of the principal vector, *An. arabiensis,* was seen in Sudan in response to the excito-repellent effect of DDT used as an indoor residual insecticide (Charlwood *et al.* 2001). Despite their promise, the long-term efficacy of ITBN for malaria control is debatable.

Arguments in support of ITBN are two-fold. Overall mosquito densities in endemic areas where bed nets have been deployed were reduced (Takken, 2002). Further, the mean age of mosquito populations was reduced after bed net deployment. For instance, in western Kenya, there was a 71.5% reduction in the indoor-resting densities of blood fed *An. gambiae* and *An. funestus* in areas with ITBN compared to areas lacking them (Gimnig *et al.* 2003a). Similar results were observed in the Gambia, Burkina Faso and coastal Kenya. With the reductions in mosquito densities, a parallel decrease in the entomological inoculation rates of 78-95% was also seen (Gimnig *et al.* 2003b). Additionally, in control villages, the overall sporozoite rate (a measure of the number of mosquitoes infected with malarial parasites) was 3.4%, whereas in intervention villages it was 1.2% (Fanello, *et al.* 2003; Gimnig *et al.* 2003a). A 'community effect' was also observed, where villages within 300m of control areas saw lowered risk of malaria parasitemia, anemia and death, particularly among young children (Charlwood and Graves, 1987).

When compared to other control efforts, the use of ITBN is more cost effective (Kweku Aikins *et al.* 1998). Although most ITBN are currently provided free of charge in some programs, the aim is to encourage local participation and appreciation of these nets so that they may later purchase their own and responsibly maintain them. In localized studies, however, the challenge of instilling the value and ownership of nets has proven

difficult (Mathenge *et al.* 2001). Efficacy trials were conducted in four African locales and were followed by dramatic reductions in malaria mortality. For instance, in endemic regions, the incidence of malaria declined from 63% to 25% and 0% in the first and second years respectively following the distribution of ITBN (Knols and Takken, 1998). However, the sustainability of this intervention without external support is questionable. For example, in the Gambia, a region with a long history of ITBN usage, fewer than 15% of nets are regularly re-treated with insecticides by their local owners, possibly due to the associated costs (Clarke, et al 2001). This lack of re-treatment may facilitate the development of resistance among targeted mosquito populations because individuals will begin to receive sub-lethal doses once the effective concentration of the insecticide has fallen.

As with previous insecticide-based treatments, that relied upon DDT for example, there is the potential for the development of resistance to common synthetic pyrethroid insecticides used on bed nets (Charlwood *et al.* 2001; Asidi *et al.* 2005; Enayati and Hemingway, 2006). Resistance to pyrethroid insecticides is particularly wide spread across West Africa where the *kdr* (knock-down resistance) gene has been detected among some target *An. gambiae* populations (Fanello, *et al.* 2003). However, other members of the *An. gambiae* complex in the region have not shown the *kdr* mutation; additional mutations may be involved in the development of resistance. Current research has led to the design of pyrethroid formulations that are less excito-repellent and irritant to extend the contact/exposure time, thereby increasing the probability that targeted vectors acquire lethal doses (Corbel *et al.* 2004).

Mosquito feeding and resting behavior has been altered by the use of ITBN and other insecticide-based treatments. Alteration of vector behavior may impact control efforts if the imposed shifts cause mosquitoes to feed when protection is not afforded. For example, regular indoor residual application of DDT in the Solomon Islands altered the feeding time of *An. farauti* Laveran from later in the evening to earlier when human hosts were free from treatment areas in their homes (Charlwood *et al.* 1986; Charlwood and Graves, 1987). Similar shifts in preferred feeding times were seen with *An. farauti* in Papau New Guinea, and with *An. gambiae* in Tanzania and coastal Kenya (Knols and Takken, 1998; Maxwell *et al.* 1998; Mathenge *et al.* 2001). In Tanzania, the percentage of bites received before bed increased from 30% to more than 60% and in some cases to more than 80% after ITBN deployment. Following six years of continued use of ITBN in Tanzania, mosquitoes were selected that feed earlier in the evening and later in the morning when hosts are active and not protected (Braimah *et al.* 2005).

Residual application of DDT in Sudan caused a shift in resting behavior of *An. gambiae*, from endophily (preference to rest indoors) to exophily (preference to rest outdoors) in as little as three years (Haridi, 1972). Additionally, widespread application of DDT on China's Hainan Island from the 1950s onwards caused a shift in resting behavior from endophily to exophily (Clements, 1992). Host selection may also be influenced by insecticide use. For instance, residual DDT application also caused a shift in host preference from humans to cattle. DDT application in the Solomon Islands and Thailand caused a similar shift in feeding preference among *An. farauti* and *An. minimus* Theobald (Charlwood *et al.* 1986; Chareonviriyaphap *et al.* 2001).

Due to their hosts' highly sensitive skin, anthropophilic *An. gambiae* usually feed in the late evening and early morning when human hosts are typically sleeping and thus less defensive. In villages equipped with ITBN, *An. gambiae* blood feed earlier in the evening compared to villages without the control devices (Bockarie, *et al.* 1996; Mathenge *et al.* 2001). Further, *An. gambiae* shifted their feeding and resting sites from indoors to outdoors in response to insecticides. If female mosquitoes fail to locate a blood meal the previous night, they may seek hosts earlier the following evening to permit oogenesis (Mathenge *et al.* 2001). Widespread bed-net use may alter the age structure through the elimination of later-feeding, parous females thereby leaving predominantly nulliparous individuals which tend to feed earlier in the evening (Gillies, 1957; Bockarie *et al.* 1996). Also, shifts in the feeding cycle may be due to alterations in the genetic structure of local populations that favour those females that access hosts earlier and hence successfully engorge.

Host selection patterns result from a combination of intrinsic host preferences and host availability (Burkot *et al.* 1988). Although *An. gambiae* is strongly anthropophilic, it will use non-human hosts when they are readily available and access to preferred hosts is restricted (Mwangangi *et al.* 2003). For example, in Sao Tome, people tend to live in houses constructed on stilts above the ground to avoid ground level humidity and mosquito bites (Charlwood *et al.* 2003). In these regions, malaria reaches only meso-hyperendemic levels because nocturnally feeding *An. gambiae* are restricted to 2 – 3 m above the ground and are thus unable to access preferred human hosts regularly (Charlwood *et al.* 2003). *Anopheles gambiae* feeding shifted to domestic animals, such as dogs and pigs, kept beneath the houses. Similarly, in Ethiopia where individuals sleep

above ground level (3-4m) and where domestic animals are housed beneath, only 3.1% of *An. arabiensis* blood meals came from humans, whereas most blood meals were obtained from cattle, sheep and goats (Habtewlold *et al.* 2001).

Anopheles gambiae is highly anthropophilic and synanthropic, and as such it is one of the most effective human malaria vectors. This synanthropic behavior has arisen in conjunction with the evolution of agriculture in human communities (Besansky *et al.* 2004). *Anopheles gambiae*, *An. arabiensis* and *An. funestus* may have evolved synanthropic and anthropophilic behaviors due to the presence of reliable blood sources and novel breeding sites found in human communities engaged in agricultural practices. As a result of their close association with humans, these species are of great concern.

While selective pressures (natural and artificial) have been shown to alter feeding preferences (Takken, 2002) and preferred feeding times (Charlwood and Graves, 1987; Mathenge *et al.* 2001) of *An. gambiae* and other species, it is uncertain what effect a selective force may have upon mosquito feeding duration. If there is a genetic basis associated with variation in blood feeding duration in *An. gambiae*, the dynamics of malaria transmission and vector control efforts could potentially be affected by changes in feeding duration selected for by these same interventions. Rapid engorgement may benefit individuals, so long as fecundity is not significantly compromised, as they will be able to continue accessing preferred human hosts. For instance, given the excito-repellent effects of ITBN, and that they protect sleeping humans, the ability to engorge rapidly should prove beneficial because anthropophilic mosquitoes such as *An. gambiae* will only be able to access preferred hosts during their active hours when they are most sensitive to mosquito irritation (Knols and Takken, 1998; Gimnig, *et al.* 2003a). Shortened blood

feeding duration may also permit individuals to access protected sleeping hosts without acquiring a lethal dose of insecticide if the dose accumulates over the time of exposure. This possibility is particularly problematic given the lack of adequate bed-net retreatment. Although any reduction in feeding duration may only be slight (a few seconds), if it facilitates engorgement, then, provided that overall fecundity is not significantly compromised, *An. gambiae* could still acquire and transmit *Plasmodium* parasites. Consequently, ITBN could act as a selective force for feeding duration if there is a genetic basis associated with variation for this trait.

Experimental assessment of genetic variation in blood feeding duration and feeding dynamics in the human malaria vector *Anopheles gambiae* sensu stricto (Diptera: Culicidae).

ABSTRACT

This study was carried out to test the hypothesis that some of the observed variation in feeding duration of *An. gambiae sensu stricto* can be ascribed to genetic differences among individual mosquitoes with respect to time spent feeding. Females were reared in iso-female lines with comparisons made between mothers and daughters for gut-filling duration, prediuresis duration, fecundity and wing length. Based on 1530 individuals tested, 34 mother-daughter pairs, with data for all parameters, were compared. Due to small sample size, potential lack of variability in the studied colony, non-measured anatomical and physiological parameters and rearing discrepancies, it is not possible to say definitively that there is a genetic basis for variation in blood feeding duration in *An. gambiae* s. s. Further research is thus warranted.

A second aim of this study was to determine the nature of the relationship between fecundity and total feeding duration. Complete data for 185 individuals was obtained. Using regression coefficients, with wing length treated as a covariate, a curvilinear relationship between fecundity and total feeding duration was observed. A functional response model, comparing fecundity and total feeding duration, revealed a decelerating payoff in terms of fecundity with increased feeding duration. These findings may have implications for the continued effectiveness of current and future control efforts.

INTRODUCTION

Since the 1970s and the conclusion of the global campaign to eradicate malaria, the impact of this parasite-borne disease has been steadily increasing in severity worldwide, particularly in under-developed, tropical regions (Takken, 2002). Malaria parasites (*Plasmodium falciparum, P. vivax, P. ovale and P. malariae*) are estimated to infect 300 to 500 million people (Sachs and Malaney, 2002; Knols *et al.* 2002; Wanji *et al.* 2003; Keiser *et al.* 2004) and kill 0.7 to 3 million people annually (Phillips, 2001; Hougard *et al.* 2002; Ito *et al.* 2002; Bremen *et al.* 2004; Ferguson *et al.* 2005). Nowhere is the impact of these pathogens more strongly felt than in sub-Saharan Africa, where it is estimated that 90% of all malaria-related mortalities occur, where 450 to 500 million individuals inhabit endemic regions (Greenwood and Mutabingwa, 2002; Knols *et al.* 2002a; Besansky *et al.* 2004). Approximately 50 million people in this area are subjected to periodic malaria epidemics. It has also been postulated that the number of malaria cases, particularly in sub-Saharan Africa, may double within the next 20 years in the absence of improved control strategies and new drug development (Keiser *et al.* 2004).

The impact of malaria in sub-Saharan Africa is complicated by a combination of factors such as poverty and lack of adequate health sources (Phillips, 2001) as well as the strongly synanthropic and anthropophilic nature of the three primary vector species of mosquitoes, *Anopheles gambiae*, *An. arabiensis* and *An. funestus* (Besansky *et al.* 2004). Given this increased burden, there is renewed interest in vector control research to minimize human-vector contact and hence exposure to the disease (Alnwick, 2001). Despite these efforts, there are biological, behavioral and ecological questions that require further study.

Recent vector control efforts have been focused significantly on the development of genetically modified mosquitoes that posses genes coding for refractoriness to malaria parasites (Holt *et al.* 2002; Ito *et al.* 2002; Tabachnick, 2003). There are many unanswered questions regarding the feasibility of introducing transgenic mosquitoes into the wild. Given these uncertainties, current control programs rely upon the use of ITBN and indoor residual spraying (IRS). Both of these tools use residual insecticides to restrict mosquito access to human hosts and thus reduce the transmission and acquisition of malaria parasites by mosquitoes. Despite their feasibility (Kweiku Aikins *et al.* 2003), the application of residual insecticides may select for a shift in vector feeding time (Charlwood and Graves, 1987), resting behavior (Haridi, 1972; Clements, 1992) and preferred host (Habtewold *et al.* 2001; Charlwood *et al.* 2003). Further study is required to determine what impact these shifts in behavior may have upon malaria transmission cycles (Mathenge *et al.* 2001). One of the key uncertainties relates to the impact that residual insecticides may have in terms of blood feeding-dynamics, in particular blood feeding duration (Lengeler *et al.* 1996). If targeted mosquito populations are forced to feed earlier in the evening when preferred human hosts are active and more sensitive to mosquito probing, their feeding dynamics may be altered. Mosquitoes may have to feed more rapidly to avoid eliciting defensive responses from blood hosts (Gillett, 1967). This rapid feeding, however, may affect both the size and quality of blood meal and consequently, overall fecundity, and thus be subject to strong selection.

