ABSTRACT

Title of Thesis: FITNESS OF THE TRANSGENIC MALARIA-
                     REFRACTORY MOSQUITO ANOPHELES
                      GAMBIAE

                     Deborah Tillman Ladner

Thesis directed by: Professor David J Hawthorne
                     Department of Entomology

The African mosquito *Anopheles gambiae* is the insect vector responsible for half of

worldwide malaria infections. One option for reducing malaria is genetic

manipulation of the vector. By creating transgenic mosquitoes incapable of spreading

the malaria pathogen, the human-mosquito-human cycle of infection may be

interrupted. In order for the engineered *Plasmodium*-refractory mosquitoes to be

effective they need to be able to thrive in nature and to compete successfully with

nontransformed conspecifics. Two lines of transgenic *An. gambiae* were used in this

study to evaluate the fitness consequences of transgenesis in this insect. Life table

characteristics of two transformed *An. gambiae* lines were compared to a control line

to determine if fitness costs were present. Specific traits measures between transgenic

and control mosquitoes were not significantly different, and the transgenic lines had

no evidence of position effects.

FITNESS OF THE TRANSGENIC MALARIA-REFRACTORY

MOSQUITO ANOPHELES GAMBIAE

by

Deborah Tillman Ladner

Thesis submitted to the faculty of the Graduate School of the
University of Maryland, College Park in partial fulfillment
Of the requirements for the degree of
Masters of Science
2004

Advisory Committee:

    Professor David J. Hawthorne, Chair
    Professor Robert Denno
    Professor David O' Brochta

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

INTRODUCTION

The malaria problem

Malaria is one of the deadliest diseases worldwide, affecting an estimated 200 to 300 million victims each year (Budiansky 2002). Two million of those infected, most of them children, die of this disease each year. Cases are concentrated in tropical and sub- tropical regions, which cover over 100 countries (Gallup and Sachs 2001, Budiansky 2002). Malaria has heavy costs not only in human life but also in economic losses. In 1995, countries sustaining high rates of malaria had incomes only 35% of non- malaria countries (Gallup and Sachs 2001). This impact is especially noticeable in Africa, where up to 90% of all worldwide malaria cases occur (Breman 2001, Moreira et al. 2002).

While local residents either die in childhood or attain a level of resistance, non-natives are left extremely vulnerable to the disease. Once contracted, malaria is not always curable. Even if the infected individual has medical access, anti-malarial drugs may be ineffective because of widespread pathogen resistance. The disease pathogen has largely become resistant to chloroquine and pyrimethamine, the most common anti- malarial drugs(Cotran et al. 1998). New medicines have not been developed for decades (Cotran et al. 1998, Hemingway et al. 2002).

In addition, strategies aimed at controlling the mosquito vectors of malaria, have been hampered by insecticide resistance. Previous malaria eradication efforts had emphasized eradication of all potential mosquito vectors. Beginning in the 1940s, the

insecticide DDT was thought to be an ideal way to eliminate mosquitoes and thus the diseases they vector. Worldwide efforts of mosquito control using insecticides were initially successful at reducing mortality due to malaria (Sachs 2002). However, this approach was soon hampered by the evolution of insecticide-resistant mosquitoes. By the 1960s, pesticide effectiveness was substantially reduced due to widespread mosquito resistance of DDT, malathion and other insecticide eradication tools. The rates of malaria infection rebounded to the levels observed before the use of DDT (Hemingway et al. 2002, Sidhu et al. 2002, Wellems 2002). Recently, insecticide-treated bed nets have been distributed as a means of mosquito control. They has been shown to reduce malaria mortality in children by 15 to 25 percent in heavily infected areas (Vogel 2002). Although insecticide resistance and other issues complicate vector-control, pesticides are still considered the most effective method of reducing malaria.

Malaria transmission

Plasmodium are the intracellular protozoan parasites that cause malaria. Several *Plasmodium* pathogens, the deadliest of which is *P. falciparum*, are capable of infecting humans. While *P. vivax* and *P. malariae* infections are associated with mild anemia, *P. falciparum* induces severe anemia and renal failure. The *Plasmodium* parasite depends on *Anopheles* mosquitoes to find human hosts. Sixty species of *Anopheles* mosquito vectors exist worldwide and mosquito species vary by region. Common species include *An. stephensi* and *An. culicifacies* in India and *An. darlingi*

in South America. The deadliest *Anopholes* species, *An. gambiae*, is found in Africa.

This species carries several *Plasmodium* pathogens of humans, including *P.*

falciparum. *An. gambiae* is responsible for an estimated 1 million malaria deaths each

year, roughly half the total malaria-caused mortality (Cotran et al. 1998, Budiansky

2002, Roberts 2002).

　　An. gambiae are highly anthropophilic mosquitoes. As a result of this

specialization, this species is only found in human- populated areas where females

exclusively take human blood meals. *An. gambiae* females prefer to oviposit in

shallow, temporary pools such as irrigation ditches, small puddles or even the water

pooled in human or livestock tracks.   The mosquitoes are active blood feeders at

night; during the day most can be found taking shelter in human dwellings (Budiansky

2002).

　　Female Anopheline mosquitoes require a blood meal to induce and provision her

eggs (Cotran et al. 1998, Roberts 2002). They locate potential human hosts through

cues such as heat, carbon dioxide, and odors (Hurd 2003). Anophelines have specially

formed mouth parts and unique salvitory enzymes to optimize human bloodmeal

acquisition (Hurd 2003). Two pairs of cutting stylets line the proboscis, which

contains modified feeding tubes. This allows for cross directional cutting of the skin

surface and smooth insertion of the proboscis. Once inserted in the skin, one set of

stylets withdraws blood and the other injects salvia into the wound. The salvia

includes anti-inflammatory and anti- clotting enzymes that keep the blood vessel open

and delay pain response from the host (Budiansky 2002, Enserink 2002).

The *Plasmodium flaciparum* pathogen has a complex human-mosquito-human life cycle (Figure 1). *Anopheles* females become infected with *Plasmodium* gametocytes picked up from human malaria victims. In order to obtain the *Plasmodium* pathogen and thus begin the infection cycle, a human host must have become infected at least 2 weeks prior to the Anopheles blood meal. This time-span is needed for gametocytes, the only stage that mosquitoes can aquire from humans, to form in the human host (Bannister and Mitchell 2003).

Multiple infections are common; sexual reproduction of *Plasmodium* occurs inside the mosquito vector (Elliot et al. 2002). Once ingested, *Plasmodium* develops in the mosquito midgut. Fertilization of gametocytes to form ookinetes takes place in the mosquito mid-gut. The ookinetes then penetrate the mosquito gut wall, forming oocysts in the basement membrane of the gut. After 10-18 days, the oocysts rupture. This releases human- infective sporozoites, which migrate to the salivary glands of the mosquito. The infected mosquito then transmits the pathogen to new human hosts during subsequent blood meals. This transmission process is enhanced by Plasmodium- induced behavioral changes in the mosquito. *Plasmodium* has been shown to reduce the vector's feeding efficiency and thus increase the frequency of blood meals required (Hogg and Hurd 1995, Koella et al. 1998, Anderson et al. 2000, Hurd 2003). The vectoring female *Anopheles* is only malaria infective if takes another blood meal at least 10-18 days after it acquires *Plasmodium.* This time span is needed for the *Plasmodium* parasite to develop into sporozoites, the form that can be passed to humans. Thus, this transmission cycle is highly dependent on mosquito longevity.

Once sporozoites are injected into the human bloodstream, they infect the host's liver cells and develop into merozoites. After 8 days, merozoites rupture and infect red blood cells. While in red blood cells, they change into trophozoites, develop into the ring form, and finally become mature schizonts. In this form *Plasmodium* ruptures, releasing merozoites and causing exponential infection of red blood cells. This rupture causes the human's symptoms: violent chills followed by fever and headache. Over a few weeks, the merozoites become gametocytes and thus available for mosquito transmission. If left untreated, anemia, blocked vessels, and even kidney failure will result (Cotran et al. 1998, Bannister and Mitchell 2003).

Mosquito transformation

Genetic alteration of mosquitoes offers a possible solution to the complex malaria problem. Though genetic manipulation as a vector control method has been discussed since the 1960s (Curtis 1968), improvements in the last 15-20 years associated with genetic technologies have revived the concept (Atkinson et al. 2001). Genetic transformation is the introduction of heritable genetic material from one individual to another. There has been previous success with genetic control programs, such as screwworm fly (*Cochliomygia hominovorax*) eradication through release of sterile males (Krafsur et al. 1986). The concept behind genetically modified mosquitoes (henceforth GMM) is the idea that potential vectors can be modified through the introduction of novel genes that prevent parasite transmission.

Three main challenges impede the creation of transgenic mosquitoes (O'Brochta 2002). The first challenge is delivering the desired DNA (via fine glass needle) into

the germ cells of a mosquito egg without physical damage. The second challenge is that, once inserted, this introduced DNA plasmid must rapidly recombine with the mosquito's chromosomal DNA. Significant research has been dedicated to discovering new gene insertion methods. In the past two decades, techniques involving transposable elements, viruses and in vivo recombination systems have all been studied as potential gene vectors (Atkinson et al. 2001). The third challenge is integration of the inserted DNA. A transposable element introduced with the desired gene (a.k.a. "transposon") solves this problem.

Several transposable elements have been isolated from various insect species and successfully used to shuttle genes into mosquitoes including: *Hermes*, *Mariner*, *Minos* and *piggyBac* (Atkinson and Michel 2002, Moreira et al. 2002, O'Brochta 2002). These transposable elements have the additional advantage of potentially creating a genetic drive system. By their nature, transposable elements may insert themselves into non-carrier gametes and thus be over-represented in following generations. This drive system allows the desired transgene, in this case a *Plasmodium* refractory protein, to be expressed above a Mendelian ratio (Kidwell and Ribeiro 1992).

After the transposon- injected egg develops into an adult and mates with no non-transformed conspecifics, transgenic progeny may be produced. However, due to the inexact nature of transposon insertion, only 10% of the resulting offspring will be fully transgenic (O'Brochta 2002). As a result, successfully transformed offspring must be easily identified and isolated. Mutant genes with an associated phenotype, such as the white-eye mutation in *Aedes aegypti*, have used to create and distinguish transgenic

germ-lines (Lobo et al. 2002). However, many mutations bear a fitness cost and thus are not widely used. More commonly, new transgenes with obvious phenotypes, such as autoflouresent proteins, are used (O'Brochta 2002). The enhanced green fluorescent protein (EGFP, Figure 2) is the most popular for mosquito transformation and has been used in the transformation of several species (Catteruccia et al. 2000, Pinkerton et al. 2000, Allen et al. 2001, Grossman et al. 2001, Perera et al. 2002). The fitness cost of these markers are considered to be neutral, though some evidence indicates otherwise (Liu et al. 1999).

An. gambiae was the first mosquito species for which genetic transformation was reported (Miller et al. 1987). Miller et al. attempted transformation with the *Drosophila* transposable P-element. After thousands of attempts, one embryo was transformed with a gene that confers resistance to a neomycin analog, G-418. Despite this success, the results indicated that this transformation was not a result of the P-element transposition. Clearly, further gene vector development was needed (Moreira et al. 2002).

The advent of mosquito gene vector and recombination technology reignited genetic modification efforts for *An. gambiae*. In addition, the *An. gambiae* genome was published in 2002, which revealed new potential targets for genetically- based control (Holt et al. 2002). Furthermore, the mosquito genes and pathways involved in vectoring malaria can be pinpointed; this discovery will elucidate previously unknown details about malaria transmission. In 2000, Grossman et. al reported stable transformation of *An. gambiae* to express the EGFP marker using the newly-

developed *piggyBac* transposable element (Grossman et al. 2001). Recently, this success was extended when *Plasmodium* refractory genes were inserted into a transformed *An. gambiae* germ-line(Kim et al. 2004) .

In addition, other Anopheline species have been genetically modified in the effort to eliminate malaria. *An. stephensi*, the major malaria vector on the Indian subcontinent, has also been a subject of genetic control research. This species has been transformed with the *Minos* transposon to express an EGFP marker (Catteruccia et al. 2000) and with the *piggyBac* transposon to express the red fluorescent protein, dsRED (Nolan et al. 2002). *An. albimanus* is a significant South American malaria vector, also present in the southwestern United States. Germ-line transformation of this species was also achieved with a *piggyBac* transposon containing the exogenous EGFP marker (Perera et al. 2002).