Blood feeding by mosquitoes has been well studied at the population level (Gillett, 1967), but there is little work to address variation among individual vectors as a basis for understanding the evolution of behavioral traits that facilitate blood feeding

success (Chadee and Beier, 1997; Chadee *et al.* 2002). Individual mosquitoes that exhibit an extended feeding duration increase the risk of eliciting potentially fatal defensive responses from the blood host (Edman and Scott, 1987; Vaughan *et al.* 1991; Klowden and Briegel, 1994; Anderson and Roitberg, 1999). In nature, some *Culex* species alter their feeding behavior to avoid particularly defensive hosts such as rodents (Day and Edman, 1984). Moreover, in laboratory experiments, *An. stephensi* and *An. quadrimaculatus* fed more successfully on restrained hosts than on unrestrained ones; thus, feeding time may be limited by host responses (Day and Edman, 1984). Given that blood feeding is costly and potentially associated with an elevated risk of mortality, there may be a dynamic trade-off inherent in blood feeding as blood volume is maximized but constrained by risks posed by vertebrate defensive behaviors. Consequently, any benefit associated with additional blood obtained may be minimized due to the potential linear relationship between increasing risk and feeding duration (Anderson and Roitberg, 1999).

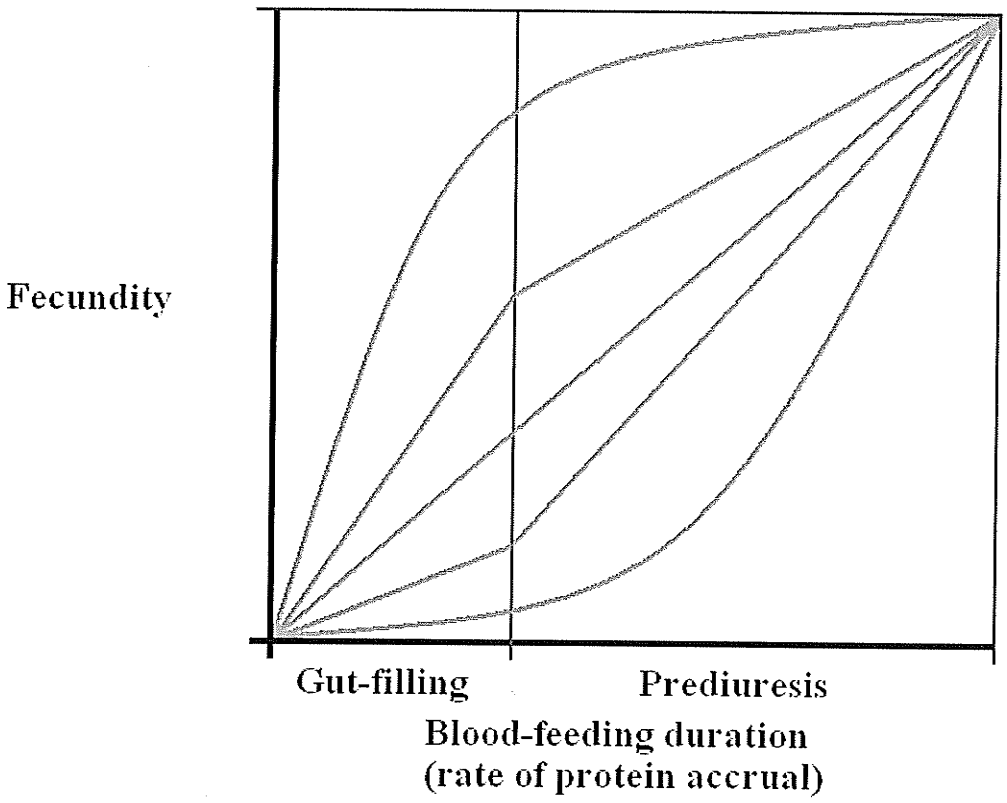
As a result of malaria interventions and altered mosquito behaviors, such as the evolution of behavioral resistance to insecticides, mosquito populations may be expected to encounter more active and hence potentially defensive human hosts if feeding is diverted to non-sleeping times. Consequently, successful mosquitoes should be those that are capable of engorging more rapidly than others, thereby minimizing their feeding associated risk, so long as their fecundity is not significantly compromised. A behavioral polymorphism for feeding duration may arise in several ways. The rate at which blood enters the mosquito mid-gut is a function of local availability of blood vessels in the skin at the feeding site, size of the mosquito (in particular the diameter of their feeding tube), ingestion rate and blood-host haematocrit. Thus, one would expect to observe significant

variation in feeding duration among individual mosquitoes as a function of these variables, but it is also possible that some variation in feeding duration is due to genetics as demonstrated by both Gillett (1967) and Chadee and Beier (1997) for *Ae. aegypti*. Thus, host-defensive behavior may be expected to select for individuals that are capable of completing their engorgement prior to eliciting host reaction, thereby minimizing their risk.

Mosquitoes that shift their feeding times to access preferred human hosts may feed more rapidly to avoid eliciting defensive responses from blood hosts in response (Gillett, 1967). This rapid feeding, however, may affect both the size and quality of blood meal and consequently overall fecundity, and thus be subject to strong selection. Given this, what proportion of time is spent by *An. gambiae* in prediuresis and how does this correspond to fecundity? Potential relationships between fecundity and total feeding duration (comprised of gut-filling duration and prediuresis duration) vary and some of these are outlined in Figure 1. It is particularly important to foster a better understanding of this relationship for two reasons. First, given the importance of blood in terms of fecundity, it is essential to assess where the greatest accumulation of protein occurs. Moreover, at what point does blood ingestion begin to plateau, and hence little further accumulation occur with additional feeding? Second, an improved understanding of the relationship between fecundity and total feeding duration is also required to evaluate the potential for vector control tools, such as ITBN and IRS, to alter the feeding behavior of target populations of *An. gambiae*.

The purpose of this study is thus two fold. The first aim is to determine if there is genetic basis for variation in feeding parameters, such as gut-filling duration and

Figure 1 – Diagram outlining potential patterns of protein accrual over time for *Anopheles gambiae* and the corresponding potential effects on fecundity during the two feeding phases.



prediuresis duration in *An. gambiae*, the primary vector of human malaria in sub-Saharan Africa. The second aim is to determine the nature of the relationship between fecundity and feeding duration. Both aims are critical to improving our understanding of the behavior of *An. gambiae* and thereby improve current and future control efforts.

MATERIALS & METHODS

Colony Rearing:

The colony used in these experiments was *Anopheles gambiae* sensu stricto from the Kilombero Valley region in Tanzania. This species has been maintained in captivity for approximately seven years with access to restrained blood hosts, human volunteers or artificial membrane systems for egg development.

Larvae and adults, including those forming the stock, parental and daughter colonies, were maintained in simulated tropical conditions. The temperature was kept at approximately 28°C ± 1°C. The relative humidity was maintained at 60% ± 1%. The photoperiod was 12 hours light and 12 hours dark, with sunrise at 22:00hr C.S.T. and sunset at 10:00hr C.S.T.. Larvae were reared in a small upright controlled environment chamber, while adults were reared in a walk-in controlled environment chamber, both maintained at identical conditions.

To maintain the stock colony, females were blood fed twice during each gonotrophic cycle. Adults forming stock and parental colonies were kept in one of two sizes of Perspex® cage (39 X 39 X 39cm or 34 X 34 X 34cm). Daughters were kept in smaller Perspex cages (15 X 15 X 15cm). Three days after the second blood feeding, an oviposition container (6.5 cm diameter X 4 cm deep; with 90 ml of distilled water) was placed inside the Perspex cage and eggs were collected.

First instar larvae were transferred to small Tupperware® containers (12 cm long X 8 cm wide X 6 cm deep; with 250 ml of distilled water) until they developed into second and third instars where they were transferred to larger plastic rearing pans (43 cm long X 28 cm wide X 6 cm deep; with 1500 ml of distilled water). Larvae from the stock and parental colonies were reared at low densities, approximately 300 individuals per pan (or 4.01cm^2 surface area per larva), given the importance of body size to development and subsequent adult blood-feeding behavior and fecundity (Blackmore and Lord, 2000; Koenraadt *et al.* 2004). To maintain iso-female lines (discussed later in this section), daughters were reared in small oviposition containers (6.5 cm diameter X 4 cm deep; with 90 ml of distilled water). Daughter rearing densities varied between a low of 26 larvae per oviposition container (1.63cm^2 per larva) and a high of 112 larvae per oviposition container (0.38cm^2 per larva). The average larval rearing density was 66 larvae per oviposition container (0.64cm^2 per larva). Larvae were fed non-ground, flaked Tetramin® fish food which increased in quantity, ad libitum, to accommodate growth. Pupae were collected using plastic pipettes and placed in oviposition containers, then transferred to large Perspex® cages for emergence.

To ensure that individuals were within a narrow age range, adults that formed the parental generation were only placed within the same cage if they emerged over a three day period. Adults were given access to a 5 % sucrose solution to increase the likelihood that oogenesis and oviposition occurred (Gary and Foster, 2001; Awono-Ambene *et al.* 2001).

Iso-female lines (offspring of individual females reared in isolation from other larvae to permit identification) were used in this experimental design to assess possible

correlations for variation in blood feeding parameters between mothers and daughters. Based on published experimental data for laboratory reared *Aedes* and *Anopheles* species, mothers were isolated from the parental colony two to nine days post eclosion, for experimental trials (Briegel and Horler, 1993; Chadee and Beier, 1997; Ribeiro, 2000; Tseng, 2003; Andreassen *et al.* 2004). Females were provided with the opportunity to mate, as mating status influences egg maturation and thus fecundity (Uchida et al 2003; Klowden and Russell, 2004). Mating status was not assessed, as blood-fed virgin females would not have matured eggs. Once emerged, females were permitted a minimum of two days to mate within their respective cages, thereby increasing the likelihood that blood feeding and oviposition might be observed (Uchida *et al.* 2003). Daughters were only able to mate with their siblings, although no offspring from such matings were retained for subsequent analysis.

Females were isolated arbitrarily using a mouth aspirator. Individuals were each placed in a plastic *Drosophila* vial (a standard culture tube for rearing *Drosophila* cultures; 9.5 cm height X 3 cm opening diameter) containing a filter paper at its base for future oviposition. The *Drosophila* vials were covered with a single layer of wedding veil to allow probing and feeding on an artificial membrane as well as to permit observation of these behaviors. Mothers and daughters were removed from their respective colonies/cages and isolated in labeled *Drosophila* vials on the day in which trials were conducted.

Blood Source & the Artificial Membrane:

Trials were conducted using an artificial membrane system that permitted only one individual to feed at a time (Figure 2 and Figure 3). The artificial membrane,

fabricated by Lille Glass Blowers in Atlanta, Georgia had an inner diameter of 1 cm, an outer diameter of 2 cm and an approximate volume of 1.5 ml. The system was designed with two separate chambers, an outer one to permit the circulation of warm water and an inner chamber to house the warmed blood. Water warmed to 39°C was circulated around the blood-filled chamber to maintain the blood temperature at approximately 38°C ± 1°C to simulate natural host body temperature and encourage engorgement (Cosgrove and Wood, 1995; Andreassen *et al.* 2004). Parafilm® was stretched and warmed by gloved hands and placed over the blood storage portion of the membrane feeder to serve as the feeding membrane itself (Mourya *et al.* 2000; Andreassen *et al.* 2004). Parafilm® was also stretched over the top end of the flask to reduce air contact and to prevent agglutination of the blood during the course of the trials.

Fresh blood was obtained from an independent contractor who collected from two bulls. Blood was obtained from the first male from April 2004 to December 2004, and from the second male from September 2005 to October 2005. The cattle were kept drug and pesticide-free for the duration of these trials to reduce potential impact on mosquito feeding and survival. Blood was collected using citrated 3.8% vacutainers and 20 gauge needles to reduce the degree of erythrocyte damage during bleeding. Cattle were bled the day before or on the first day of a series of trials to ensure that the blood was as fresh as possible. Blood was refrigerated (4°C) in the citrated vacutainers for no longer than four days to minimize lysis of the erythrocytes. Individual vials were only removed from refrigeration prior to the use of blood for trials.

Upon removal from refrigeration, blood-filled vacutainers were warmed for a period of 15 minutes in the water bath that fed the circulation pump at a temperature of

39°C. Blood was then lightly agitated by hand for a period of 1 – 2 minutes to suspend any erythrocytes that may have settled during storage (mix both serum and erythrocytes). Syringes with a capacity of 3cc and 22-gauge needles were used to remove blood from the vacutainer and transfer it to the artificial membrane system. In each trial, approximately 1.0 to 1.2ml of blood was initially added, with additional amounts added from the same vial as required. To prevent haemosedimentation (settling of the erythrocytes at the base of the artificial membrane) the artificial membrane system was lightly shaken by hand approximately every 25 minutes for a period of 30 seconds (Shieh and Rossignol, 1992).

Trials:

Females were placed in a walk-in controlled-environment chamber for a period of 15 minutes prior to the start of experiments to permit them to acclimate to the chamber. The scotophase in this experiment was 10:00 hr to 22:00 hr C.S.T. to permit observations of feeding behavior to be conducted during the day but in such a way that the mosquitoes interpreted the time as night. Trials were generally conducted between 10:00 hr and 18:00 hr C.S.T., a period that coincided with natural times of 18:00 hr and 02:00 hr in the tropics when *An. gambiae* activity is most likely to occur (Gillies, 1957; Bockarie *et al.* 1996). A flexible fibre-optic light source was used to illuminate the vials to permit observation. This light source did not appear to affect mosquito blood feeding behavior based on comparisons with behavior under red light to which mosquitoes are not sensitive (Dr. R. Anderson, pers. comm.).

The net-covered end of each vial was placed on the artificial membrane and individual mosquitoes were permitted a total of five minutes during which to blood feed

Figure 2 – Artificial membrane system developed to assess feeding behavior and feeding dynamics in *Anopheles gambiae*. A – artificial membrane; B – water heater; C – thermometer; D – water bath; E – water outflow tube; F – water delivery tube; G - *Drosophila* vial with female mosquito.

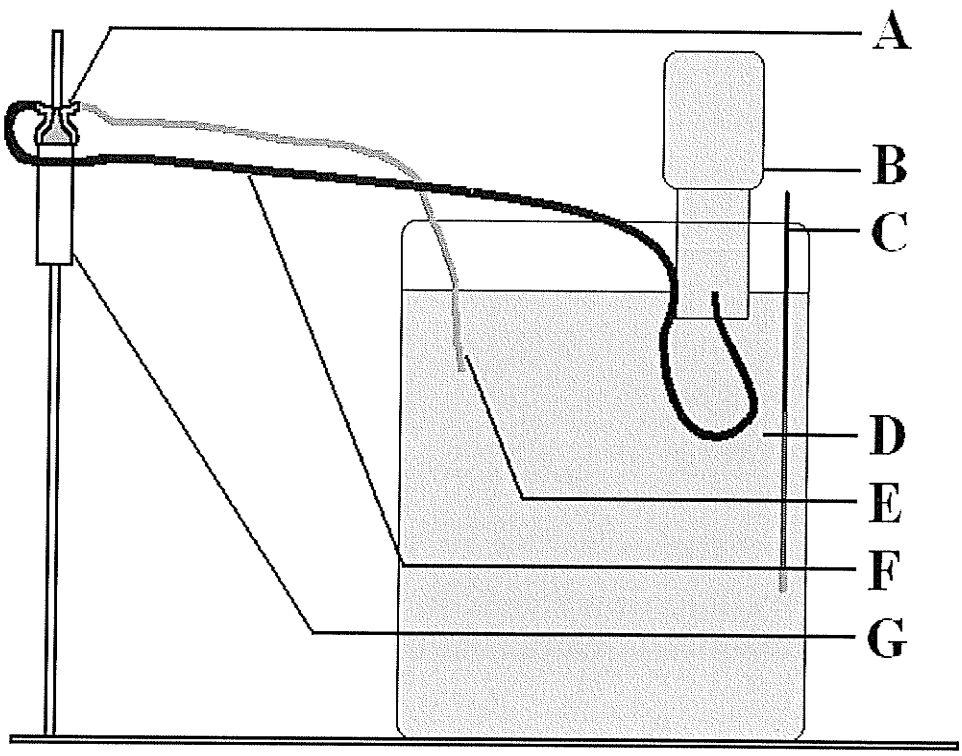
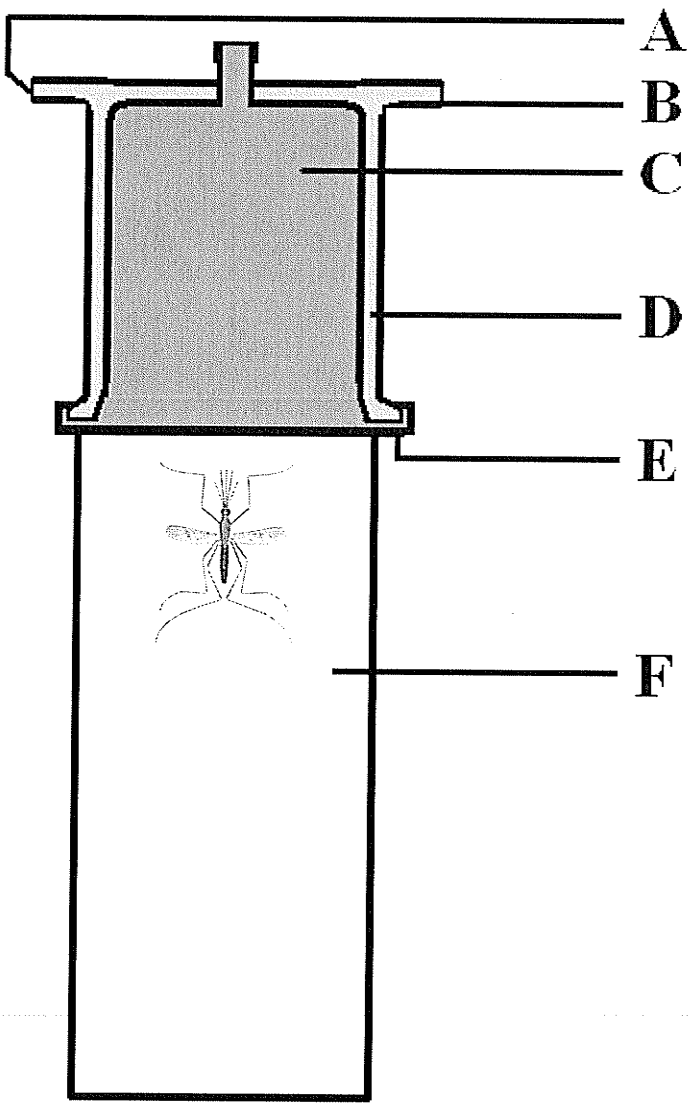


Figure 3 – Close up of the artificial membrane system developed to assess the feeding behavior and feeding dynamics in *Anopheles gambiae*. The water inlet (B) allows water to circulate within the outer chamber (D) and exit through the water outflow (A). Inner chamber houses blood (C). Note the *Drosophila* vial (F) placed flush with the Parafilm® membrane (E), this is where individuals would feed.



(Roitberg *et al.* 2003). In cases where no feeding activity was observed, females were removed after the five-minute period had elapsed and discarded. Otherwise, females that started to feed before five minutes elapsed were allowed to continue until such time as they completed engorgement indicated by the withdrawal of their mouthparts.

Blood-feeding parameters were measured with the aid of a stopwatch with a precision of 1/100 of a second. Gut-filling duration, prediuresis duration (combined = total blood feeding duration), fecundity and wing length were recorded for each mosquito where possible. Critical mosquito behaviors for timing were marked by the insertion of the proboscis, appearance of blood within the abdomen, appearance of prediuretic fluid and removal of the proboscis. Gut-filling duration was defined as the period of time between the insertion of the proboscis and the appearance of red prediuretic fluid from the anus. Prediuresis duration was defined as the period between the appearance of red prediuretic fluid and removal of the proboscis.

Upon completion of trials, blood-fed females were returned to a controlled environment chamber (temperature – 28°C, ± 1°C; relative humidity – 60%, ± 1%, photoperiod 12L:12D). Engorged individuals were given access to 5% sucrose, applied to 2 cm pieces of braided cotton rolls (dental wicks), placed on the tops of their vial for a period of 2 days post-engorgement to encourage oviposition. Once the female had oviposited, the number of eggs laid (not including the minimal number of eggs that may have remained within the ovarioles) and the individual's wing length were recorded. The number of eggs oviposited was used as a proxy or relative estimate of individual fecundity. Wing length, an important indicator of body size (Lyimo and Koella, 1992) was measured using an eye-piece micrometer at 10x magnification, and measurements

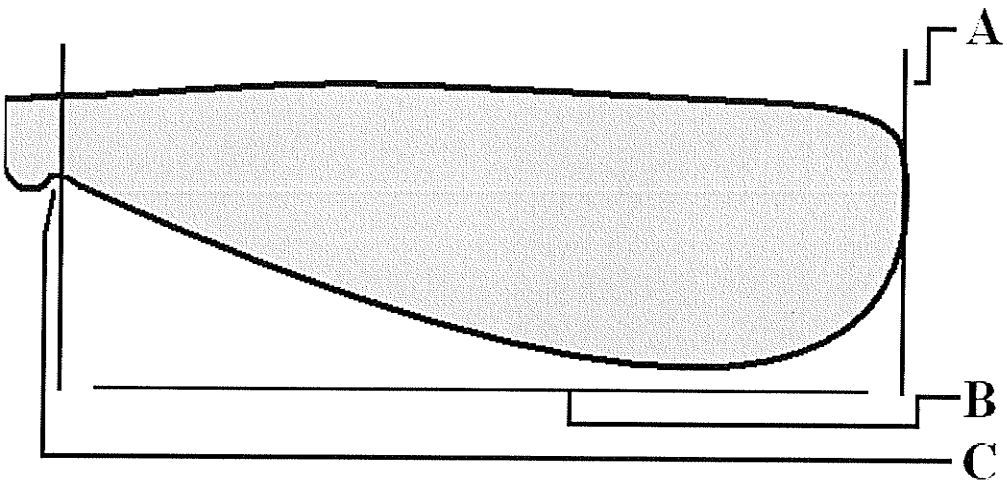
were taken from the base of the allula to the wing tip excluding the fringe scales (Figure 4).

Analysis:

Data for individual mosquitoes were used for analysis only when all blood feeding and related parameters such as gut-filling duration, prediuresis duration, fecundity and wing length were recorded. The primary tool of analysis used for these experimental results was regression analysis. This method was appropriate as it is used to determine the relationship between two variables, one independent and one dependent, such as mother and daughter feeding parameters.

To test for the presence of a genetic component associated with blood feeding parameters (gut-filling duration, prediuresis duration and fecundity), pairwise correlation was used (using JMP IN® 5.1 statistical software) as a preliminary tool to determine where potential relationships between mother and daughter parameters may exist. Regression analysis was then performed to determine the nature of the relationship between these variables both between generations and between overall pooled data. Where relationships were significant ($P < 0.05$), linear and quadratic relationships were compared with the best fit being used. Microsoft Excel® was used as a tool to graph those relationships with significant P values. It should be noted that individuals were only included in these observations if they had data points for all fields. If data for an observation was missing, the remaining data points for the given individual were not included because it may have been difficult to determine where any interaction was occurring due to the missing data.

Figure 4 – Diagram of protocol for wing length measurement in female *Anopheles gambiae*. A – distal wing tip (excluding fringe hairs); B – region of wing measured; C – tip of allula.



To examine the relationship between potential changes in selection pressure expected as a result of total blood feeding duration (comprised of gut-filling duration and prediuresis duration) and fecundity, a conceptual model was constructed from the regression results. Certain assumptions were made to generate this model. First, all individuals were assumed to have an equal ingestion rate (rate at which erythrocytes were ingested). Second, all individuals were assumed to have an equal size, indicated by wing length. Third, it was assumed that the blood volume ingested was similar between individuals. These assumptions were made to permit the analysis of the theoretical relationship between fecundity and total feeding duration. In addition, this modeling was performed to examine the average response of the population of *An. gambiae* rather than the responses of each individual. The statistical parameters (constants and coefficients) from the regression analysis of the relationship between fecundity and total feeding duration, with wing length treated as a covariate (maintained at the mean of 2.72 mm), were used to estimate the shape of the relationship. The shape of this conceptual model was then used to calculate the potential fitness cost associated with incremental reductions in total feeding duration (or given its demonstrated importance, prediuresis duration) by estimating the relative loss in fecundity due to truncated feeding. This functional response model permits analysis between fecundity and total feeding duration and permits the potential effects of selective pressures to be estimated.

$$\mathbf{F = a + w*(W) + t*(T) - ts*(TS)}$$

F = fecundity

a = intercept

w = wing length regression coefficient

W = wing length mean

t = total feeding duration regression coefficient

T = total feeding duration

ts = total feeding duration squared regression coefficient

TS = total feeding duration squared

RESULTS

A total of 1530 individual mosquitoes, 805 mothers and 725 daughters, were observed for blood feeding data. Overall, 32.20% (490 /1530) of individuals' blood fed successfully. Blood-feeding success was seen to conform to similar laboratory data obtained from research with *An. gambiae* where the feeding rate varied from 25 – 35% (B. Roitberg, pers. comm.). Complete data for all parameters were obtained for 34 mothers and 52 daughters. A series of 34 mother/daughter pairs was analyzed. Where there was more than one daughter per mother, mean values were taken and used for subsequent analysis. The number of daughters per mother varied from 1 to 4 (64.71% of mothers produced one viable offspring, 20.59% produced 2, 11.76% produced 3 and 2.94% produced 4). Data used for the functional response model, were obtained from 185 individuals (pooled from both mothers and daughters).