Genetical modification of mosquito vectors from other disease systems has also been accomplished. *Aedes aegypti*, a vector of yellow fever and dengue virus, has been a focus of genetic modification efforts. In 1998, stable integration of the *Drosophila melanogaster* cinnabar gene with the transposon *Mariner* (*Mos1-cn+*) into an *Aedes aegypti* germ-line was one of the first successful mosquito transformation experiments (Coates et al. 1998). *Ae. aegypti* has also been transformed to express markers *Drosophila melanogaster cinnabar (cn)* (Jansinskiene et al. 1998), *luciferase (luc)* (Coates et al. 1999) and EGFP (Pinkerton et al. 2000) with the*Hermes* transposon. The *piggyBac* transposable element has also been a gene vector for EGFP transformation (Kokoza et al. 2001) and for*cn* , the *Drosophila* eye color gene.

Finally, *Culex quinequesfasciatus*, a vector for West Nile Virus, has been transformed to express EGFP with the *Hermes* transposable element (Allen et al. 2001).

That many species that have been successfully transformed in the past few years demonstrates the feasibility of genetic transformation (O'Brochta 2002). The ultimate goal in the development of transgenic *An. gambiae* is their release into a natural system. Instead of attempting to eradicate all malaria mosquitoes, this approach proposes to eliminate mosquitoes' ability to vector the disease. Ideally, genetically modified mosquitoes would displace or out- compete endemic mosquito populations in hyper-endemic areas of Africa. For this strategy to succeed, the released transgenic mosquitoes must have fitness equal or greater to their wild counterparts (Scott et al. 2002).

Genetic drive is a general term referring to situations of increased transmission patterns. Transposons can be "driven" by virtue of their super-Mendelian enhanced transmission rates. The characteristics and potential use of transposable elements and transposons has been described and modeled (Kidwell and Ribeiro 1992, Ribeiro and Kidwell 1994). The enhanced transmission of transposons is the consequence of the replicative nature of transposition. Genetic drive describes any alteration of the genetic transmission system that may enhance spread of the transgene. For example, if a transgene's replication is pre-meiotic, the transgene will be over-represented in the resulting gametes. Thus, an increased fraction of the gametes will contain the transgenic allele. This greatly increases the transgenic allele's rate of spread in the population to a rate significantly higher that Mendelian expectations. Even if there is a fitness cost associated with the transgenic allele, its spread through a population may

still be rapid because of the "drive" provided by the transposable elemnet. However,

it is unknown if this phenomenon will occur in transgenic mosquito species. Still,

transposable element genetic drive must be considered, along with fitness, in

understanding the dynamics of introduced genes in natural populations (Ribeiro and

Kidwell 1994).

Kim, et al. describe the methods used to genetically transform the *An. gambiae*

mosquito lines used in this study (Kim et al. 2004). These mosquitoes have been

genetically transformed to block the development of *P. falciparum*, the deadliest

malaria pathogen. To create the transgenic lines, a *piggyBac* transposable element that

contained EGFP and a *Plasmodium* refractory gene was injected into developing

mosquito embryos. The *piggyBac* gene vector was co-injected with a helper plasmid,

phsp-pBac. This makes the gene vector motile and, because this plasmid can be

activated via heat shock, it adds another level of regulation to the transgenic

expression.   The refractory gene, *An. gambiae cecropin A*(AngCecA), is a natural

part of the A*n. gambiae* immune system. It has known anti-*Plasmodial* activity

(Gwadz et al. 1989, Vizioli et al. 2000). To gain entrance to the insect hemolymph,

Plasmodium exploits the *An. gambiae* midgut, where cecropin is not expressed.

However, when the AngCecA gene is inserted and regulated with the *Aedes aegypti*

carboxypeptidase A (CP) promoter, it exhibits ectopic expression (i.e. occurring in a

novel position) in the female mosquito's mid gut after bloodmeals. This blocks

Plasmodium passage through the midgut. Because peptide expression is up- regulated

in the female mosquito digestive track to aid in digestion post-bloodmeal, the CP A

promoter linked to AngCecA is only transcribed after bloodmeals. This time and site

specific expression ensures that the extra cecropin is only present when the female

mosquito is vulnerable to infection.

Once transcribed, the cecropin is either secreted or released intercellularly.

Cecropin is then able to destroy *Plasmodium* via cell membrane disruption (exact

mechanism is unknown) (Gwadz et al. 1989, Vizioli et al. 2000). Bioassays

performed on transformed insects reveal that cecropin transcription in the *An. gambiae*

midgut causes a 60% decrease in relative *Plasmodium* infection (Kim et al. 2004).

Two plasmids (*piggyBac* and a helper plasmid) were injected into embryos of G3/

wild-type *An. gambiae*. The injected embryo ultimately produces transformed germ-

line cells that, following mating with a nontransformed male, develop into transformed

F1 individuals. The EGFP fluorescent gene used as a marker provides a phenotypic

indication that mosquitoes contain the *Plasmodium* refectory gene. It is controlled

through separate regulation and thus is expressed for all transgenics, not just females

post-bloodmeal. EGFP screens can therefore be used to identify transformed

mosquitoes as larvae. The transformed founders of the G1 and G2 *An. gambiae* lines

underwent several generations of full- sib mating in order to create individuals with

homozygous genotypes at the cecropin gene and EGFP marker. This entire process

was performed twice, once to form the transformed G1 line and again to produce the

transformed G2 line (Kim et al. 2004).

Fitness costs

Fitness costs associated with transgenic alleles may have several causes.

Transposable elements used in creation of transgenic lines are known to cause

mutations and may disrupt normal gene function. Because two independently transformed lines (G1 and G2) are being compared to the control line (G3), position effects are possible. This effect is the difference between transgenic lines due to insertion site of the transgene. Detecting potential position effects between G1 and G2 is another goal of my study. Fitness costs may be associated with the gene expression; excess production of cecropin may lead to an energy or resource trade-off that lowers fitness. Also, the location of transgene insertion may cause an interruption of important pre-existing genes in the chromosome. This disruption would also ostensibly lead to reduced transgene fitness. Also, high levels of EGFP expression have been shown to induce cell apoptosis, indicating a toxic effect that may have fitness consequences (Liu et al. 1999). Furthermore, transformed mosquitoes must be isolated and full-sib mating must occur to establish a transgenic germ-line (Yan et al. 1997, Catteruccia et al. 2003). This requires at least two generations of inbreeding, which accelerates allele fixation and may, via inbreeding depression, reduce offspring growth and increase offspring mortality via inbreeding depression (Armbruster et al. 2000).

Past work with transgenic *Drosophila* suggests that genetic manipulation has an impact on fitness (Woodruff 1992). Recent work examining transgenic mosquitoes supports the claim that creating a transgenic insects have a considerable fitness costs (Tiedje et al. 1989, Yan et al. 1997, Irvin et al. 2002, Catteruccia et al. 2003) (but see: (Moreira et al. 2003).

A recent study by Catteruccia et al. found reduced fitness of transgenic *An. stephensi* (Catteruccia et al. 2003). Competitive ability of transgenic *An. stephensi*

was measured by establishing a free-breeding population with equally represented transgenic and non- transgenic mosquitoes. In the presence of competition, the frequency of the transformed allele was lower than expected (based on Hardy-Weinberg Equilibrium) and decreased for 4-16 generations, when all transgenic lines became extinct. Thus, wild- type *An. stephensi* mosquitoes were found to be superior competitors to transgenic mosquitoes. This suggests that transgenic alleles contain some sort of selective disadvantage in a competitive situation.

With the exceptions of *An. gambiae* (Kim et al. 2004) and *Ae. Aegypti* (Coates et al. 1999, Kokoza et al. 2001, Lobo et al. 2002), few mosquito species have been successfully transformed to include a marker and a *Plasmodium* refractory gene. In few cases have the fitness consequences of transformation been evaluated. A life table analysis has been performed on *Ae. aegypti* transformed to express EGFP with *Hermes* and *Mos1* transposable elements. The study revealed fitness deficits in transgenic lines of this insect compared to wild, non-transformed types (Irvin et al. 2002). No published studies address the fitness consequences of transformation of *An. gambiae*, the major African malaria vector responsible for half of all malaria deaths.

The goal of this experiment is to determine the fitness costs associated with two genetic transformation events in *An. gambiae*. This study is a detailed life-table analysis designed to identify the life stages causing fitness differences in transformed *An. gambiae*. The variables measured included larval and adult duration, adult survivorship, fecundity and estimated intrinsic rate of increase for two strains of genetically modified *An. gambiae* and the non-transformed strain. Because previous studies indicated that transgenesis reduces fitness, it was predicted that non-

transformed mosquitoes would have a higher mean fitness. It was expected that

significantly higher survivorship, fecundity and/or growth rate would indicate this

higher relative fitness. In addition, life table statistics were compared between the two

transformed lines to see if different strains bear significantly different fitness costs,

possibly indicating position effects.

METHODS

Establishing transformed lines

A lab strain of *An. gambiae* named G3 was genetically transformed using the *piggyBac* transposable element, following techniques described by Kim et al. (Kim et al. 2004).The vector, pPBMG-CEC , contained EGFP and *An. gambiae cecropin A*(AngCecA), a *Plasmodium* refractory gene located downstream of the promoter *Aedes aegypti carboxypeptidase A* (CP). Eggs were collected from *An. gambiae* females 72-120 hours after blood meal. The eggs were desiccated and covered with Halocarbon oil. The gene vector pPBMG-CEC was co-injected with the *piggyBac* helper element phsp-pBac into *An. gambiae* embryos. After injection, the oil was removed and larvae were reared using standard techniques (Kim et al. 2004). Founder adults that developed from the transformed eggs were backcrossed to wild type G3 adults. The offspring from these matings were screened for expression of the transgene via EGFP. Those individuals expressing the EGFP were sib-mated to create homozygous transgenic lines. Two independent transformations were preformed to produce two separately transformed lines, designated G1 and G2. These lines contain the same transgenes but they likely differ in the location of the transgenes integration in the mosquito genome.

Experimental design

To compare the lifetime fitness of transformed and non-transformed lines, the life table characteristics of the three *An. gambiae* lines, the non-transformed G3, and the transformed lines, G1 and G2, were examined in controled environmental

conditions. One day after emergence, two hundred first instar larvae from each line were placed into two larval pans (100 larvae each) (Figure 3). Each tray contained 20 identical cups holding 5 larvae each in equal volumes of distilled water (Figure 4). The number of live larvae in each cup was recorded daily.   Once they began to appear, the numbers of pupae per cup were also recorded daily. All of the pupae from the same line were collected daily and placed in a new cup of distilled water within a separate adult cage. Each cage was then labeled with the mosquito line, number of individuals, and date of pupation (Figure 5). Each cage was checked to see when pupae eclosed. After all individuals successfully emerged as adults or died, the pupal cup was removed. The adults were fed *ad libitum* with cotton soaked in 10% sugar solution. The cotton was replaced every other day until all adults had died. Larvae and adults were held in 26.5 C temperature and 85% humidity growth chambers with a 16 hour light 8 hour dark photocycle. This experiment was replicated three times, beginning on July 14th, August 7th and September 9th, 2003 (referred in analysis as "Replicates").

Fecundity of *An. gambiae* females was measured for the individuals in adult cages that contained a minimum of 5 females and 5 males as potential mates. Blood meals are required for female egg production. Because *An. gambiae* females are anthropophilic, human blood meals were offered. Cages 4-6 days old with the required number of individuals were given a human blood meal. In order to perform this, cages were placed in darkness to simulate night feeding conditions. The human host's arm (always the same host) was held up to the mesh top of the adult cage, allowing the females to feed freely. Each cage was exposed to the blood meal for 10-

15 minutes, until all females had completed feeding. The sex ratio was also known, so the number of females available for oviposition was recorded each day.

One day following the blood meal a cup with a moistened filter paper substrate for oviposition was added to these cages. The filter paper was removed (covered with eggs) and replaced daily until no eggs were produce for two consecutive days. Each filter paper was preserved in a labeled plastic bag and stored at -20˚C. The number of eggs per female per day was counted for each line. Fecundity measures were taken during replicates 1 and 3, when a human host was available to administer blood meals.