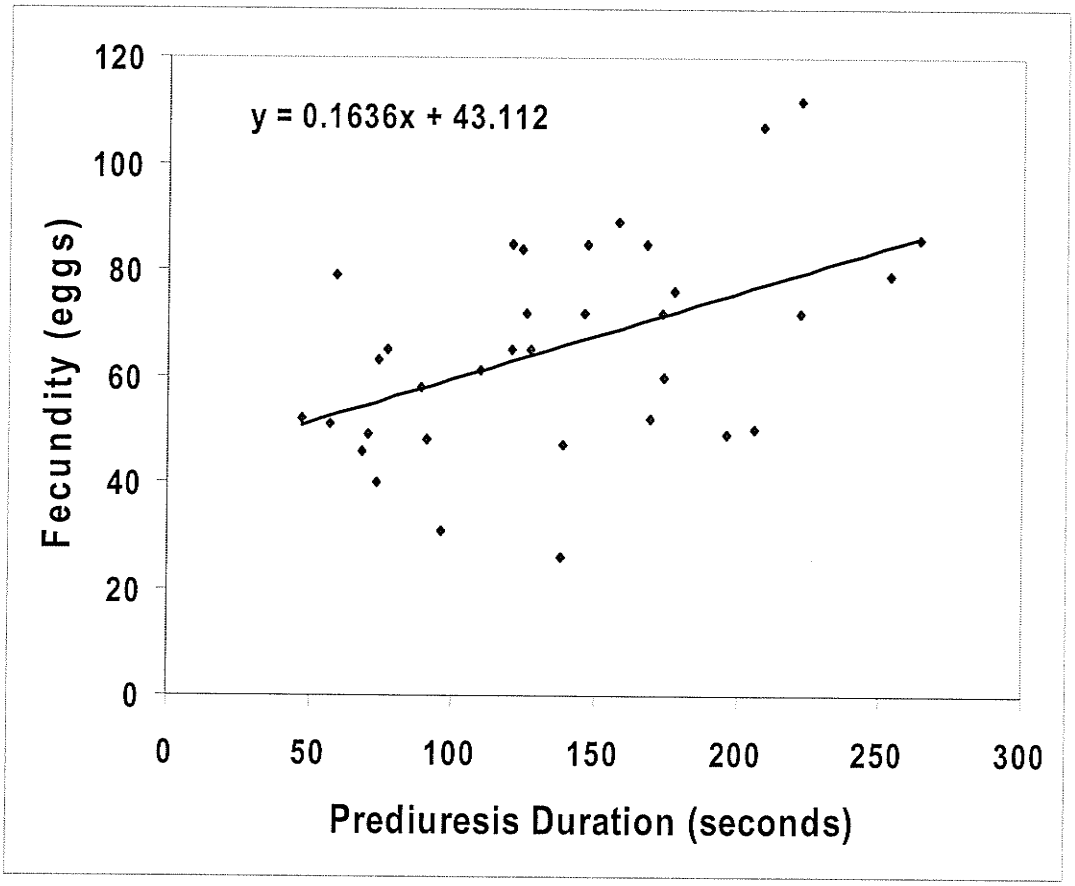
Summary statistics of measurements for the 34 mothers that were compared with their daughters are given below. The mean gut-filling duration for mothers was 65.85 ±2.18 seconds (mean ± SE). Gut-filling duration had a minimum and maximum value of 45.99 and 93.79 seconds respectively producing a range of 47.80 seconds. Average prediuresis duration was 137.94 ±10.05 seconds. Minimum and maximum prediuresis duration values were 47.04 and 263.91 seconds respectively producing a range of 216.87

seconds. The average fecundity was 65.68 ± 3.39 eggs. The minimum and maximum fecundity values were 25 and 112 eggs respectively to produce a range of 86. Mean maternal wing length was 2.79 ± 0.05 mm. Minimum and maximum wing length values were 2.35 and 3.50 mm respectively producing a range of 1.15 mm.

The average gut-filling duration for 52 daughters was 64.02 ± 2.23 seconds. The minimum and maximum values were 42.59 and 121.81 seconds respectively yielding a range of 79.22 seconds. Mean prediuresis duration was 143.52 ± 10.00 seconds. Minimum and maximum values were 30.32 and 373.07 seconds respectively producing a range of 342.75 seconds. The mean fecundity was 54.60 ± 2.72 eggs. The range was 79 with a minimum and maximum value of 25 and 104 eggs respectively. Average wing length was 2.78 ± 0.03 mm. The minimum and maximum wing length values were 2.30 and 3.40 mm respectively producing a range of 1.10 mm.

Correlation analysis was conducted using mother wing length as a weighted variable for the 34 mother daughter pairs. No significant correlations were detected between mother and daughter parameters, such as gut-filling duration ($r = -0.0232$; $P = 0.8964$) and prediuresis duration ($r = -0.1934$; $P = 0.2732$), when controlled for variation in wing length, a proxy for body size. However, there was a significant negative correlation between mother and daughter fecundity ($r = -0.3916$; $P = 0.0220$). Likewise there was a significant negative correlation between mother prediuresis duration and daughter fecundity ($r = -0.4819$; $P = 0.0039$). In addition, a significant positive correlation was seen between mother prediuresis duration and mother fecundity ($r = 0.4659$; $P = 0.0055$).

Figure 5 – Regression plot illustrating the effect that increasing prediuresis duration has on fecundity in mothers, in *Anopheles gambiae* (F = 9.8550; P = 0.0036; n = 34).



These correlation results were subsequently analyzed using regression analysis. A significant positive relationship was observed between mother prediuresis duration and mother fecundity (P = 0.0036; Figure 5). Similarly, a negative relationship was observed between maternal prediuresis duration and daughter fecundity (P = 0.0042; Figure 6). A significant relationship was also observed between maternal fecundity and daughter fecundity (P = 0.0131; Figure 7). Wing length parameters were compared, given the potential influence of this parameter on behavior. A significant quadratic relationship (P = 0.0130) was observed between mother and daughter wing length values (Figure 8).

Based on the 185 individuals for which complete data were obtained, irrespective of mother/daughter relationships, no statistically significant differences were observed between mother and daughter mean data for gut-filling duration (P = 0.5831) and fecundity (P = 0.4641). There were significant differences between mother and daughter means for prediuresis duration (P = 0.0610) and wing length (P = 0.0036).

Data were thus pooled (n = 185) to study temporal feeding dynamics over the total range represented by value for mothers and daughters. Mean gut-filling duration was 64.21 +/- 1.09 seconds (mean ± SE). The minimum and maximum values were 35.40 and 124.17 seconds. Average prediuresis duration was 135.11 ± 4.66 seconds. Minimal and maximal values were 14.11 and 373.03 seconds. Mean fecundity was 57.76 ± 1.51 eggs. Fecundity range was 96 eggs with a minimum and maximum value of 16 and 121 eggs. Average wing length was 2.72 ± 0.02 mm. Minimum and maximum values were 2.30 and 3.50 mm. Based on the mean pooled data, gut-filling duration accounted for approximately 1/3 (~ 32.22%) of the total feeding duration, while nearly 2/3 (~ 67.78%) was allocated to prediuresis duration.

Figure 6 - Regression plot comparing the effect that maternal prediuresis duration has upon the fecundity of their daughters in *Anopheles gambiae* (F = 9.4998; P = 0.0042; n = 34).

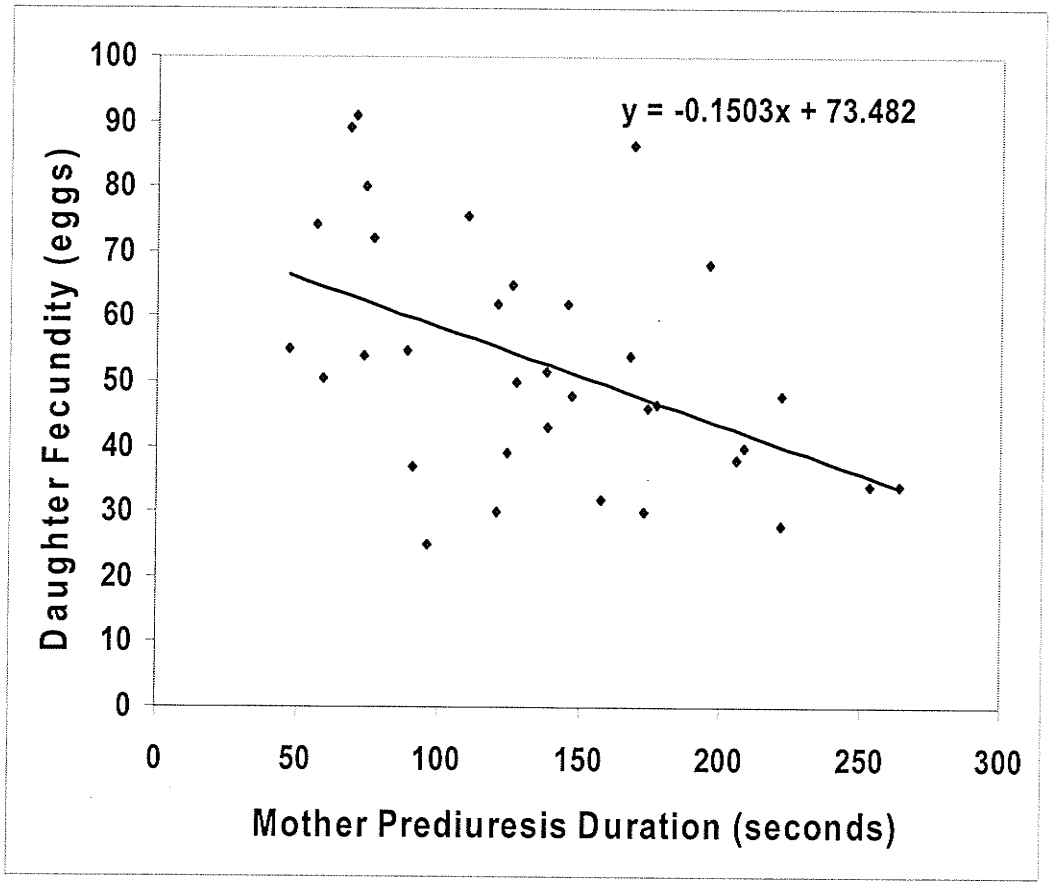


Figure 7 - Regression plot comparing the effect that mother fecundity has on the subsequent fecundity of their offspring in *Anopheles gambiae* (F = 5.0005; P = 0.0131; n = 34). Note that this fit was best described by a quadratic relationship (t = -1.99; P = 0.0550).

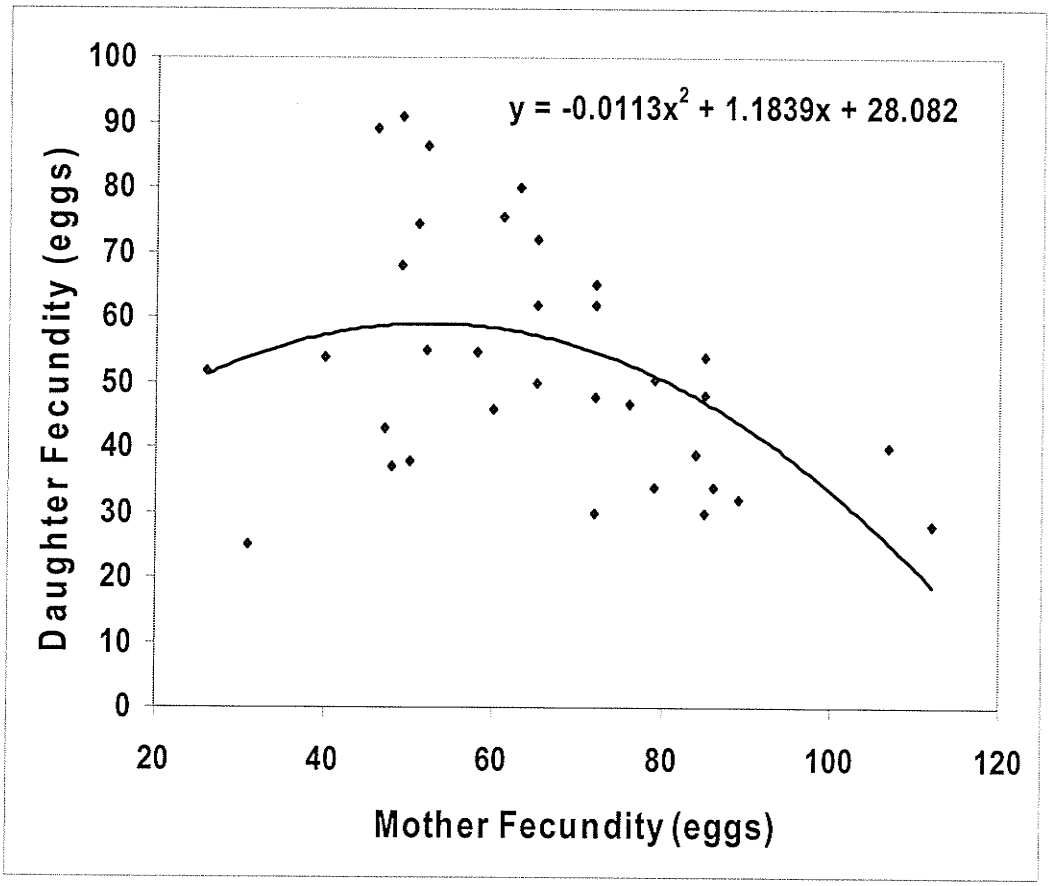
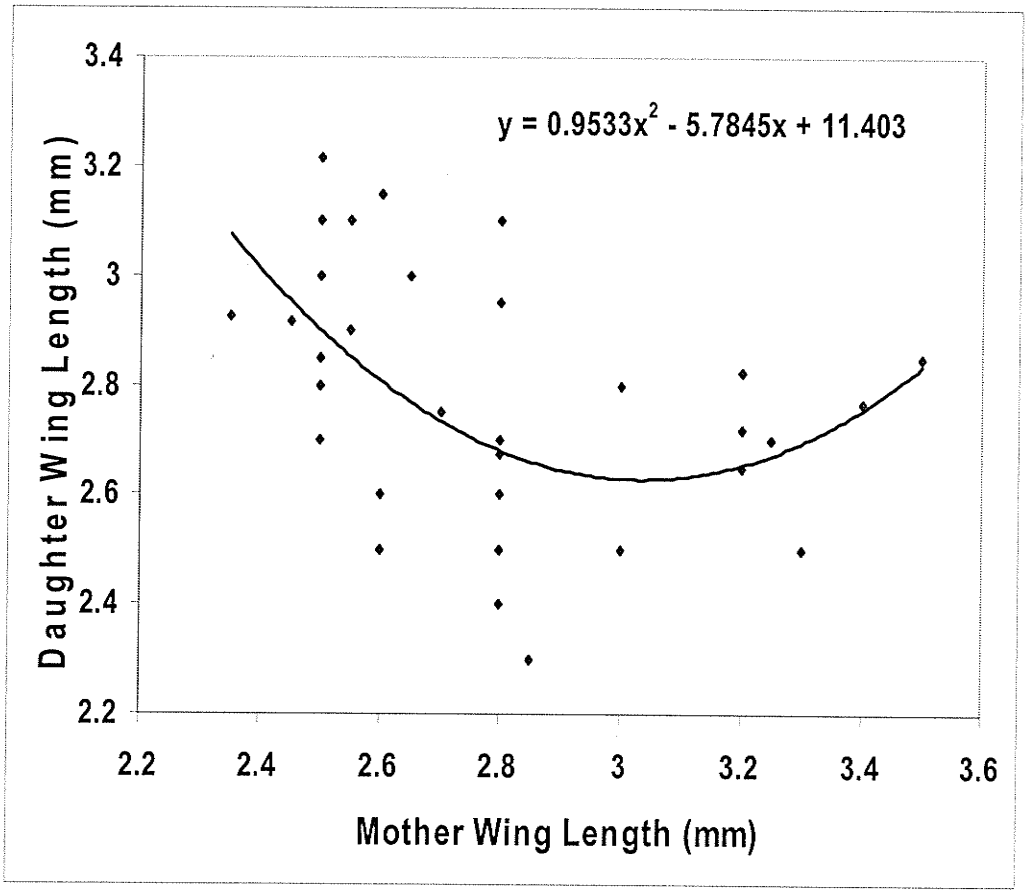


Figure 8 - Regression plot comparing the effect of maternal wing length on the size (indicated by wing length) of their daughters in *Anopheles gambiae* (F = 5.0127; P = 0.0130; n = 34). Note that this fit was best described by a quadratic relationship (t = 2.39; P = 0.0231).



Regression analysis was conducted to determine the nature of the relationship between various general parameters, using the generated regression coefficients (Table 1). A significant positive relationship ($P < 0.0001$) was recorded between fecundity and prediuresis duration (Figure 9). No significant relationship ($P = 0.3985$) was recorded, however, between gut-filling duration and fecundity. There was a significant positive relationship ($P < 0.0001$) between fecundity and wing length (Figure 10). Fecundity residuals were taken from the fecundity vs. wing length comparison and corrected, so as to generate positive values. The relationship between residual fecundity and prediuresis duration was significant (($P = 0.0005$) Figure 11). No significant interaction ($P = 0.8035$) was seen between gut-filling duration and fecundity.

A significant relationship ($P = 0.0006$) was also observed between the corrected residual fecundity and total feeding duration (Figure 12). The effect of wing length on feeding parameters was examined using regression analysis. No significant relationship ($P = 0.1262$) was observed between gut-filling duration and wing length. Yet, a significant positive relationship ($P = 0.0153$) between prediuresis duration and wing length was observed (Figure 13).

Based on the conceptual model derived from the regression coefficients of the analysis of fecundity and total feeding duration, a decelerating payoff curve is seen (Figure 14). As a result, a functional response curve was generated as a function of the per cent predicted change in feeding duration (Figure 15). It should be noted that the estimated curve was used only to the point at which fecundity was maximized. There was a marked difference as a function of percent change in feeding duration relative to the change in fecundity.

Table 1 – Effects table showing coefficients generated by regression analysis used in the development of the fecundity and total feeding duration model for data obtained from a colonized *Anopheles gambiae* population.

Term	Estimate	Std Error	t Ratio	Prob > t
Wing Length	28.681495	5.247409	5.47	<0.0001
Total Feeding Duration	0.268127	0.104239	2.57	0.0109
Total Feeding Duration Squared	-0.000418	0.000221	-1.89	0.0601

Figure 9 – Regression plot comparing the effect of prediuresis duration on fecundity in *Anopheles gambiae* with pooled data ($F = 11.5589$; $P < 0.0001$). Note that the relationship is best fit by a quadratic curve. ($t = -2.14$; $P = 0.0334$).

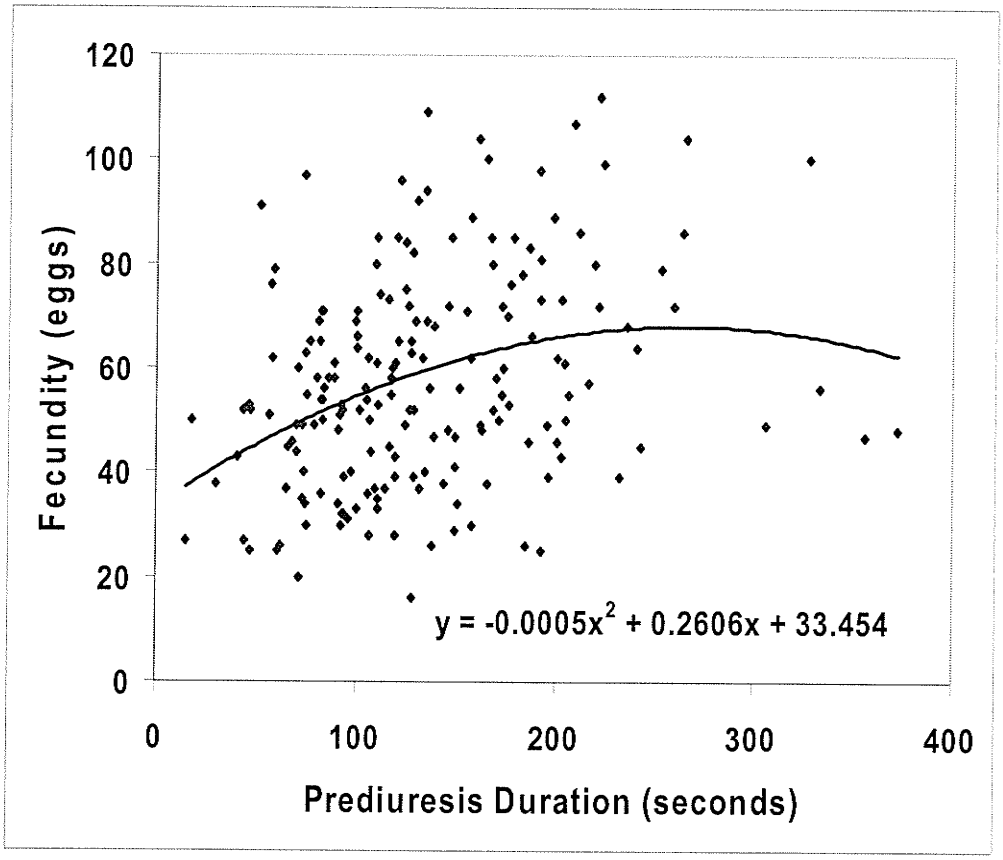


Figure 10 – Regression plot comparing the effect of wing length on fecundity in *Anopheles gambiae* with pooled data ($F = 35.7046$; $P < 0.0001$).

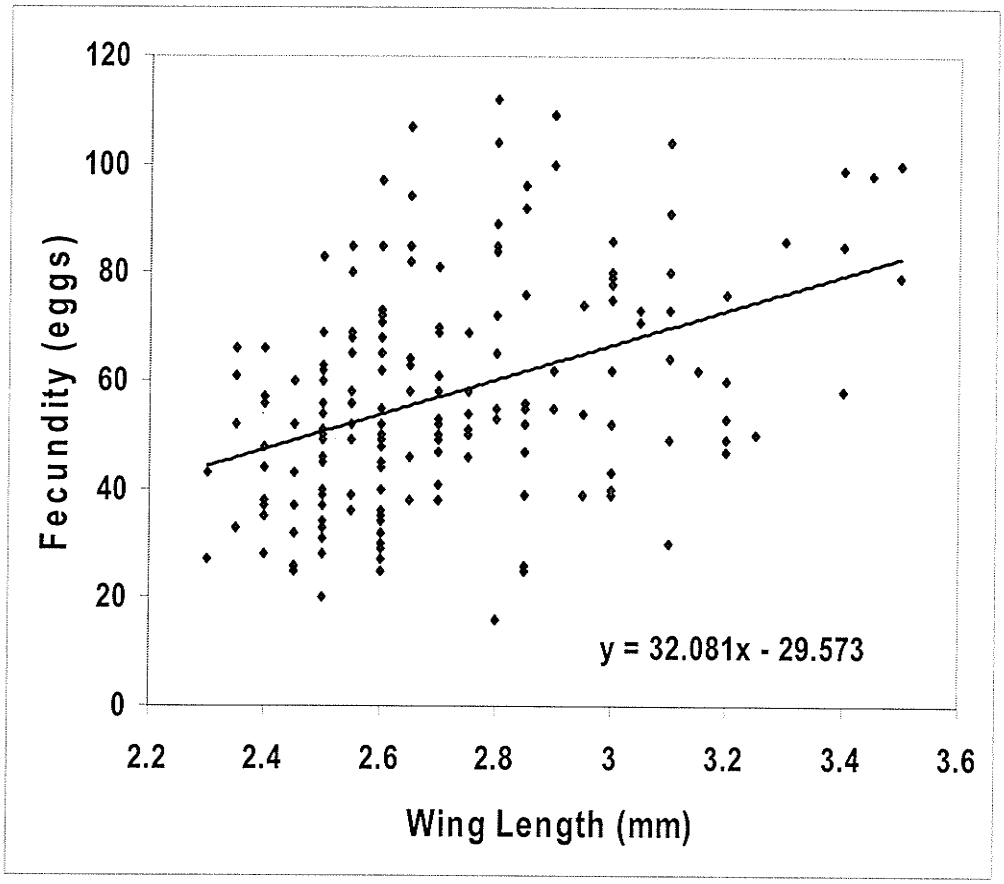


Figure 11 - Regression plot comparing the relationship between residual fecundity and prediuresis duration in *Anopheles gambiae* with pooled data (F = 7.8595; P = 0.0005). Residual fecundity was obtained using wing length as a covariate, and corrected for by adding 44.25 to generate positive fecundity values. Note that the relationship is best fit by a quadratic curve (t = -1.84; P = 0.0679).

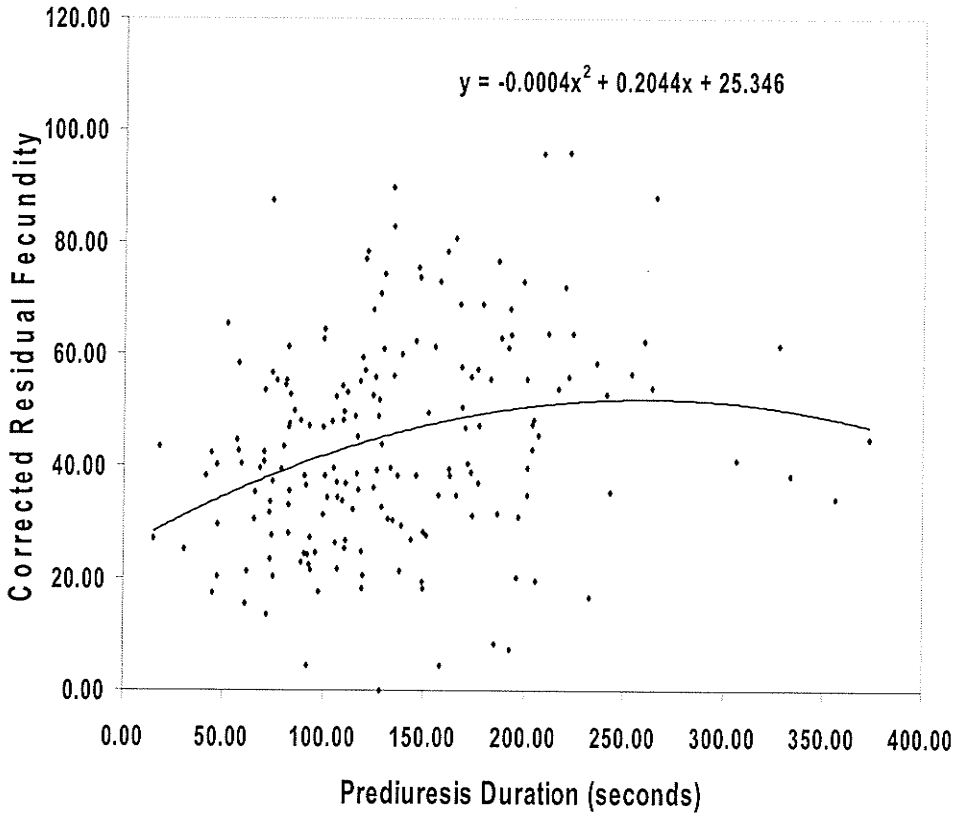


Figure 12 – Regression plot comparing the relationship between residual fecundity and total feeding duration in *Anopheles gambiae* with pooled data (F = 7.7425; P = 0.0006). Residual fecundity was obtained using wing length as a covariate, and corrected for by adding 44.25 to generate positive fecundity values. Note that the relationship is best fit by a quadratic curve. (t = -1.86; P = 0.0647).