Egg hatch rate of the stock populations of G1, G2 and G3 mosquitoes was also measured. Adult cages from each line with over 150 bloodfed females were sampled. Two replicates of this experiment were performed. For replicate 1, adult females 7 days post pupal emergence were used. For replicate 2, adult females were 4 days post emergence. The younger females were thought to have enhanced fecundity compared to the replicate 17 day old females because fecundity decreases as adult females age (Gilles and Wilkes 1965). Samples were taken from the filter paper used for oviposition for each available cage. From the papers containing over 1,000 eggs, three subsets of 50 eggs from each line were sampled. The eggs were chosen on the day of oviposition. The groups of 50 eggs per line were placed on separate pieces of moistened filter paper and allowed two days to hatch. After two days, the numbers of newly hatched larva from each batch were counted.

Analysis

I tested for effects of several variables related to fitness. Variables examined included r values, larval and adult duration, adult survivorship, fecundity and egg hatch rate.   The intrinsic rate of growth of a population, defined as $r \sim = \ln(R_0)/T$, is an overall fitness indicator.   It combines information from both survivorship and fecundity, termed net reproductive rate per generation (R_0), as well as age at reproduction (T). This value was used because it is the most comprehensive life table parameter and therefore the most comprehensive with which to compare populations.

ANOVA was used to test for effects of experimental replicate and mosquito line on intrinsic rate of growth (r).   Replicates 1 and 3 only were used to calculate mean r value because bloodmeals were given during these replicates. Consequently, fecundity could only be determined for these replicates.   Fecundity was measured as (total number of eggs produced / number of females) per cage each day.

The initial 200 larvae for each mosquito line were subdivided into 40 groups of 5 to test for larval cup duration (Figure 3, 4). ANOVA was used to test for effects of larval cup duration and mean duration. Larval cup duration was determined by the number of days a cup was occupied by at least one individual larva.   Mean larval duration was measured as the mean number of days larvae survived per line.

To determine adult cage duration and longevity, newly ecolosed adults were caught daily by line. Because the availability of adults varied each day, cages contained a range of individuals; some held only one adult while others contained over 50. To evaluate the effects of adult density on longevity, ANOVA was performed

based on data from individuals from line G1 (replicate 1). The effects of initial density on longevity were tested.

Adult cage duration was defined as the number of days at least one individual was present per cage and longevity was measured as the mean number of days that adults survived. Each replicate was individually analyzed via ANOVA to determine if there was an effect of line during any given replicate.

Survivorship curves were formed to represent the survivorship of adults from each line. All survivorship graphs display replicate 1 and 3 data, the replicate where selected adult females received bloodmeals. The graphs were standardized so that lines start at 100 percent. Survivorship is shown beginning the day after bloodmeals were administered. The first survivorship curve (Figure 6) displays the survivorship of all adults in replicates 1 and 3.   The second survivorship curve (Figure 7) is the most specific because it isolates the female mosquitoes from all lines that have received a bloodmeal. Mean survivorship for all bloodfed females was also analyzed with ANOVA.

Egg hatch rate (mean number of eggs hatched form each subset of 50) was analyzed by two-factor ANOVA without replication. This tested the mean hatch rate for each line during two independent replicates.

RESULTS

Intrinsic rate of increase (r)

The intrinsic rate of increase (r) was similar for all lines during experimental replicates 1 and 3 (Table 1a). The control line, G3, had an r value of 0.151 (SE ±0.28), which is intermediate to G1 (0.145 ±0.31) and G2 (0.164 ±0.43), the transgenic lines. The variance of G3 was 2.7^4, larger than the variance of the transgenic lines G1 (2.80^-5) and G2 (1.18^-4). Neither line (p= 0.08) nor replicate (p= 0.15) was a significantly less varied within groups (Table 1b). Even within each replicate, line was not a significant factor (p= 0.10 for replicate 1, p=0.93 for replicate 3) (Table 1c).

Larval duration

Mean larval cup duration was similar for all lines during replicates 1, 2 and 3 (Table 2a). Lines averaged about 12 days (SE range ± 0.2-0.28) of larval duration for replicate 1. Variance was highest for transgenic line G2 at 40.80 days; variance was just 1.74 for G1 and 3.05 for G3. Although the non-transgenic line went through the larval stage about 0.5 day faster than either transgenic lines, that difference was not significant (p= 0.48) (Table2b).

Because of improved mosquito rearing techniques from the final two replicates, there was increased uniformity within lines for the second and third replicate. Variances was small during replicates 2 and 3, all lines were at or below 1.3. The mean larval cup duration decreased by almost half for replicates two and three (Table 2a). Though this created a highly significant effect of replicate (p<0.001)

(Table 2b), duration remained similar across all lines. More importantly, ANOVA indicated no interaction between replicate and line (p= 0.70) (Table 2b). The difference in replicate did not disrupt the trends found between and within lines on any replicate.

G1 averaged 6.38 ± 0.094 and 6.65 ± 0.161 days in larval cup duration for replicates 2 and 3; G2 averaged 6.15 ±0.0865 and 7.18 ± 0.183 days. The non-transgenic line (G3) averaged 6.03 ± 0.026 days, below the transgenic lines, for replicate 2. For the final replicate, G3 averaged 6.85 ± 0.086 days, above both transgenic lines. There were no significant differences between larval cup duration, though G3 did avergae about a half day shorter than either transgenic. All lines had a reduced larval duration and variance for replicates 2 and 3 (Table 2a).

Mean duration displayed similar trends (Table 3a). The mean number of days that individuals spend in the larval stage was the shortest, 6.46 ±0.14 days, for replicate 1. The variance, 0.05, was also the highest in replicate 1. Mean duration during replicate 3 was 5.58 ± 0.07 days, close to replicate 1, but with a variance of only 0.01. Duration dropped to an mean of 3.37 ± 0.04 days during replicate 2 with a variance of only 0.004, contributing to the significance of replicate (p<0.001) (Table 3b).

All lines had very similar mean duration within and between lines; the mosquito line was never found to be a significant factor (p=0.88) (Table 3b). The transgenic line G1 had the longest larval duration of 5.18 ±0.96 days; G1 averaged 5.11 ±0.86 days (Table 3a). The control line, G3, averaged 5.13 ±0.95 days, slightly

closer to line G2. The variance for G1 and G3 was 2.75 and 2.71, respectively, slightly longer than the G2 variance of 2.23.

Adult duration, longevity and survivorship curves

Analysis of the effects of density on longevity in line G1 did not reveal a significant density effect (Table 4a, b). Adult cage duration did not differ significantly among replicates (p=0.075) (Table 5b). For replicates 1 and 3 adults cages were occupied a mean of 36.05 ±2.11 and 36.50 ±1.26 days. Mean cage duration for replicate 2 was 43.01 ±1.54 days, several days longer than the other replicates (Table 5a). When characterized by line, adult cage duration is also comparable; lines are within about three days of each other. The transgenic lines G1 and G2 averaged 36.66 ± 2.32 and 39.21 ± 2.12 days while the control line G3 averaged 39.21 ± 3.32 days (Table 5a). ANOVA revealed that there is no effect of line on adult cage duration (p=0.469) (Table 5b). Even when replicates are isolated and lines are analyzed via single factor ANOVA, there are still no significant differences in cage duration. The effects of line were p = 0.28, p = 0.42 and p = 0.29 for replicates 1,2 and 3 (Table 5c).

Mean adult longevity was similar between lines for each replicate (Table 6a). During replicate 1, adults lived a mean of 16.29 ± 0.78 days, with a variance of 1.82. Longevity increased to a mean of 17.75 ± 0.37 for replicate 2, but variance decreased to 0.42. Longevity and variance were both lowest for replicate 3; adults lived a mean of 15.07 ±0.16 days with a variance of 0.08. This decrease in variance between replicates 1 and 2-3 is due to a learning curve; longevity was more uniform with each

successive replicate. This caused replicate to be a significant factor in the ANOVA (p=0.04) (Table 6b).

Lines did not have significantly different adultlongevity (p = 0.33) (Table 6a). All lines had means within about one day of each other. Transgenic line G2 had the shortest mean longevity of 15.73 ±0.67 days with the smallest variance, 1.34. Transgenic line G1 and control line G3 had similar mean longevities, 16.54 ±1.02 with variance of 3.12 and 16.84 ±0.88 with variance of 2.31, respectively. Line was not a significant factor (p=0.33) (Table 6b).

Mean longevity for females post- bloodmeal was similar across lines and replicates (Table 7a). Mean longevity decreased from 11.51 ±0.23 days during replicate 1 to 11.48 ±0.57 days during replicate 3, a difference of only 0.03, while variance increased from 0.17 to 0.96. These differences between replicate were not significant (p=0.98) (Table 7b). The mean longevity between lines of bloodfed females differed by less than one day. The transgenic line, G2, averaged 11.34 ±0.29 days with a variance of 0.28 (Table 7a). The control line, G3, averaged 11.13 ±0.70 days, similar to G2, but with a higher variance of 0.97. Transgenic line G1 had the highest adult longevity post bloodmeal, a mean of 12.01 ±0.37 days with a variance of 0.28. Lines were not significantly different (p=0.63) (Table 7b).

Adult survivorship curves also show the relative survivorship of adults from each line for replicates 1 and 3 (Figures 6,7). Survivors are plotted as a fraction of the founding population. The overlapping survivorship curves reinforce the idea that only slight, random variations existed between the lines (Figure 6). Survivorship curves show the highest divergence when displaying transgenic lines expressing both EGFP

and ectopic cecropin verses the control line (Figure 7). In fact, this plot of bloodfed females over time shows transgenic lines with a 3 or 4 day increase in survivorship as compared to the control line G3 (replicate 3). This agrees with the mean longevity of bloodfed females (Table 7), which also shows slightly higher survival for transgenic lines.

Egg hatch rate

The mean egg hatch rate did not differ among lines (p=0.53) (Table 8b). The control line averaged 26.7 ±0.33 eggs hatched out of 50, slightly smaller than 28 ±0.67 eggs for G2 and virtually the same as 26 ±0.33 eggs for G1 (Table 8a). Females 4 days post-emergence (used in replicate 2) had an mean of 27.1 ±0.78, which was not a significantly higher hatch rate when compared to 26.7 ±0.51 for 7 day females (used in replicate 1). About 27 eggs out of each subset of 50 hatched regardless of if the mothering female was transgenic (line effect) or past peak reproductive peak (replicate effect).

DISCUSSION

Overview

The object of this research was to determine if *Anopheles gambiae* mosquitoes transformed with the *piggyBac* transposable element to express EGFP and ectopic cecropin experience a reduction in fitness. It was predicted that all life table characteristics would be reduced for transgenic lines as compared to a non-transgenic control line. However, larval development time, adult longevity, fecundity, survivorship and egg hatch rate did not differ significantly between transgenic and control lines for any given experimental replicate.

The intrinsic rate of increase, an overall fitness measure, was not significantly different between transgenic and control lines. In fact, the quantitatively highest r value was 0.164 ±0.43 for G2, a transgenic line. The cecropin transgene and EGFP in lines G1 and G2 were both engineered to be fully expressed in adult females post-bloodmeal; this was expected to reduce intrinsic growth rates for transgenic populations. Instead, all mosquito lines had quantitatively similar rates of intrinsic growth.

In addition, there were no significant differences between any larval or adult growth variables. Interestingly, adult longevity was longer during the second experimental replicate, when no mosquitoes received bloodmeals. Mean adult longevity was about 4 days longer, and adult cages were occupied an about 7 days longer in replicate 2 (for replicates p = 0.04, p = 0.075). The acquisition of human bloodmeals and/ or egg production by females may involve an obligate energy and

fitness tradeoff. Studies of reproductive tradeoff indicate that longevity tends to increase when reproductive efforts are minimized (Schluter et al. 1991). During replicate 2, females may have increased in fitness because they were not allowed to expend energy towards reproductive efforts. Investigating energy tradeoff between reproduction and fitness was not a goal of my study, though this is an important topic in malaria transmission research.

Survivorship curves also show trends of adult survivorship. The first survivorship curve shows mean survivorship across all individuals in each line (Figure 6). Replicates 1 and 3 were selected because mosquitoes were administered bloodmeals during these runs. For transgenic mosquitoes, females that received bloodmeals were the only individuals engineered to express both EGFP and ectopic cecropin. When survivorship of all individuals is displayed, the lines are nearly indistinguishable. Instead of G3 showing enhanced survivorship, it slowly declined over time at approximately the same rate as the two transgenic lines.

A higher level of differentiation is seen when examining only the transgenic mosquitoes expressing cecropin and EGFP verse the control line (Figure 7). The standardized graph of bloodfed females survivorship displays a much more pronounced divergence between all lines in replicates 1 and 3. In fact, replicate 3 survivorship curves indicate *enhanced* survival of transgenic lines G1 and G2 relative to non-transgenic G3. The mean adult survivorship of bloodfed females is also slightly longer for transgenic lines G1 (12.01 ±0.37 days) and G2 (11.34 ±0.29 days) than for the control line (11.13 ±0.70 days). This improved survivorship of transgenic lines when expressing both a refractory and marker is also contradictory to the fitness

costs predicted. This increased survivorship of transgenics is a non- significant (mean

longevity p= 0.63) but highly relevant trend.

Egg hatch rate was also an exclusive product of bloodfed females and thus

expected to be influenced by both the EGFP marker and the ectopic cecropin

transgene. Thus, the hatch rate may be considered an estimate of adult fitness as well

as a larval trait. However, there were no differences in egg hatch rate between

transgenic and control mosquitoes. Therefore, throughout all aspects of the adult life

stage, there was no detectable fitness load of transgenics to compromise the fecundity

or survivorship. Adult survivorship and fecundity data only provides further evidence

to refute the prediction that transgenes bear fitness costs.

Expression of transgene may influence costs

Several aspects of the transgene are potential sources of fitness costs. The use

of the effector gene, cecropin, as well as the EGFP marker gene may have inherent

costs due to expression or position effects associated with the insertion site. Though

cecropin is a natural aspect of mosquito immunity, increased expression of this

defensive compound may involve energy or fitness trade-offs (Cotter et al. 2004). To

compensate for this, ectopic cecropin is regulated for expression at a specific time and

tissue. In addition, the protein itself is part of innate mosquito immunity, which may

help reduce fitness costs associated with the transformed mosquito lines. Transgenic

adult females that received bloodmeals expressed *Plasmodium* refractory cecropin in

their mid gut; these individuals were expected to bear the highest fitness costs due to

expression of additional transgenes. However, no significant fitness differences were

found that suggest costs associated with cecropin transgene expression. Thus, ectopic cecropin does not appear to be costly to produce.

The EGFP marker gene is an excellent way to identify and screen transgenic mosquitoes, but it may also be a source of fitness costs. While the time and location of the cecropin gene was highly regulated, the EGFP marker had more generalized expression throughout all life stages. The fluorescence is expressed in the neural tissue and anal papillae of larva and limited to expression in the optic lobes of the brain for adults (O'Brochta 2004). Some work has suggested that EGFP marker expression induces apoptosis (programmed cell death) and thus bears insidious fitness costs (Liu et al. 1999). In addition, a life table study of *Aedes aegypti* and a competition study of *An. stephensi* found that mosquitoes transformed to express EGFP were less fit than their non transgenic counterparts (Irvin et al. 2002, Catteruccia et al. 2003). While the EGFP marker may be somewhat costly to produce, it does not appear to have any negative fitness impacts in this study.

Two transgenes (EGFP and cecropin) with separate regulation were used to create these transgenic lines. Because G1 and G2 were independently transformed germ-lines, it is likely that the transgenes were inserted into different locations of the *An. gambiae* genome. The location of insertion may disrupt other gene expression, causing different position effects in each line. Despite this possibility, there were never significant differences in fitness traits between the two transgenic lines. Thus, there is no evidence that position effects are present between these two transgenic lines.

A recent life-table study of transgenic *An. stephensi* also found minimal fitness costs associated with transgenic lines (Moreira et al. 2003). Moreira et al. concluded that fitness costs were due to the product of the transgene, not its site of insertion. A population cage experiment testing competition as well as a life table study compared mosquito lines transformed with different effector genes (SMI and PLA2) to a non-transformed control line. SMI lines (transformed with the SMI peptide tetramer) showed no reduced fitness or indications of position effects between lines (Moreira et al. 2003). This SMI transgene, similar to the ectopic cecropin produced in the transgenic *An. gambiae* of our study (AngCecA), is time and tissue specific.

Additional, unobserved components of fitness may be affected by transgenesis. Resistance to pathogens and extreme conditions, such as heat, should also be considered before a full conclusion can be drawn. In addition, a competition study that tracks the frequency of the AngCecA transgene over several generations may reveal subtle differences between G1, G2 and G3 not measured here. This type of competition study was able to detect differences in fitness between transgenic and wild type *An. stephensi* (Catteruccia et al. 2003). Though the ectopic cecropin transgene does not appear to have any fitness costs, it may affect transgenic mosquito behavior, such as competitive mating ability. In fact, assortative mating is considered to be a major hurdle that must be overcome before transgenic mosquitoes are released (Reisen 2002, Scott and Morrison 2002). Future work should compare the mating success of transgenic and control mosquitoes as well as determine the frequency of matings between transgenic and control lines.

Factors that may outweigh fitness costs

Because this study was conducted under the benign conditions of a laboratory, its field application is limited. For instance, this study showed relatively slight mortality in stages prior to adult emergence (about 50-75% for all lines) whereas field studies indicate that 91.5% of *An. gambiae* mortality occurs during this period (Aniedu et al. 1993). One significant ecological factor is field exposure to the malaria pathogen, *Plasmodium falciparum*. This can actually benefit transgenic mosquitoes; the fitness costs associated with carrying this pathogen may counteract any competitive advantage that non-transgenic mosquitoes have. While it is possible that the non-significant fitness differences found in the laboratory may be magnified in a field situation, there may also be an additional advantage for transgenics in the field.

Costs of carrying *Plasmodium*

Our experiment focused on the fitness costs of transgenic mosquitoes. None of the lab strains were infected with the *Plasmodium falciparum* parasite. However, non-transformed mosquitoes would be exposed to this pathogen in a field situation. These vulnerable mosquitoes must bear the substantial cost of carrying the *Plasmodium* pathogen. This may counteract whatever costs are associated with genetic transformation and thus create a higher relative fitness for transgenic mosquitoes.

The burden of vectoring *Plasmodium* has been found to impact the fitness of Anopheline mosquitoes in several studies. Infected *An. stephensi* were found to have reduced fecundity for three gonotrophic cycles, even when oocyst levels were low

(Hogg and Hurd 1995). *P. falciparum* sporozoite level has also been linked to decreased survival of blood- feeding *An. gambiae* females (Anderson et al. 2000). The presence of this parasite is correlated with reduced blood-feeding efficiency; fewer anti-clotting enzymes are produced by infected mosquitoes (Hurd 2003).

Host manipulation

Parasites manipulate infected females in order to maximize dispersal. Apyrase enzyme, used by females to induce blood flow from human hosts, is an essential part of acquiring blood meals. However, this enzyme is present in a much lower concentration when the mosquito is infected with *Plasmodium*. Reduction in the enzyme makes bloodmeals more difficult to obtain from hosts; this increases the number of probing attempts, which corresponds with an increase in mortality. Infected *An. gambiae* are more likely to take several bloodmeals from multiple hosts, which is risky for the mosquito but beneficial for *Plasmodium* dispersion (Koella et al. 1998). Thus, the malaria parasite manipulates its mosquito host to infect humans more often. This fitness reduction in infected wild mosquitoes will increase the relative fitness of transgenic mosquitoes that cannot carry pathogen

However, some work has suggested that *An. gambiae* survivorship is not reduced due to parasite infection, perhaps because the mosquito has evolved to minimize these costs (Robert et al. 1990). Despite this, *Plasmodium* infection is still generally thought to reduce the fitness of *An. gambiae* and other vectors (Schwartz and Koella 2001, Ferguson and Read 2002). In addition, even a small fitness load inflicted by the parasite may translate into a large transgenic advantage in the field.

Ecological models

Modeling simulations give an indication of the relative importance of fitness in the dispersion of transgenic mosquitoes (for review: Gould 2004). A model by Kiszewski and Spielman incorporated spatial and seasonal discontinuity to determine the spread of released transgenic mosquitoes (Kiszewski and Spielman 1998). Their model emphasized fitness as the most important factor of transgene dispersal. In an ideal scenario of a 0% fitness reduction of released transgenic mosquitoes relative to the wild population, fixation of the transgene may still require up to 200 generations. A minimum fitness requirement for a model without environmental structure allows for a 50% reduction in fitness of transgenic mosquitoes. However, once spatial and temporal structure (such as village distribution and seasonality) is incorporated, the minimum fitness reduction for GMM is limited to 30%. However, fixation with a 30% fitness reduction of transgenic may require over 2,000 generations, depending on length of breeding season and wet or dry conditions. Only models assuming a 20% or smaller reduction in fitness consistently predicted replacement of wild mosquitoes with released transgenic mosquitoes. Fixation of the transgene in the wild population would occur only after 150 generations. It was predicted that high release ratios and annually repeated releases only aid in dispersion if fitness was above a minimum threshold of 70% relative to wild (no more than 30% reduction). When transposon drive (i.e. "genetic drive") was taken into account for simulations, the minimum

relative fitness was only reduced to 70% (30% reduction) and 140 generations were needed to achieve fixation.

However, other models predicted more optimistic outcomes once the genetic drive of the transposon was taken into account. Ribeiro and Kidwell created a simple population model that varied fitness costs and integration transmission rates of the transgene. The results indicated that transgenic mosquitoes could spread through wild population even with 50% fitness costs.

A simulation model by Boete and Koella also predicts the outcome of a GMM release aided by transpose drive (Boete and Koella 2002). In addition to a transposon drive mechanism, this model assumed a single refractory gene and a reduction in fitness due to transgenesis. Two alternates of the model determined the spread of the refractoriness; one assumed fixed (constant) costs while the other assumed conditional (dependent upon *Plasmodium* infection) costs of the transgene. In both versions, the transposon may drive refractory genes into the population, even if there are substantial fitness costs as long as the benefits of refractoriness outweigh the costs. In fact, in the presence of a drive system, there is even the potential to drive the refractory gene to fixation in the wild population. There is a caveat; effective refractoriness will reduce the number of infected mosquitoes, but fewer infected mosquitoes means less selection pressure for refractoriness. Thus, epidemiological feedback would lessen selection pressure for refractoriness that is not close to 100%. As a result, this model predicts that the introduced gene must be nearly 100% refractory in order to have a large impact on malaria.

Transgenic Mosquitoes: Challenges and Promises

Lowered fitness of transgenic mosquitoes is only one of many concerns about the potential release of these organisms into the wild. A myriad of other ecological concerns have been raised regarding the topic. A 2002 conference at Wageningen, The Netherlands was held exclusively on the topic of ecological challenges to releasing transgenic mosquitoes. The proceedings of this conference outline several major problems with the assumption that transgenic mosquitoes will be able to eradicate disease (2002). Yet there is still an overall deficiency of work in ecology, population genetics and simulation models to compliment the exhaustive genetic work devoted to creating transgenic mosquitoes.

The problem of a reliable genetic drive mechanism, which is integral to a successful transgenic program, is a concern often raised in the literature e.g.(Alphey et al. 2002, Scott et al. 2002, Boete and Koella 2003). Genetic drive is needed to ensure a high frequency of the *Plasmodium* refractory transgene in the wild mosquito population (see "Introduction: Mosquito transformation" for discussion of drive system theory). Mosquito germ-lines can now be reliably transformed using transposable elements that have inherent genetic drive. Genes linked to transposable elements are capable of being represented in a disproportionately large number of gametes (Kidwell and Ribeiro 1992, Ribeiro and Kidwell 1994). Ideally, this drive mechanism will compensate for small fitness reductions associated with the transgene. Though transposable elements solve the problem of finding a drive mechanism, there is still a possibility that the elements will dissociate from transgenes engineered to

refract *Plasmodium*. This would circumvent its intended purpose of spreading the novel gene through a wild mosquito population.