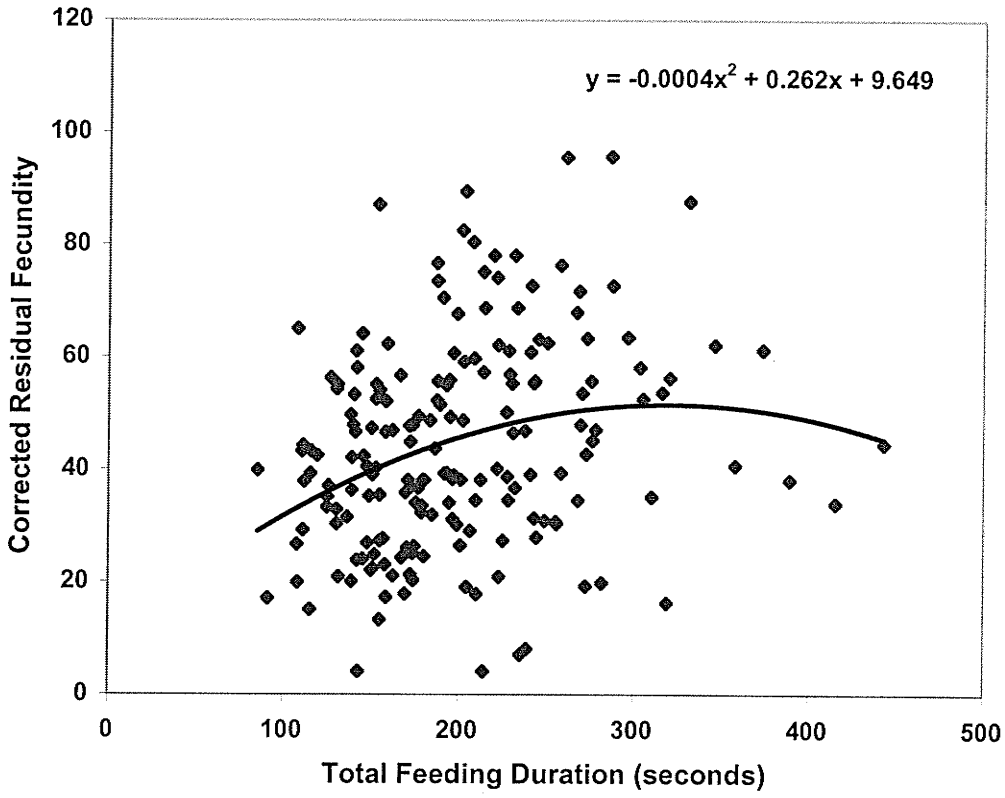


Figure 13 - Regression plot comparing the effect of wing length on prediuresis duration in *Anopheles gambiae* with pooled data (F = 5.9935; P = 0.0153).

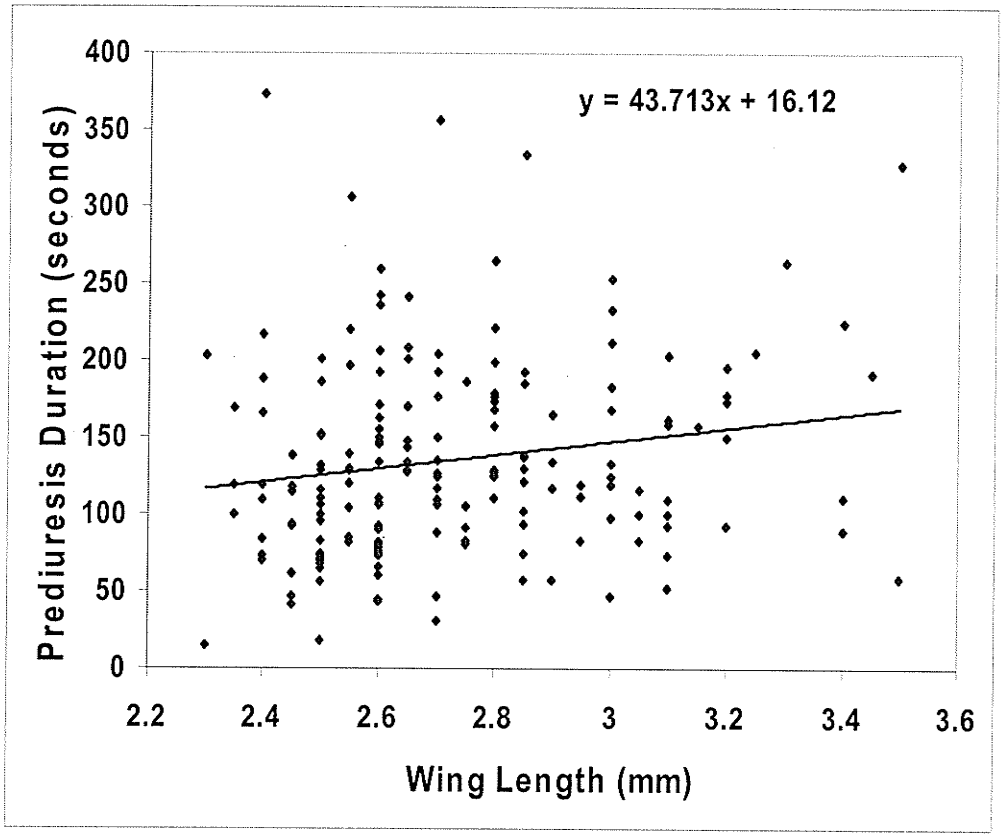


Figure 14 – Modeled relationship between predicted fecundity and total feeding duration based on the quadratic relationship indicated by regression for colonized *Anopheles gambiae.* Note that the curve was cut at 320 seconds, the point at which fecundity maximizes, as predicted fecundity should plateau. Note that minimal point on the x-axis is where prediuresis begins, and this was chosen as the minimum threshold for predicted fecundity.

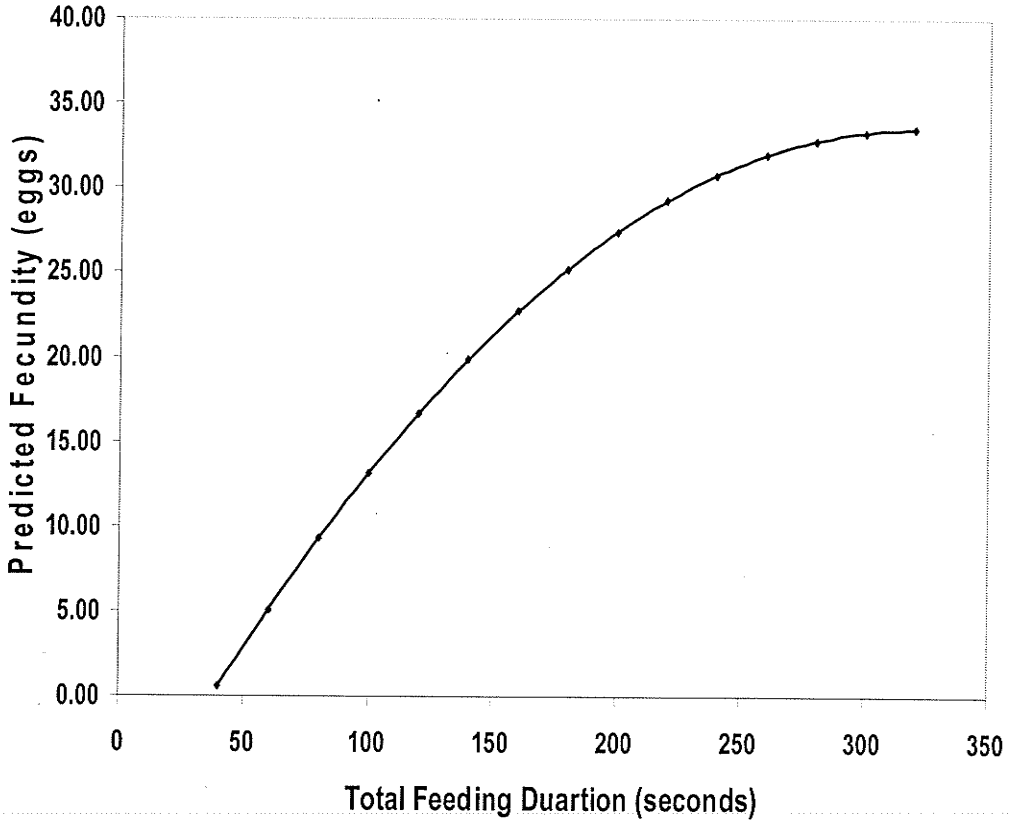
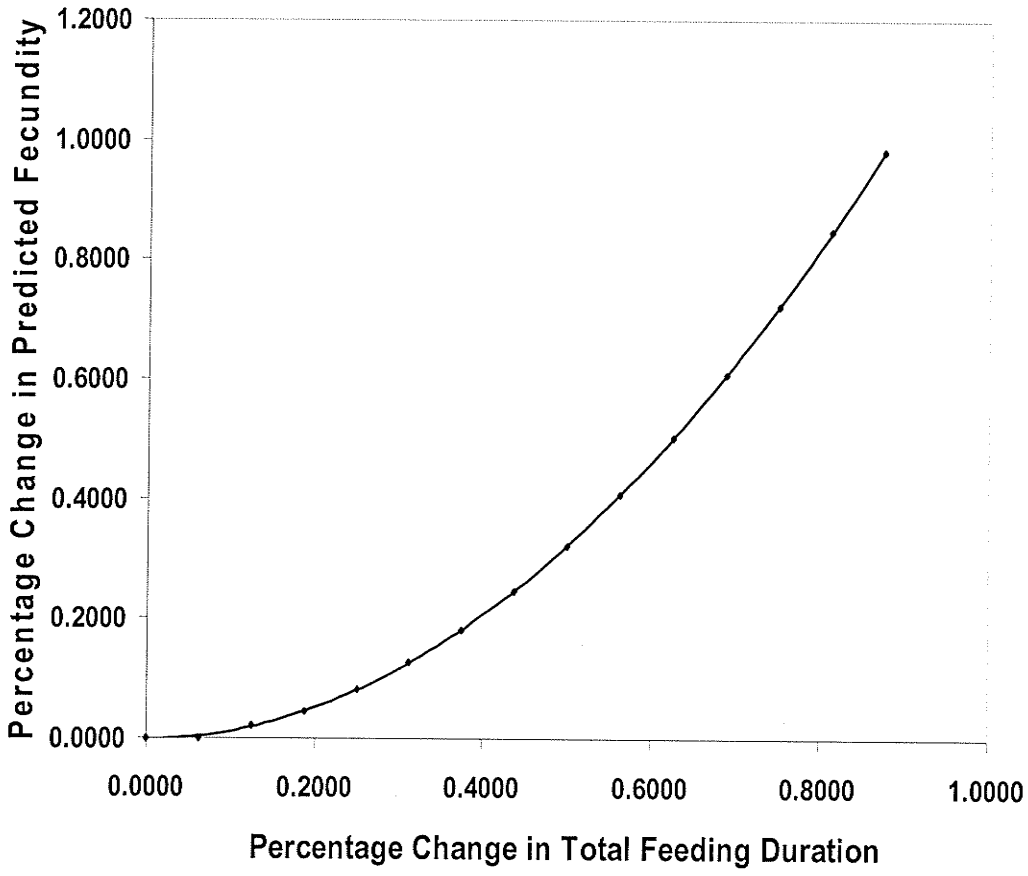


Figure 15 – Functional response model illustrating the relative change in fecundity in response to per cent change in feeding duration for colonized *Anopheles gambiae*.



The per cent change in fecundity was approximately 1% in response to a 10% reduction in feeding duration. However, when feeding duration was reduced by 50%, the per cent change in predicted fecundity was approximately 30%. When the feeding duration was reduced by 80%, the change in fecundity was approximately 90%. This functional relationship best described the quadratic fit of the feeding duration and fecundity data.

DISCUSSION

Given, the uncertainties regarding the potential for mosquito evolution to reduce the efficacy of control tools such as ITBN and the use of IRS, an improved understanding of vector behavior and biology is required. One of the key parameters that may enable vectors, such as *An. gambiae*, to circumvent current and future control efforts pertains to the flexibility in feeding duration, a critical determinant of mosquito survival and hence overall fecundity. Yet it remains unclear whether feeding duration is regulated by genetic factors, environmental factors or a combination thereof.

Based on the measured parameters, which focused on behavioral characters, there was no evidence of a genetic basis for variation in blood feeding and related parameters such as gut-filling duration, prediuresis duration and fecundity. If there is a genetic basis underlying blood feeding parameters, it is expected that significant correlations and/or relationships between mother and daughter feeding times should be apparent. In these trials, there were no significant correlations or relationships between the measured parameters among the 34 mother and daughter pairs that were tested.

This inability to detect any correlation may have been due to the small sample size that would have yielded weak statistical power. Additionally, this inability may also have been related to the absence of measured physiological and anatomical parameters

which may have influenced blood feeding behavior. Thus, a combination of small sample size and non measured physiological and anatomical parameters may have prevented the detection of a genetic basis associated with variation in blood feeding parameters.

If there is a heritable component associated with blood feeding parameters, as has previously been postulated (Gillett, 1967; Chadee and Beier, 1997), when individuals are maintained under laboratory conditions, rapid feeding durations should be selected against. Unlike wild situations, where brevity in host contact is beneficial as it reduces the risk of eliciting anti-mosquito behaviors (Ribeiro *et al.* 1985; Edman and Scott, 1987), laboratory conditions should be conducive to the selection of prolonged feeding times. When confounding variables, such as the interaction of potentially defensive blood hosts that commonly results in interrupted feeding (Clements, 1992; Chadee and Beier, 1997; Chadee *et al.* 2002), and the heterogeneous nature of larval development conditions (Terizan and Stabler, 1949; Clements, 1992; Hurd *et al.* 1995; Gimnig *et al.* 2002), a factor that may significantly influence adult body size, health and behavior, are removed individuals should be expected to utilize a majority of their blood meal to initiate oogenesis. Individuals that emerge smaller or undernourished often seek a pre-gravid blood meal to be used to build up minimal teneral reserves rather than oogenesis (Briegel and Horler, 1993). However, despite controlling for these variables, a combination of significant and non-significant relationships between mother and daughter parameters was observed.