Another argument against the release of transgenics stems from the shortcomings of sterile male release programs in the 1970s and 80s. The sterilization and release of *Anopheles*, *Culex* and *Aedes* disease vector mosquitoes proved to be a failure, mainly due to the dearth of ecological considerations, such as mating and oviposition preference (Lounibos 2002, Reisen 2002). However, these difficulties may be avoided if field ecology is incorporated into GMM research and employed in any attempted release strategy. Further research on understudied areas of mosquito ecology, such as male biology and mating behavior, would help elucidate the conditions needed to encourage wild-transgenic mosquito matings and thus aid the spread of a transgene through a wild population. Sterile male release programs found that little to no cross breeding occurred between wild mosquito populations and the sterile males released (Reisen 2002). Thus, assortative mating is likely to be a substantial obstacle that must be overcome to ensure dispersal of a *Plasmodium* refractory transgene.

Colonization efforts may be thwarted by lab selection, which favors different phenotypes than the natural mosquito environment. For example, the lab-adapted mosquitoes is the *Aedes aegypti* sterile male release failed to colonize village dwellings or oviposit in clay pots like wild mosquitoes (Lounibos 2002). Enclosed greenhouse experiments that reproduce the environmental conditions a mosquito in the wild would encounter (i.e. Semi-Field tests) may be the next step to determine if lab adaptation will be a problem for transgenic mosquitoes. Raising transgenics in this type of setting may also be the best way to reintroduce selection for field- related

fitness traits. Semi-field tests have already been successfully used to investigate the behavioral ecology of non-transgenic mosquito vector species (Knoles and Scott 2002). These tests could be used to expand current laboratory work and determine relative fitness of transgenic and wild mosquitoes in a realistic environment.

Semi-field experiments as well as observational studies of wild mosquitoes are needed to gain insight about optimal release conditions. Any potential release must be carefully predetermined to give genetically modified mosquitoes the best possible chance of mating success with their wild counterparts.   Precise ecological recommendations must be followed in order to maximize mating success. Time, location, and photoperiod, for example, must be appropriate to optimize cross matings between wild and genetically modified mosquitoes (Reisen 2002).

There are large wild populations of mosquito vectors such as *An. gambiae*, yet only about 2%-6% of these mosquitoes are infective (Gilles and Wilkes 1965). As a result, there must be a very large number of transgenic mosquitoes released in order to effectively reduce this small fraction of infected wild vectors.   As a result, release ratios suggested based on previous experiments are as high as 10 transgenic mosquitoes for every wild mosquito (Spielman 2002). However, this estimate assumes no genetic drive and fitness costs to transgenesis, release ratios may be substantially lower without these limitations.

Population subdivision is a key factor in the success of any vector control strategy. Even if an introduced transgene is 100% refractory and bears no fitness costs, simulation models show that its spread is highly dependent on metapopulation structure (Taylor and Manoukis 2002). Review studies suggest that *An. gambiae* has a

high level of genetic diversity and one major subdivision that separates north-western and a south-eastern African populations (Lanzaro and Tripet 2002). In addition to *An. gambiae sensu stricto*, The *An. gambiae* complex incorporates 6 species with varying degrees of genomic differences. The overall population structure consists of co-occurring species and various intermediate chromosomal forms. In West Africa, gene flow was found to be high within chromosomal forms and moderate between forms. Gene flow was limited between species (Taylor et al. 2001). If this is true for most endemic areas, the inconsistency of gene flow will be a challenge that should be considered in transgenic mosquito programs. For instance, genetically distinct groups may require separate releases in order to ensure sufficient spread of the transgene. Despite this complication, the subdivision present in *An. gambiae* populations may present an advantage: it would reduce the introduction threshold (number of mosquitoes released to transform a population) (Lanzaro and Tripet 2002). Population-specific introductions of transformed *An. gambiae* may overcome the release ratio problem and enhance spread of the transgene.

Once in the natural environment, transgenic mosquitoes may alter the human-mosquito-human transmission cycle. Environmental cues may modify the physiology of gene expression of cause selection for or against a transgene (Billingsley 2002). Evolutionary responses may also alter parasite virulence, vector longevity and human infection rate. For instance, the introduction of transgenic mosquitoes would likely lower the degree of multiple *Plasmodium* infections in humans (Elliot et al. 2002). This would decrease *Plasmodium* transmission, which may result in reduced pathogen virulence. Ideally virulence to the human host would be diminished; however, the

burden of carrying *Plasmodium* may be reduced for mosquito vectors (Elliot et al. 2002). If the later occurs, there would no longer be a relative fitness benefit for mosquitoes to parasite-free. This may ultimately reduce the spread of transgenic mosquitoes (Boete and Koella 2002).

The risk of contracting malaria can be quantified using the entomological inoculation rate (EIR), the number of "infective" mosquitoes (with sporozoites) biting a person per unit time (Charlwood et al. 1998). Research shows that if transmission is lowered, there is the potential for a rebound effect (Smith et al. 2001). Normally children contract infections that give them a degree of immunity as an adult; primary malaria infection as an adult is more likely to be fatal. Lower EIR creates unstable malaria, or a reduction in "human herd immunity." The result is a rebound in the number of cases once the non-immune children become infected as adults. However, studies of bed-nets treated with insecticides have thus far shown no evidence of rebound effects, suggesting that refractory transgenes would not be likely to exacerbate malaria morbidity (Curtis 2002). In addition, if genetically modified mosquitoes are limited to one type of malaria vector such as *An. gambiae*, children may acquire fewer infections and eventual resistance from other, less fatal vectors (Scott et al. 2002). Though there is some concern about malaria spreading via non GMM vectors, no Anopheline vectors are as anthropophilic and thus as deadly as *An. gambiae*.

Finally, the ethical, legal and social concerns inherently linked to a potential release must be fully addressed (Touré et al. 2002). The public must be involved in the process in order to be convinced that this method of malaria control is a safe and

effective one. Thus far, there has been a high public investment in the development of genetically modified mosquitoes.   Unfortunately this has been, to some extend, at the expense of ecological and behavioral research. It is now imperative that the ecological as well as social considerations now take center stage in the GMM quest for malaria control.

There is enough evidence to support cautious optimism about the potential of release of genetically modified *An. gambiae*. Finding fitness to be similar for wild and transgenic mosquitoes is an encouraging step, not an indication that GMM are ready for release. Hopefully concern over the ecological aspects of introducing transgenics will incite ecologist and geneticists to work together to do work in behavioral ecology, population genetics and enclosed field trials. Though the ecological concerns brought up in the Wageningen conference are undoubtedly valid, the concept of genetic modification should not be abandoned. While genetic modification alone may not be sufficient to eradicate malaria, it may have a powerful impact when combined with other transmission control techniques in hyper-epidemic areas (Koella 2002).

TABLES

(a)

Line	Mean	Variance
G1	0.145 ±0.31	2.80E-5
G2	0.164 ±0.43	1.18E-4
G3	0.151 ±0.28	2.74E-4

(b)

Source of Variation	df	MS	P-value
Replicate	1	3.6E-4	0.08
Line	2	1.8E-4	0.15
Error	2	3.2E-5	

(c)

Replicate 1 Source of Variation	df	MS	P-value
Between Groups	2	9.4E-05	0.10
Within Groups	6	0.005	

Replicate 3 Source of Variation	df	MS	P-value
Between Groups	2	0.0003	0.93
Within Groups	9	0.0047	

TABLE 1:
(a) Intrinsic rate of growth comparison. The intrinsic rate of growth for three lines of *An. Gambiae*, two transformed (G1, G2) and one untransformed control (G3) for replicates 1, 2 and 3.
(b) P- values. ANOVA of effects of lines and replicate and error on the intrinsic rate of growth. Only replicates 1 and 3 were analyzed because bloodmeals were given during these replicates. (Bloodmeals are required for fecundity to be measured)
> **(c) P-values by replicate.** ANOVA of effects of line on intrinsic rate of growth for individual replicates 1 and 3.

(a)

Line	Replicate 1	Var.	Replicate 2	Var.	Replicate 3	Var.
G1	12.83 ±0.208	1.74	6.38 ±0.094	0.34	6.65 ±0.161	1.05
G2	12.63 ±0.243	40.80	6.15 ±0.0865	0.28	7.18 ±0.183	1.27
G3	12.08 ±0.277	3.05	6.03 ±0.026	0.03	6.85 ±0.086	0.28

(b)

Source of Variation	df	MS	P-value
Replicate	2	1.44E 3	< 0.001
Line	2	4.04	0.48
Interaction	4	3.03	0.70
Within	351	5.43	

TABLE 2:
(a) Larval cup duration comparison. Larval cup duration of three lines of *An. Gambiae*, two transformed (G1, G2) and one untransformed control (G3) for replicates 1, 2 and 3.
(b) P-values. ANOVA of effect of replicate, line and their interaction on larval cup duration.

(a)

	Number of Days	Variance
Replicate 1	6.46 ±0.14	0.05
Replicate 2	3.37 ±0.04	0.004
Replicate 3	5.58 ±0.07	0.01
G1	5.18 ±0.96	2.75
G2	5.11 ±0.86	2.23
G3	5.13 ±0.95	2.71

(b)

Source of Variation	df	MS	P-value
Replicate	2	7.63	<0.001
Line	2	0.00	0.88
Error	4	0.03	

TABLE 3:
(a) Mean larval duration. The mean larval duration of three lines of *An. Gambiae*, two transformed (G1, G2) and one untransformed control (G3) for replicates 1, 2 and 3.
(b) P-values. ANOVA of effect of replicate and line on larval duration. The mean of each line per replicate was treated as a unit, thus, error degrees of freedom are 4.

(a)

Cage	Survivorship	Initial density	Variance
111	25.64 ±7.32	11	107.11
112	27.28 ±3.41	34	23.28
113	27.44 ±20.27	68	822.51
114	26.08 ±6.54	13	85.50
115	10.80 ±2.10	15	8.82

(b)

Source of Variation	df	MS	P-value
Cage	4	382.52	0.34
Survivorship	1	56.97	0.66
Error	4	247.56	

TABLE 4:
(a) Adult cage density comparison. Adult cage density comparison transformed *An. gambiae* line G1 for replicate 1. This is representative of other lines and replicates.
(b) P- values. ANOVA of effects between initial density survivorship on adult density.

(a)

	G1	G2	G3	Mean	Variance
*R 1	32.28 ±3.64	36.28 ±3.15	39.57 ±2.61	**36.05** ±2.11	13.32
R 2	40.20 ±2.52	43.33 ±2.40	45.50 ±0.50	**43.01** ±1.54	7.10
*R 3	37.50 ±2.50	38.00 ±1	34.00 ±0	**36.50** ±1.26	4.75
mean	**36.66** ±2.32	**39.20** ±2.12	**39.69** ±3.32		
Variance	16.19	13.50	33.07		

(b)

Source of Variation	df	MS	P-value
Replicate	2	45.54	0.075
Line	2	7.94	0.469
Error	4	8.61	

(c)

R 1 Source of Variation	df	MS	P-value	R 2 Source of Variation	df	MS	P-value
Between Lines	2	93.19	0.28	Between Groups	2	22.81	0.42
Within Lines	18	69.80		Within Groups	7	23.13	

Replicate 3 Source of Variation	df	MS	P-value
Between Groups	2	9.50	0.29
Within Groups	3	4.83	

TABLE 5:
(a) Adult cage duration comparison. Adult cage duration of three lines of *An. Gambiae*, two transformed (G1, G2) and one untransformed control (G3) for replicates 1, 2 and 3.
(* indicates full transgene expression by bloodfed females.)
(b) P-values. ANOVA of effect of replicate and line on adult cage duration.
(c) P- values by replicate. ANOVA of effect of line on adult cage duration during individual replicates.

(a)

	Number of Days	Variance
Replicate 1	16.29 ±0.78	1.82
Replicate 2	17.75 ±0.37	0.42
Replicate 3	15.07 ±0.16	0.08
G1	16.54 ±1.02	3.12
G2	15.73 ±0.67	1.34
G3	16.84 ±0.88	2.31

(b)

Source of Variation	df	MS	P-value
Replicate	2	5.43	0.04
Line	2	0.98	0.33
Error	4	0.67	

TABLE 6:
(a) Mean adult longevity. Mean adult longevity of three lines of *An. Gambiae*, two transformed (G1, G2) and one untransformed control (G3) for replicates 1, 2 and 3.
(b) P-values. ANOVA of effects of replicate and line on adult longevity. The mean of each line per replicate was treated as a unit, thus, error degrees of freedom are 4.

(a)

	Number of Days	Variance
Replicate 1	11.51 ±0.23	0.17
Replicate 3	11.48 ±0.57	0.96
G1	12.01 ±0.37	0.28
G2	11.34 ±0.29	0.17
G3	11.13 ±0.70	0.97

(b)

Source of Variation	df	MS	P-value
Replicate	1	7E -4	0.98
Line	2	0.42	0.63
Error	2	0.71	

TABLE 7:
(a) Mean longevity for bloodfed females. Mean longevity for bloodfed females of three lines of *An. Gambiae*, two transformed (G1, G2) and one untransformed control (G3) for replicates 1 and 3. (No bloodmeal occurred during replicate 2)
(b) P-values. ANOVA of effects of replicate and line on mean longevity of bloodfed females. The average of each line per replicate was treated as a unit, thus, error degrees of freedom are 4.

(a)

	Percent	**Mean**	**Variance**
Replicate 1	0.533	26.7 ±0.51	0.778
Replicate 2	0.542	27.1 ±0.78	1.81
G1	0.52	26 ±0.33	0.222
G2	0.56	28 ±0.67	0.889
G3	0.533	26.7 ±0.33	0.222

(b)

Source of Variation	df	MS	P-value
Replicate	2	2.33	0.96
Line	6	51.89	

TABLE 8:
(a) Egg hatch rates. Egg hatch rate for three lines of *An. Gambiae*, two transformed (G1, G2) and one untransformed control (G3) for 2 replicates. The mean number of successfully hatched eggs is out of subsets of 50.
(b) P- values.   ANOVA of effects of replicate and line on egg hatch rate.
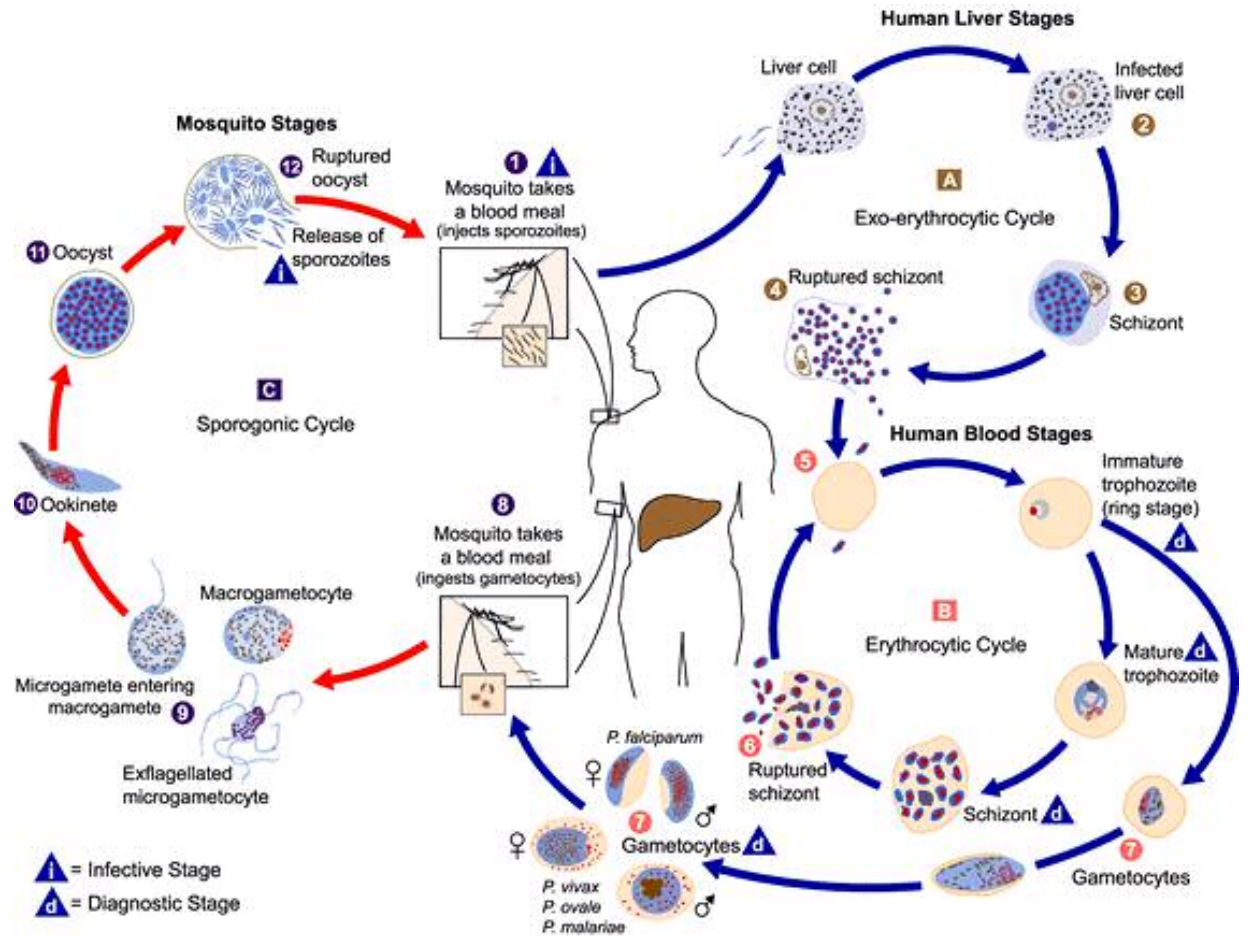
FIGURE 1: The *Plasmodium* life cycle. This complicated malaria disease cycle involves obligate human and mosquito stages.
http://www.dpd.cdc.gov/dpdx/HTML/Image_Library.htm
CDC Parasite Image Library

FIGURES
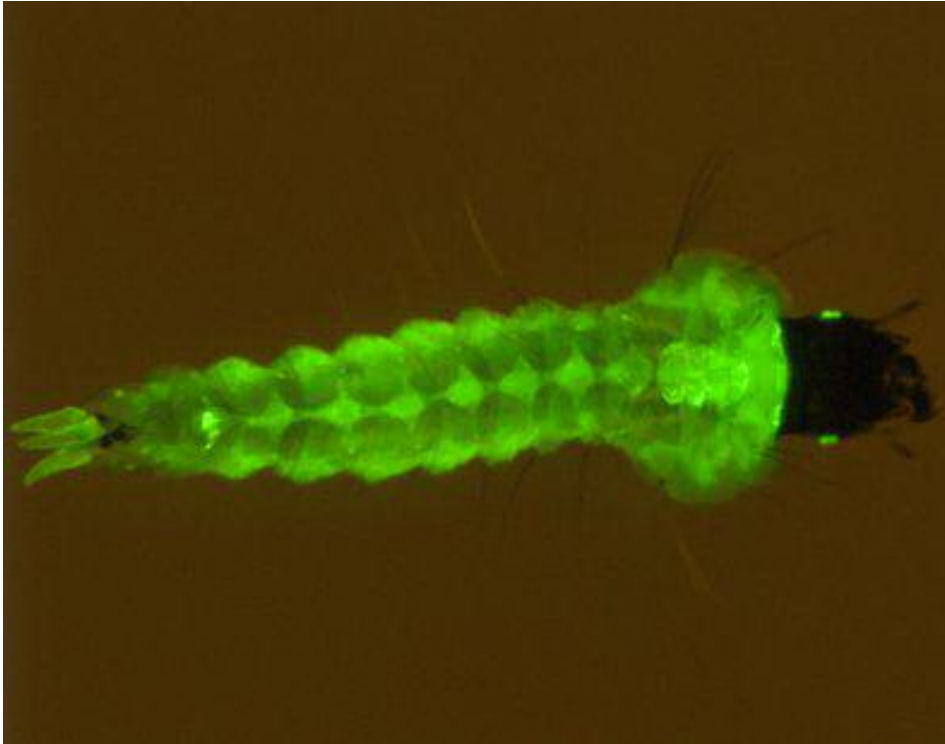
FIGURE 2: **Transgenic mosquito larvae**. This *An. stephensi* individual is expressing enhanced green fluorescent protein (EGFP), the same marker gene used for the *An. gambiae* in this study.
http://library.wur.nl/frontis/malaria/index.html
Proceedings of the Frontis workshop on ecological challenges concerning the use of genetically modified mosquitoes for disease control
Wageningen, The Netherlands 26-29 June 2002
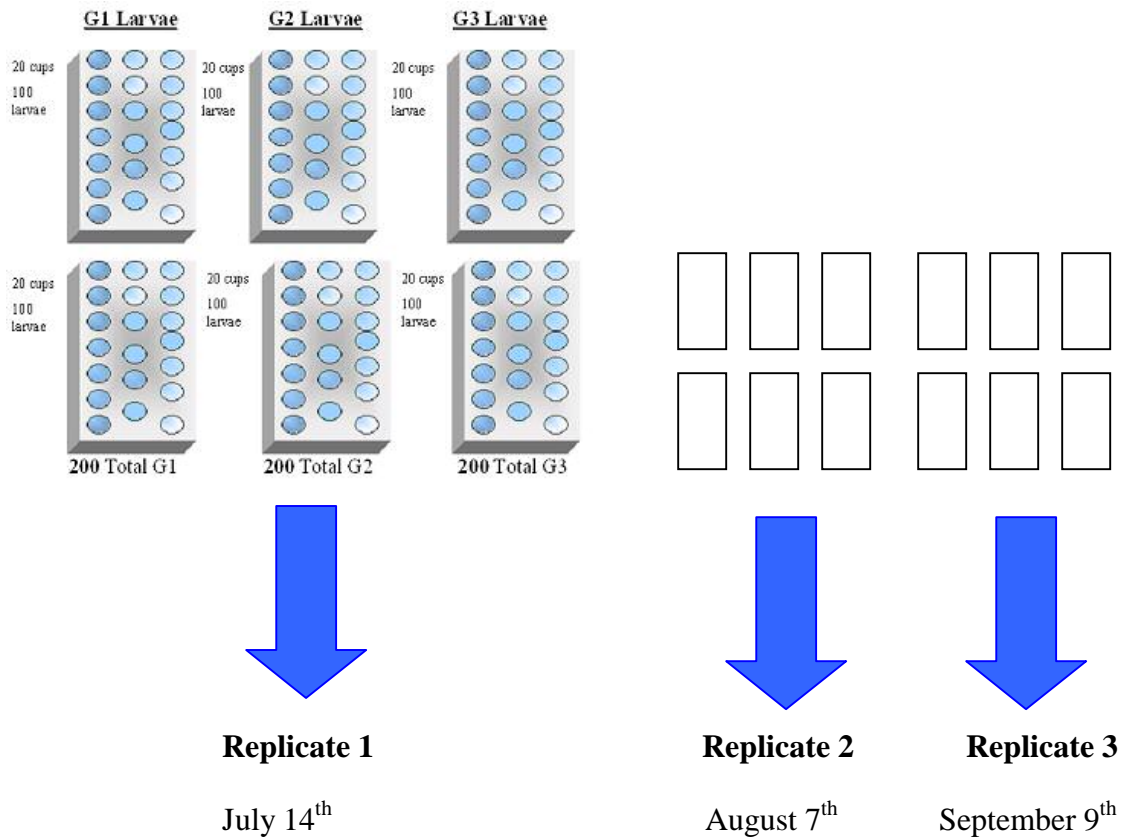
FIGURE 3: Larval tray set-up. The entire setup was repeated for three replicates.
Five first-instar larvae were placed in each cup. There were two trays for each
mosquito line. There were 20 cups/100 larvae in each tray, summing to 40 cups/200
larvae per line. Survivorship was recorded daily by determining the number of larvae
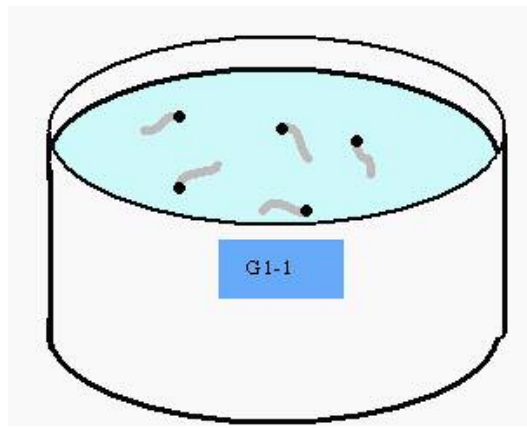in each cup. Pupae were counted, removed, and placed in an adult cage.