Based on the correlation analysis, it appears that the only significant positive relationship was between mother prediuresis duration and fecundity. This relationship is expected given that the greater amount of time spent engaged in prediuresis, the greater

the number of erythrocytes that may be concentrated and hence the greater the potential fecundity. Prediuresis has been shown to enable anophelines to increase the amount of protein and other nutrients available for oogenesis (Briegel and Rezzonico, 1985; Hogg *et al.* 1986). When the effect of maternal prediuresis duration was compared to daughter fecundity a negative correlation was observed. A similar negative correlation was also observed between maternal fecundity and daughter fecundity. Where the maternal generation had large fecundity values, a function of prolonged prediuresis duration, they laid a greater number of eggs. Their daughters subsequently developed in overcrowded conditions and emerged smaller. Smaller offspring had reduced prediuresis duration and subsequently reduced fecundity values. These observations may better explained by the impact posed by body size in terms of adult behavior.

There was a significant relationship between mother and daughter wing length. It appears that as the size of the mothers increased, the size of their offspring declined. This effect could be due to the observed rearing densities. The size of the oviposition containers was fixed, but the density of daughter larvae in these was driven by the number of eggs laid by the mothers, thus larger, more fecund mothers produced more offspring. Greater numbers of offspring reared at higher densities would be expected to emerge as smaller adults (Briegel, 1990). Additionally, small mothers produced fewer eggs such that their offspring developed in less crowded situations. As mother wing length increased (> 3.10 mm), so too did their daughters. This effect may be partially explained by the fact that in overcrowded conditions, there is high mortality and hence density is reduced over time (Terizan and Stahler, 1949). However, while overcrowded conditions may yield fewer, larger offspring, few of those that emerge will survive to

engage in blood feeding, a factor that may account for the relatively low daughter feeding success observed in these trials (55 daughters compared to 130 mothers).

These observations may be explained by rearing discrepancies that are capable of producing differences in wing length (Koenraadt *et al.* 2004). Intra-specific competition among larvae, particularly in crowded conditions, reduces larval development rate, lowers fecundity, lowers larval survival rates and most importantly, produces smaller adults (Terizan and Stahler, 1949; Chadee and Beier, 1997; Ramasamy *et al.* 2000; Koenraadt *et al.* 2004). Further, Gimnig *et al.* (2002) demonstrated a relationship between wing length and fecundity. They estimated that when *An. gambiae* larvae were reared at a density of 200 per container, females produced 50% fewer eggs compared to females reared at a density of 20 larvae per container. When reared at low densities, larger adults with greater energetic reserves emerge, and a positive correlation has been demonstrated between adult size and the numbers of ovarioles, an indicator of potential fecundity (Hurd *et al.* 1995). Larger adults are capable of ingesting larger blood meals that may permit them to produce more eggs (Briegel and Rezzonico, 1985; Lyimo and Koella, 1992; Hogg *et al.* 1996). When reared at high densities, adults are smaller, with minimal energetic reserves and fewer ovarioles and are capable of developing fewer eggs. This negative correlation between mother and daughter fecundity may be associated with wing length, which itself may be a reflection of environmental constraints (i.e. crowded development conditions). In other words, fit mothers, those with lengthened feeding duration and/or large size, tended to produce unfit daughters as a result of the high rearing densities experienced by their offspring.

Terizan and Stahler (1949) demonstrated the effect of rearing density when they created two populations, one designated as under populated (containing 150 larvae per pan, or 14.49 cm^2/ per larva) and one as overpopulated (containing 1500 larvae per pan or 1.25 cm^2/ per larva). Larvae developing in less populated situations emerged larger with nearly double the rate of blood feeding compared to those reared in crowded conditions (25.0% +/- 8.3 (Mean +/- SE) for less populated: 12.7% +/- 11.2 for crowded). In the current study, daughters, to preserve iso-female lines, were reared in smaller containers (surface area = 42.25 cm^2) compared to mothers that were reared in larger containers (surface area = 1,204 cm^2). Mothers were reared at a fixed density of 4.01cm^2/ per larva, while daughters rearing density varied from 0.38 cm^2/ per larva – 1.63 cm^2/ per larva. These discrepancies may have facilitated the development of biting irregularities and size differentials between generations that may have hampered the ability to detect a genetic component for variation in blood feeding duration within this colony.

The inability to detect the presence of a genetic component associated with variation in blood feeding may also be due to the measured parameters. Measured parameters could be characterized as being the behavioral results of anatomical and physiological processes. Consequently, the interaction between both behavior and mosquito blood feeding anatomy and physiology was not examined. One anatomical factor that was not measured was prediuresis ability. A series of anatomical spines within the posterior mid gut act in concert with the peristaltic contractions to trap erythrocytes while expelling fluid. This ability varies across species for instance, as *An. stephensi* permitted more intact human erythrocytes to pass through their anus compared to *An.*

gambiae (Vaughan *et al.* 1991). Although the concentration ability is similar between these two species, their filtration ability differs.

An additional physiological process that may have prevented the detection of a genetic basis associated with variation in blood feeding parameters may be blood haematocrit levels. Haematocrit levels have been shown to influence the rate of blood ingestion (Daniel and Kingsolver, 1983; Kingsolver, 1987; Rossignol and Shieh, 1993; Taylor and Hurd, 2001). For instance, individuals with malaria have a low haematocrit that permits relatively easy and rapid blood engorgement. However, there is a tradeoff. While blood is more easily ingested, the number of erythrocytes engorged is considerably less over time compared to healthy individuals, thereby compromising fecundity (Shieh and Rossignol, 1992). Minor declines in host haematocrit levels, often associated with early stages of malaria infection, have been demonstrated to significantly improve engorgement and hence reduce feeding duration (Taylor and Hurd, 2001). Haematocrit levels of the two cattle hosts were not measured during this study because they were reared under identical and controlled conditions and as a result, it was assumed that these levels would not have changed considerably over the course of the trials. The lack of data for haematocrit levels prevents the assessment of the role of this physiological factor in the blood feeding duration in this study.

One physiological factor that may have prevented the detection of a genetic basis associated with variation within blood feeding parameters may have been related to the salivary compounds in the individuals themselves. Salivary apyrase facilitates engorgement on blood hosts (Ribeiro, 1984; Ribeiro *et al.* 1985; Ribeiro, 2000). Ribeiro *et al.* (1985) demonstrated that salivary-apyrase levels found within the mosquito salivary

glands, was a critical determinant of blood location ability. Individuals possessing greater amounts of salivary apyrase were able to locate and ingest blood more rapidly thereby increasing the likelihood of their survival. The amounts of salivary apyrase per individual mosquito tested were not measured and as a result it is not possible to state what influence this factor may have had on individual blood feeding durations.

A further confounding factor that may prevent the detection of a genetic basis associated with variation in blood feeding duration may be linked with the history of the colony itself. The colony of *An. gambiae* has been reared in captivity for nearly seven years and over the course of this study in particular has gone through two bottlenecks. Consequently, the presence of natural variation in blood feeding duration and related parameters may have been removed through colonization. Thus, it may not be possible to detect variation within this population as it may not actually contain any naturally occurring variation as a result of its long period of colonization.

While it was not possible to state conclusively whether there was a genetic basis associated with variation in blood feeding duration, the data obtained permitted a theoretical analysis of the relationship between fecundity and blood feeding duration. Most tropical anopheline species, such as *An. gambiae*, are faced with an anatomical constraint posed by their limited mid-gut capacity that restricts their ability to ingest large blood meals, and as a result they are forced to engage in prediuresis to improve their fecundity (Briegel and Rezzonico, 1985; Briegel, 1990). For instance, the mid-gut capacity of *An. albimanus* is approximately 3 µl, and this species was seen to consume between 1.5 and 4.2 µl with an average concentration factor of 1.9 times, thereby increasing its fecundity to a point matching that of larger species (Briegel and Rezzonico,

1985). The importance of prediuresis, which accounts for 2/3 of the total feeding duration, was further demonstrated by a significant correlation between fecundity and the amount of haematin (a measurement of prediuretic fluid) excreted (Briegel, 1990; Hogg *et al.* 1996). Briegel (1985) demonstrated a significant positive correlation between the size of the blood meal ingested and the number of eggs developed for several species of mosquitoes including *An. elutus*. However, increased blood meal sizes in tropical anophelines require a longer blood feeding duration which may increase their risk of eliciting anti-mosquito behaviors from hosts (Roitberg and Gordon, 2005).

Blood feeding represents a complex tradeoff between increased fecundity and a decreased probability of survival (Anderson and Roitberg, 1999). Roitberg *et al.* (2003) theorized that the fecundity benefit associated with increased feeding duration should positively decelerate in parallel with increasing risk of mortality resulting from prolonged feeding duration. In other words, the benefit of extra feeding duration declines rapidly while the risk of eliciting host response increases. Using blood meal volume as an indicator of time spent blood feeding (individuals were allocated with pre-determined feeding durations that translated into increasing blood meal volumes and hence fecundity), Roitberg and Gordon (2005) generated a curvilinear relationship between fecundity and blood meal volume, that revealed that fecundity payoff declines with increasing feeding duration (Roitberg and Gordon, 2005). This decline in fecundity returns may be due to either the increased risk of eliciting a response from the blood host or rather the rate of protein accrual slowing down as repletion nears its absolute maximum.

A similar observation is seen in the current study where blood meal volume is replaced by total feeding duration. A curvilinear relationship between fecundity and total feeding duration was observed, as the benefits in terms of increased fecundity begin to decline after a certain time engaged in blood feeding. In other words, a change in fecundity did not correspond to a similar change in feeding duration and vice versa. This decelerating return was illustrated with the generated functional response curve that compared the percent change in fecundity in response to the percent change in total feeding duration. When the percent change in feeding duration is 10%, the corresponding reduction in fecundity is approximately 1%. However, when the percent change in feeding duration is 50%, the corresponding reduction in fecundity is 30%. Thus, a 10% decline in feeding duration may not significantly compromise fecundity but it may significantly increase the likelihood that an individual mosquito survives engorgement to potentially oviposit, or in come cases, potentially continue to transmit malaria parasites. As a result, if the average feeding duration of a target population is lowered by 10%, selection for faster feeding individuals may not be seen. Furthermore, individuals may be able to truncate their total feeding duration by as much as 10%, or approximately 30 seconds, prior to eliciting a significant selection effect if the cost of feeding to absolute repletion is significantly increased by the danger. This ability to truncate feeding duration without compromising fecundity may have the potential to reduce the effectiveness of control programs relying upon ITBN and IRS

This decelerating payoff curve may be explained by several factors. First, although mid-gut capacity does not vary within an individual, its ability to concentrate erythrocytes does (Vaughan *et al.* 1991). Thus, the ability to concentrate erythrocytes

may decline over time because, as effective erythrocyte concentration rises in the mid-gut, the ability to force additional blood through the mid-gut decreases. The rate of blood flow through the mid-gut declines with increasing feeding duration. Second, the decline in fecundity, may be a function of the increased difficulty pumping blood into the mid-gut as the contents effectively thicken as the erythrocyte:plasma ratio would be expected to increase. Third, mosquitoes experience an increased risk of eliciting a potentially fatal response, through the irritation of blood hosts, when feeding is prolonged and this would contribute to decreasing the marginal value of incremental fecundity. Blood hosts generally have a tolerance threshold above which they will react to nuisance mosquitoes and potentially exhibit responses that may be detrimental to mosquitoes (Ribeiro *et al.* 1985a; Anderson and Roitberg, 1999). Consequently, many species feed as rapidly as possible, or like *An. gambiae*, feed on inactive hosts to avoid the likelihood of disturbing their host (Gillett, 1967; Vaughan *et al.* 1991; Takken *et al.* 2002).

The functional response curve permits some potential conclusions to be drawn with regard to the feeding behavior of *An. gambiae*. *Anopheles gambiae* have been observed to shift their resting and feeding behaviors in response to ITBN and IRS use. For instance, in response to the prolonged deployment of ITBN in areas of Tanzania, the feeding times of *An. gambiae* has shifted to earlier in the evening and later in the morning (2000hr – 2200hr and 0500hr – 0700hr) when human hosts are not protected (Braimah *et al.* 2005). As a result, *An. gambiae* should encounter active human hosts that are potentially more sensitive. Successful individuals in this situation should be those that are capable of rapidly engorging without compromising fecundity. It may be expected that the selection differential favouring a reduction in feeding time would increase as relative

decreases in fitness due to smaller blood meals accelerate. This selection differential may be dependent on a significant benefit with respect to risk reduction as a result of truncated feeding duration. However, this flexible period of approximately 32 seconds (based on a maximum of 320 seconds in the fecundity-feeding duration graph) permits engorgement with minimal compromise to fecundity. Any additional truncations to feeding duration greater than 32 seconds may begin to select for individuals that are capable of more rapidly engorging without compromising fecundity. If a genetic basis associated with variation in blood feeding duration exists, then selection for this trait would become more apparent in response to control interventions where fecundity is compromised, thus circumventing these interventions and potentially allowing for the continued transmission of malaria parasites.

The decelerating nature of the fecundity/feeding duration relationship may provide sufficient biological plasticity to favour the evolution of alternative feeding strategies, including alternative hosts and different feeding periods. For example, *An. gambiae* may still be able to engorge on active human-hosts with little impact on fecundity. Similarly, the minimal reduction in fecundity may enable individuals to engorge on hosts protected by ITBN faster and thus not acquire a lethal dose of insecticide. This is a particular concern because the effectiveness of ITBN decline over time as insecticides degrade. Further it has also been difficult, primarily due to the cost of the insecticides, to convince net users to re-treat nets regularly (Knols and Takken, 1998; Clarke *et al.* 2001). Greater exposure to sub-lethal doses of insecticides may also increase the development of resistance within *An. gambiae* populations (Charlwood *et al.* 2001; Fanello *et al.* 2003; Asidi *et al.* 2005).