FIGURE 4: Larval Cup. Each cup was numbered and labeled with the appropriate line. Five first- instar larvae were placed in each cup one day after hatch. Larvae were fed every other day until all individuals had died or pupated.
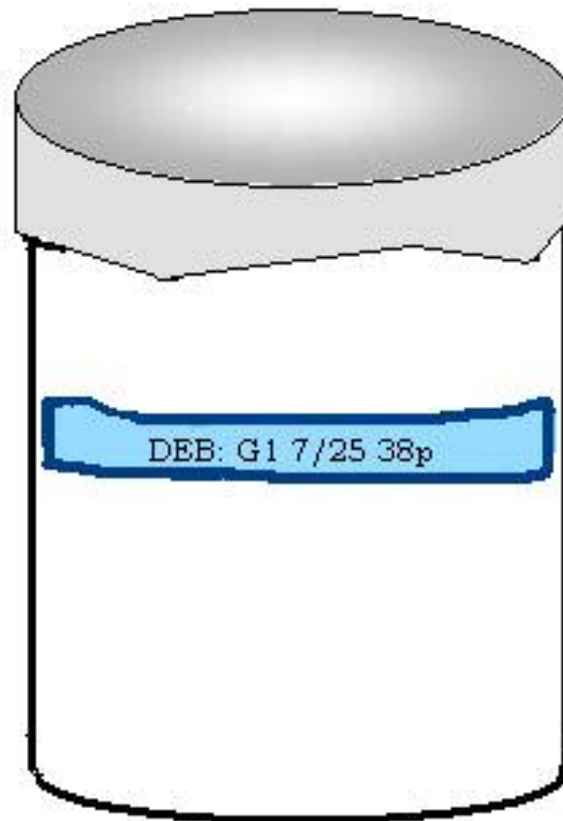

FIGURE 5: Adult cage. Adult cages were labeled with the number of pupae, date, and mosquito line. Adult survivorship was determined by counting number of individuals each day. Adults were fed every other day with a sugar pa soaked in 10% sugar solution. Certain adult cages with sufficient females were chosen for blood meals. The human hosts arm was placed over the mesh top of the cage to allow for feeding. The quantity of eggs standardized by the number females in the cage each day was recorded and used to estimate fecundity.
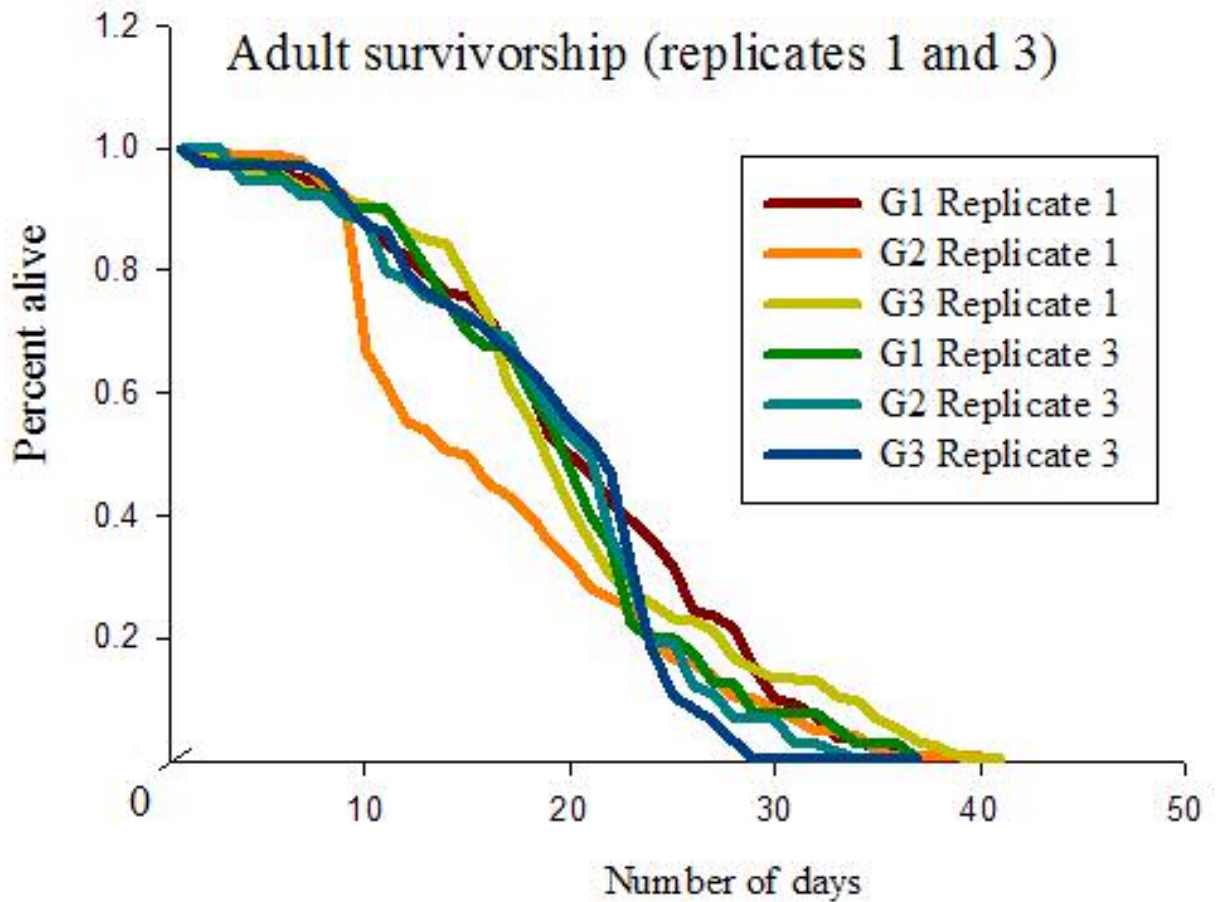
FIGURE 6: Adult survivorship curve; Percent alive vs. day (Replicates 1 and 3).
Survivorship curves display mean survivorship of all individuals from of three lines of
An. Gambiae, two transformed (G1, G2) and one untransformed control (G3) for
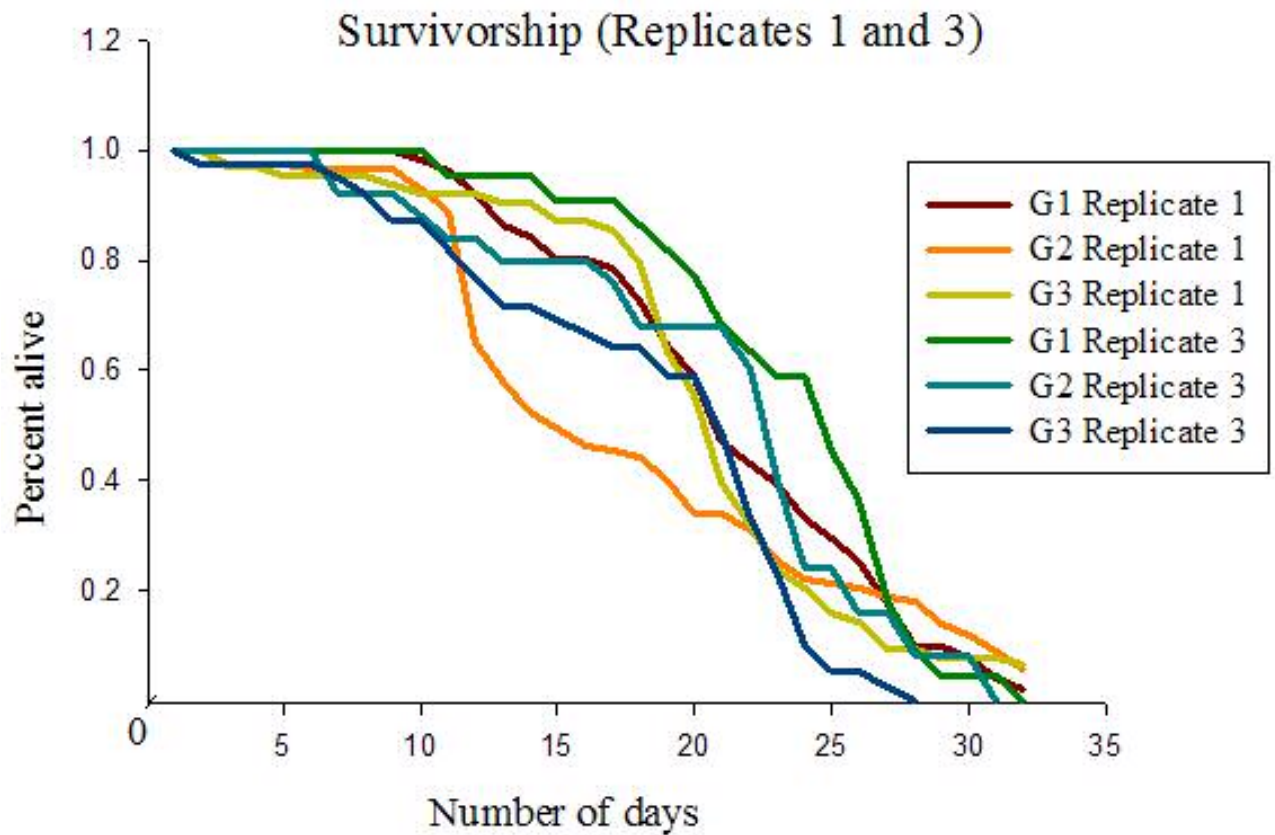replicates 1 and 3. (No bloodmeal occurred during replicate 2)

FIGURE 7: Adult survivorship curve; Bloodfed females (Replicates 1 and 3).
Survivorship curves display mean survivorship of bloodfed females from three lines of
An. Gambiae, two transformed (G1, G2) and one untransformed control (G3) for
replicates 1 and 3. (No bloodmeal occurred during replicate 2)

REFERENCES

2002. Ecological aspects for application of genetically modified mosquitoes. *In* W. Takken and T. W. Scott [eds.]. Kluwer Academic Publishers, Dordrecht.

Allen, M. L., D. A. O_Brochta, P. W. Atkinson, and C. S. Levesque. 2001. Stable, germ-line transformation of Culex quinquefasciatus (Diptera: Culicidae). Journal of Medical Entomology 38: 701-10.

Alphey, L., C. B. Beard, P. Billingsley, M. Coetzee, A. Crisanti, C. Curtis, P. Eggleston, C. Godfray, J. Hemingway, M. Jacobs_Lorena, A. A. James, F. C. Kafatos, L. G. Mukwaya, M. Paton, J. R. Powell, W. Schneider, T. W. Scott, B. Sina, R. Sinden, S. Sinkins, A. Spielman, Y. Toure, and F. H. Collins. 2002. Malaria control with genetically manipulated insect vectors. Science 298: 119-21.

Anderson, R. A., B. G. Knols, and J. C. Koella. 2000. Plasmodium falciparum sporozoites increase feeding-associated mortality of their mosquito hosts Anopheles gambiae s.l. Parasitology 120 (Pt 4): 329-33.

Aniedu, I., M. J. Mutinga, and C. M. Mutero. 1993. Vertical estimates of survivorship of larvae and pupae of *Anopheles gambiae* Giles complex in Baringo District, Kenya. Insect Science and its Application 14: 39-48.

Armbruster, P., R. A. Hutchinson, and T. Linvell. 2000. Equivalent inbreeding depression under laboratory and field conditions in a tree-hole-breeding mosquito. Proceedings of the Royal Society of London Series B- Biological Sciences 267: 1939-1945.

Atkinson, P. W., and K. Michel. 2002. What's buzzing? Mosquito genomics and transgenic mosquitoes. Genesis 32: 42-48.

Atkinson, P. W., A. C. Pinkerton, and D. A. O'Brochta. 2001. Genetic transformation systems in insects. Annual Review of Entomology 46: 317-346.

Bannister, L., and G. Mitchell. 2003. The ins, outs and roundabouts of malaria. Trends in Parasitology 19: 209-13.