In conclusion, fecundity increases in a curvilinear fashion with feeding duration, after controlling for wing length. Based on the functional response between reduction in fecundity relative to reduction in feeding duration (approximately 32 seconds), there may be a flexible period of an approximately 10% decline in feeding duration where minimal effect is seen in terms of fecundity. Consequently, this period may permit continued engorgement on preferred human hosts, not protected by bed-nets, potentially reducing the efficacy of ITBN. In addition, shortened feeding duration may permit engorgement through degraded/ing ITBN, allowing *An. gambiae* and other species to acquire a sub lethal dose, and thus possibly increase the rate of resistance development. Continued engorgement on preferred human hosts may permit the continued transmission of malaria parasites. However, it was not possible to state conclusively that there is a genetic basis underlying feeding parameters such as total feeding duration. This inability may be due to a number of factors such as, small sample size, non-measurement of contributing physiological factors, absence of naturally occurring variation and rearing discrepancies. Thus, given the theoretical work demonstrating that selection will not act on feeding duration so long as fecundity is not significantly compromised and the lack of certainty regarding the genetic basis associated with variation in blood feeding duration, more work should be undertaken with freshly obtained mosquitoes from the field.

GENERAL DISCUSSION

Recent malaria estimates show that some 300 to 500 million people become infected with malaria per annum (Sachs and Malaney, 2002; Knols *et al.* 2002a; Wanji *et al.* 2003). Experts have shown that morbidity and mortality rates, attributed to malaria, have increased during the past two-decades (Greenwood and Mutabingwa, 2002). Moreover, it has been suggested that if no new control initiatives are developed, and the effectiveness of current efforts are not maintained, that mortality rates related to malaria will double in the coming years (Keiser *et al.* 2004). Given these shortcomings, an improved understanding of mosquito biology is required to preserve and improve the efficiency of present control tools, such as ITBN. Among the areas that require further study are those concerning behavior, in particular, their relation to blood feeding dynamics, for the principal human malaria vector in sub-Saharan Africa, *Anopheles gambiae* sensu stricto. An improved understanding of mosquito feeding behavior is critical, particularly with the evolution of behavioral resistance that has developed in *An. gambiae* in response to the use of ITBN (Braimah *et al.* 2005).

Published evidence demonstrating a genetic basis for variation in behavioral traits has been recorded with rats, honey bees, crickets and various mosquito species (Tolman, 1924; Gillies, 1964; Rothenbuhler, 1964; Gillett, 1967; Bentley and Hoy, 1972; Mukwaya, 1977; Day and Edman, 1984; Chadee and Beier, 1997; Chadee *et al.* 2002). In their studies, Gillett (1967) and Chadee and Beier (1997) demonstrated the presence of genes that controlled traits such as blood-feeding duration for *Aedes aegypti*. Both studies found that, when selective pressures posed by the potentially negative response from irritated blood hosts were removed, engorgement times lengthened considerably.

Consequently, if feeding duration increased in the absence of selective pressure, it may also be affected by the introduction of selective pressures, such as blood-host behavior and human interventions (i.e. bed-nets). A genetic basis underlying variation for feeding duration of *An. gambiae* may thus be subject to factors such as the need to minimize both exposure to insecticide and time spent in contact with blood hosts, an ecological landscape that has changed for *An. gambiae* in recent years.

Based on the findings from this study it was impossible to state whether or not existing variation in blood feeding dynamics could be attributable to genetics for this colony of *An. gambiae* owing to a number of factors. First, the small sample size may not have permitted the presence of a genetic component associated with blood feeding duration to be detected if it actually existed. Second, naturally occurring variation may no longer be apparent in the colony used, as it has been held in captivity for nearly 7 years and has been subjected to at least two bottlenecks. Third, a series of anatomical and physiological factors, such as prediuresis ability, host haematocrit level, variability in salivary components, and erythrocyte ingestion rate, were not measured, but may have contributed to observed variation. Last, rearing discrepancies, an important factor influencing adult body size and behavior (Terizan and Stahler, 1949; Koenraadt *et al.* 2004), may have confounded results as adults that emerged larger were able to ingest a greater amount of blood (Briegel and Rezzonico, 1985; Lyimo and Koella, 1992) compared to their smaller cohorts – a fact that may influence blood feeding duration. In addition, larger mothers laid a greater number of eggs, thus creating overcrowded conditions for development of daughter larvae, which often emerged as smaller adults. As

a result, further research is required to determine whether a genetic basis is associated with variation in blood feeding duration.

A number of improvements could be made to conduct this experiment anew. First, individuals should be given two blood meals, with observations made during the second blood feed as this was done to maintain the stock colony, and other researchers have also begun to use this technique (Roitberg and Gordon, 2005). This would remove pre-gravid individuals and potentially increase the sample size, thus increasing the statistical power. Second, mosquitoes should be reared in each generation individually in small vials of equal size, thereby removing the effect of rearing conditions as a potential confounding variable. Third, given the length of time that this colony has been in captivity, naturally occurring variation may have been removed. To account for this a newly established colony should be used. To verify that this concern is valid, a series of colonies should be established with different testing methods used and results compared to verify that the assumed variation in blood feeding behavior is real. Last, a greater number of parameters need to be measured to account for any other potential effects.

The importance of detecting a genetic basis for variation in blood feeding duration in wild mosquitoes is critical because of the significant potential selective pressure imposed by the combination of human behavior and human interventions. Insecticide impregnated materials, such as ITBN and IRS, have been demonstrated to alter resting behaviors (endophilic to exophilic (Haridi, 1972; Clements, 1992; Evans, 1993; Phillips *et al.* 2001), host feeding preferences (from anthropophilic to zoophilic (Charlwood *et al.* 1986; Chareonviriyaphap *et al.* 2001; Habtewold *et al.* 2001; Sousa *et al.* 2001)) and preferred feeding times (Charlwood *et al.* 1986; Charlwood and Graves, 1987; Knols and

Takken, 1998). Mathenge *et al.* (2001) demonstrated a shift in the feeding behavior of *An. gambiae*. In villages with permethrin treated ITBN, *An. gambiae* were more likely to exit houses sooner, and engorged on preferred human hosts earlier in the evening when they were active and not protected. In rural Tanzania, six years of ITBN use precipitated a shift in the feeding times of targeted vectors, *An. gambiae* and *An. funestus* (Braimah *et al.* 2005). These species shifted their feeding times to earlier in the evening, between 20:00hr and 22:00hr, and later in the morning, between 05:00hr and 07:00hr, when their hosts are accessible, and hence not protected by ITBN. This shift in preferred feeding times has caused concern that the effectiveness of insecticide impregnated materials may be compromised in response to the change in biting rhythm as targeted vector populations may still be able to access human hosts and thus continue the transmission of malaria (Pates and Curtis, 2005). The data presented in this thesis support the hypothesis that some behavioral plasticity exists for *Anopheles gambiae* that may allow successful feeding despite potentially increased host risk from feeding while humans are not sleeping.

It is well established that blood is a critical resource for the reproductive success of mosquitoes so it would be expected that behaviors that increase the quantity and quality of the blood meal be under strong positive selection. However, a decelerating fitness curve associated with feeding effort may provide the evolutionary flexibility for significant changes in behavior as the selective landscape changes.

Mosquito control efforts relying upon insecticide-impregnated materials, like bed-nets, and application of residual insecticides to human dwellings have precipitated a shift in feeding behavior for target species such as *An. gambiae*. Behavioral resistance has

evolved within some mosquito populations, characterized as strongly synanthropic and anthropophilic, such as *An. gambiae* (Lengeler *et al.* 1996; Braimah *et al.* 2005). As previously demonstrated, behavioral resistance is commonly characterized by altered feeding times, and this may enable individual mosquitoes to circumvent control tools such as ITBN, increasing the probability of host contact and subsequent engorgement. As a consequence, behavioral resistance may not only facilitate engorgement on preferred hosts, it may also permit the continued transmission of malaria parasites.

Given the potential for continued malaria transmission, the relationship between fecundity and total feeding duration was examined because of the potentially increased interaction between target populations and human hosts, in response to intervention tools. A curvilinear relationship between fecundity and total feeding duration was observed among 185 individuals. This suggests that there is a decelerating fecundity return associated with increased feeding duration, which may likely be a physical process as the gut becomes more densely packed with erythrocytes This declining return may also be associated with the linear relation between increased feeding duration and the increased likelihood of eliciting host reaction (Chege and Beier, 1998; Anderson and Roitberg, 1999; Takken *et al.* 2002; Roitberg *et al.* 2003). In this instance, the feeding duration/fecundity curve mirrors that developed by Roitberg and Gordon (2005) examining the relationship between fecundity and blood meal volume. Fecundity does not increase in parallel with feeding duration, suggesting that benefits associated with increased feeding duration decline over time. This is a critical factor given that selection for altered feeding durations should arise in response to factors such as diminished fecundity. In other words, individuals that are more fecund will oviposit more eggs,

potentially yielding a greater number of offspring to pass on their traits, compared to individuals with compromised fecundity, a fact that may occur due to truncated feeding duration.

Based on the functional response model, there was a period of behavioral plasticity in terms of total feeding duration and its impact on fecundity. For example, a 10% reduction in total feeding duration corresponded to a 1% reduction in fecundity. What this implies is that *An. gambiae* may be able to truncate its feeding duration by 10% without significantly compromising fecundity. So long as fecundity is not significantly compromised there should be little selective pressure for individuals to maximize feeding time. Further, this shortened feeding duration may also significantly increase the survival probability of the feeding mosquito. Increased survival may facilitate increased fecundity and permit continued malaria transmission to occur. As a result, *An. gambiae* can alter their feeding duration, in response to either control tools such as ITBN or IRS or human behavior encountered when they engorge on active and hence sensitive hosts. Further, this ability to truncate feeding duration with minimal effect on fecundity may also permit *An. gambiae* to feed through degrading/ed ITBN before they acquire a lethal dose, thereby potentially contributing to the continued transmission of malaria parasites.

Continued research regarding the feeding dynamics and behavior of *An. gambiae* sensu stricto is required to foster a greater understanding of the interaction between of control tools and the potential to alter vector behavior. If there is a genetic basis associated with variation in blood feeding duration within wild populations of *An. gambiae,* selection for rapid feeding may be possible in response to control interventions and human host behavior. Shortened feeding duration will be beneficial in terms of

reduced risk of mortality due to host defensive behaviors and/or lethal exposure to insecticides, so long as the relative cost in terms of fecundity is minimal. An improved understanding of *An. gambiae* behavioral response to control interventions is thus required to maintain and ensure that current and future vector control efforts remain viable and hence continue to restrict malaria transmission in sub-Saharan Africa.

CONCLUSION

In total, 1530 individual *Anopheles gambiae* sensu stricto were examined for evidence of a genetic basis underlying variation in blood feeding duration (including both gut-filling duration and prediuresis duration). Using iso-female lines to compare mother and daughter feeding parameters, there was no evidence to suggest that variation in blood feeding duration was regulated by genetic factors for this colony of mosquitoes, but these conclusions may not be applicable to wild populations. Results may have been confounded by four factors. First, despite the total numbers of individuals tested, only 34 mother-daughter pairs, with complete data, were obtained. As a result the presence of a genetic basis underlying variation in blood feeding duration may not have been detectable with such a small sample size. Second, there may have been little genetic variation in terms of feeding duration left within the colony of *An. gambiae* as they have been kept in captivity, and not subjected to selective pressures, for approximately 7 years. Third, a series of non-measured anatomical and physiological parameters such as prediuresis ability, host haematocrit and salivary apyrase levels were not measured, and as such their role in terms of feeding duration was uncertain. Last, rearing discrepancies between mothers and daughters may have led to size differentials, a factor that can influence subsequent adult behaviors, such as blood feeding.

In addition to the experimental analysis of the hypothesis that observed variation in feeding speed was due to genetics, feeding dynamics were examined to provide information on how feeding effort vis-a-vis speed affects fitness. The primary question was to determine the nature of the relationship between total feeding duration and fecundity. Prediuresis duration, which accounted for nearly 2/3 of the time allocated to

blood feeding, appeared to be the most significant determinant of individual fecundity. The fecundity and total feeding duration relationship was best described as curvilinear. A decelerating payoff in terms of fecundity was seen with increasing feeding duration. Based on the conceptual model, a significant reduction in feeding duration would be necessary to significantly compromise fecundity. Consequently, given the shifts in *An. gambiae* behavior in response to control interventions, such as ITBN, this flexible feeding duration may be significant in terms of compromising current and future control efforts, particularly if a genetic basis underlies variation in blood feeding duration thereby permitting the continued transmission of malaria.

With regards to the objectives of my research stated earlier, the following conclusions can be stated:

1) It is impossible to conclude with certainty there is a genetic basis for variation in blood feeding parameters, such as gut-filling duration and prediuresis duration, in *Anopheles gambiae* sensu stricto.

2) The relationship between fecundity and blood feeding duration was best described as non-linear. A decelerating payoff curve, in terms of fecundity, was seen with increased feeding duration.

3) A significant decline in feeding duration is required to significantly compromise fecundity and hence precipitate selective pressure on *An. gambiae* behavior. Therefore, individuals may be able to truncate their feeding duration, thereby increasing the probability of their survival, to feed on active human hosts that are no longer protected by interventions such as ITBN. In addition, the ability to shorten feeding duration may enable *An. gambiae*, to feed through

degrading/ed bed-nets and thus acquire a sub-lethal dose of insecticide, potentially contributing to increased insecticide resistance. This behavioral plasticity in terms of feeding duration may permit continued malaria transmission.

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