Billingsley, P. F. 2002. Environmental constraints on the physiology of transgenic mosquitoes, pp. 149-161. *In* W. Takken and T. W. Scott [eds.], Ecological aspects for application of genetically modified mosquitoes. Kluwer Academic Publishers, Dordrecht.

Boete, C., and J. C. Koella. 2002. A theoretical approach to predicting the success of genetic manipulation of malaria mosquitoes in malaria control. Malaria Journal 1.

Boete, C., and J. C. Koella. 2003. Evolutionary ideas about genetically manipulated mosquitoes and malaria control. Trends in Parasitology 19: 32-8.

Breman, J. G. 2001. The ears of the hippopotamous: manifestatitions, determinations, and estimates of the malaria burden. American Journal of Tropical Medicine and Hygiene 64: 1-11.

Budiansky, S. 2002. Creatures of our own making. Science 298: 80-6.

Catteruccia, F., C. J. Godfray, and A. Crisanti. 2003. Impact of genetic manipulation on the fitness of *Anopheles stephensi* mosquitoes. Science 299: 1225-1227.

Catteruccia, F., T. Nolan, T. G. Loukeris, C. Blass, F. Savakis, F. C. Kafatos, and A. Crisanti. 2000. Stable germline transformation of the malaria mosquito, Anopheles stephensi. Nature 405: 959-962.

Charlwood, J. D., T. Smith, A. Lyimo, A. Y. Kitua, H. Masanja, M. Booth, P. L. Alonso, and M. Tanner. 1998. Incidence of Plasmodium falciparum infection in infants in relation to exposure to sporozoite-infected Anophelines. American Journal of Tropical Medicine and Hygiene 59: 243-251.

Coates, C. J., N. Jansinskiene, L. Miyashiro, and A. A. James. 1998. Mariner transposition and transformation of the yellow fever mosquito, Aedes aegypti. Proceedings of the National Academy of Sciences 95: 3748-3751.

Coates, C. J., N. Jasinskiene, G. B. Pott, and A. A. James. 1999. Promoter-directed expression of recombinant fire-fly luciferase in the salivary glands of Hermes-transformed Aedes aegypti. Gene 226: 317-25.

Cotran, R. S., T. Collins, and V. Kumar. 1998. Robbins Pathological Basis of Disease. Elsevier Science.

Cotter, S. C., L. E. B. Kruuk, and K. Wilson. 2004. Costs of resistance: genetic correlations and potential trade-offs in an insect immune system. Journal of Evolutionary Biology 17: 421-429.

Curtis, C. F. 1968. Possible use of translocations to fix desirsble genes in insect pests. Nature 218: 368-369.

Curtis, C. F. 2002. Measuring public-health outcomes of release of transgenic mosquitoes., pp. 223-234. *In* W. Takken and T. W. Scott [eds.], Ecological aspects for application of genetically modified mosquitoes. Kluwer Academic Publishers, Dordrecht.

Elliot, S. L., M. W. Sabelis, and F. R. Adler. 2002. Evolution of parasite virulence to vectors, pp. 163-171. *In* W. Takken and T. W. Scott [eds.], Ecological aspects for application of genetically modified mosquitoes. Kluwer Academic Publishers, Dordrecht.

Enserink, M. 2002. What mosquitoes want: secrets of host attraction. Science 298: 90-2.

Ferguson, H. M., and A. F. Read. 2002. Why is the effect of malaria parasites on mosquito survival still unresolved? Trends in Parasitology 18: 256-261.

Gallup, J. L., and J. D. Sachs. 2001. The economic burden of malaria. The American Journal of Tropical Medicine and Hygiene 64: 85-96.

Gilles, M. T., and T. J. Wilkes. 1965. A Study of the age-composition of poulations of Anopheles gambiae Giles and A. funestus giles in north-eastern Tanzania. Bulletin of Entomological Research 56: 237-262.

Grossman, G. L., C. S. Rafferty, J. R. Clayton, T. K. Stevens, O. Mukabayire, and M. Q. Benedict. 2001. Germline transformation of the malaria vector, Anopheles gambiae, with the piggyBac transposable element. Insect Molecular Biology 10: 597-604.

Gwadz, R. W., D. Kaslow, J.-Y. Lee, W. L. Maloy, M. Zasloff, and L. H. Miller. 1989. Effects of magainins and cecropins on the sporogonic development of malaria parasites in mosquitoes. Infection and Immunity 57: 2628-2633.

Hemingway, J., L. Field, and J. Vontas. 2002. An overview of insecticide resistance. Science 298: 96-7.

Hogg, J. C., and H. Hurd. 1995. Plasmodium yoelii nigeriensis: the effect of high and low intensity of infection upon the egg production and bloodmeal size of Anopheles stephensi during three gonotrophic cycles. Parasitology 111 (Pt 5): 555-62.

Holt, R. A., G. M. Subramanian, A. Halpern, G. G. Sutton, R. Charlab, D. R. Nusskern, P. Wincker, A. G. Clark, J. M. Ribeiro, R. Wides, S. L. Salzberg, B. Loftus, M. Yandell, W. H. Majoros, D. B. Rusch, Z. Lai, C. L. Kraft, J. F. Abril, V. Anthouard, P. Arensburger, P. W. Atkinson, H. Baden, V. de_Berardinis, D. Baldwin, V. Benes, J. Biedler, C. Blass, R. Bolanos, D. Boscus, M. Barnstead, S. Cai, A. Center, K. Chaturverdi, G. K. Christophides, M. A. Chrystal, M. Clamp, A. Cravchik, V. Curwen, A. Dana, A. Delcher, I. Dew, C. A. Evans, M. Flanigan, A. Grundschober_Freimoser, L. Friedli, Z. Gu, P. Guan, R. Guigo, M. E. Hillenmeyer, S. L. Hladun, J. R. Hogan, Y. S. Hong, J. Hoover, O. Jaillon, Z. Ke, C. Kodira, E. Kokoza, A. Koutsos, I. Letunic, A. Levitsky, Y. Liang, J. J. Lin, N. F. Lobo, J. R. Lopez, J. A. Malek, T. C. McIntosh, S. Meister, J. Miller, C. Mobarry, E. Mongin, S. D. Murphy, D. A. O_Brochta, C. Pfannkoch, R. Qi, M. A. Regier, K. Remington, H. Shao, M. V. Sharakhova, C. D. Sitter, J. Shetty, T. J. Smith, R. Strong, J. Sun, D. Thomasova, L. Q. Ton, P. Topalis, Z. Tu, M. F. Unger, B. Walenz, A. Wang, J. Wang, M. Wang, X. Wang, K. J. Woodford, J. R. Wortman, M. Wu, A. Yao, E. M. Zdobnov, H. Zhang, Q. Zhao, et al. 2002. The genome sequence of the malaria mosquito Anopheles gambiae. Science 298: 129-49.

Hurd, H. 2003. Manipulation of medically important insect vectors by their parasites. Annual Review of Entomology 48: 141-161.

Irvin, N., M. S. Hoddle, D. A. O'Brochta, and B. Carey. 2002. Assessing fitness costs for transgenic *Aedes aegypti* expressing the green fluorescent protien marker and transposase genes. Proceedings of the National Academy of Sciences 101: 891.

Jansinskiene, N., C. J. Coates, M. Q. Benedict, C. S. R. Cornel, A. A. James, and F. H. Collins. 1998. Stable transformation of the yellow fever mosquito, *Aedes aegypti*, with the Hermes elemnet from the housefky. Proceedings of the National Academy of Sciences 95: 3743-3747.

Kidwell, M. G., and J. M. C. Ribeiro. 1992. Can transposable elements be used to drive disease refractoriness genes into vector populations? Parasitology Today 8: 325-329.

Kim, W., H. Koo, A. M. Richman, D. C. J. Seeley, J. Viziolo, A. D. Klocko, and D. A. O'Brochta. 2004. Ectopic expression of a cecropin transgene in the human malaria vector *Anopheles gambiae* (Diptera: Culicidae): Effects on susceptibility to *Plasmodium*. Journal of Medical Entomology 41: 447-455.

Kiszewski, A. E., and A. Spielman. 1998. Spatially explicit model of transposon-based genetic drive mechanisms for displacing fluctuating populations of anopheline vector mosquitoes. Journal of Medical Entomology 35: 584-590.

Knoles, B. G. J., and T. W. Scott. 2002. Discussion - Ecological challenges concerning the use of genetically-modified mosquitoes for disease control: synthesis and future perspectives, pp. 235-242. *In* W. Takken and T. W. Scott

[eds.], Ecological aspects for application of genetically modified mosquitoes. Kluwer Academic Publishers, Dordrecht.

Koella, J. C. 2002. On the evolutionary ecology of mosquito immunity and the use of transgenic mosquitoes for malaria control, pp. 173-185. *In* W. Takken and T. W. Scott [eds.], Ecological aspects for application of genetically modified mosquitoes. Kluwer Academic Publishers, Dordrecht.

Koella, J. C., L. S. Flemming, and R. A. Anderson. 1998. The malaria parasite, Plasmodium falciparum, increases the frequency of multiple feeding of its mosquito vector, Anopheles gambiae. Proceedings of the Royal Society of London Series B- Biological Sciences 265: 763-768.

Kokoza, V., A. Ahmed, E. A. Wimmer, and A. S. Raikhel. 2001. Efficient transformation of the yellow fever mosquito Aedes aegypti using the piggyBac transposable element vector pBac[3xP3-EGFP afm]. Insect Biochemistry and Molecular Biology 31: 1137-43.

Krafsur, E. S., H. Townson, G. Davidson, and C. F. Curtis. 1986. Screwworm eradication is what it seems. Nature 323: 495-496.

Lanzaro, G. C., and F. Tripet. 2002. Gene flow among populations of Anopheles gambiae: a critical review, pp. 109-132. *In* W. Takken and T. W. Scott [eds.], Ecological aspects for application of genetically modified mosquitoes. Kluwer Academic Publishers, Dordrecht.

Liu, H. S., M. S. Jan, C. K. Chou, P. H. Chen, and N. J. Ke. 1999. Is green fluorescent protein toxic to the living cells? Biochemical and Biophysical Research Communications 260: 712-7.

Lobo, N. F., A. Hua-Van, X. Li, B. M. Nolen, and M. J. Fraser. 2002. Germ line transformation of the yellow fever mosquito, Aedes aegypti, mediated by transpositional insertion of a piggyBac vector. Insect Molecular Biology 11: 133-9.

Lounibos, L. P. 2002. Genetic-control trials and the ecology of Aedes aegypti at the Kenya coast, pp. 33-43. *In* W. Takken and T. W. Scott [eds.], Ecological aspects for application of genetically modified mosquitoes. Kluwer Academic Publishers, Dordrecht.

Miller, J. H., R. K. Sakai, P. Romans, R. W. Gwadz, P. Kantoff, and H. G. Coon. 1987. Stable integration and expression of a bacterial gene in the mosquito Anopheles gambiae. Science 237: 776-781.

Moreira, L. A., A. K. Ghosh, A. E. G., and M. Jacobs-Lorena. 2002. Genetic transformation of mosquitoes: a quest for malaria control. International Journal for Parasitology 32: 1599-1605.

Moreira, L. A., J. Wang, F. H. Collins, and M. Jacobs-Lorena. 2003. Fitness of anopheline mosquitoes expressing transgenes that inhibit Plasmodium development. Genetics 166: 1337-1341.

Nolan, T., T. M. Bower, A. E. Brown, A. Crisanti, and F. Catteruccia. 2002. piggyBac-mediated Germline Transformation of the Malaria Mosquito Anopheles stephensi Using the Red Fluorescent Protein dsRED as a Selectable Marker. Journal of Biological Chemistry 277: 8759-8762.

O'Brochta, D. A. 2002. Transgenic mosquitoes: the state of the art, pp. 15-24. *In* W. Takken and T. W. Scott [eds.], Ecological aspects for application of genetically modified mosquitoes. Kluwer Academic Publishers, Dordrecht.

O'Brochta, D. A. 2004. Description of drive mechanisms, the ectopic cecropin and EGFP transgenes. *In* D. T. Ladner [ed.], College Park, Maryland.

Perera, O. P., R. A. Harrell, and A. M. Handler. 2002. Germ-line transformation of the South American malaria vector, Anopheles albimanus, with a piggyBac/EGFP transposon vector is routine and highly efficient. Insect Molecular Biology 11: 291-7.

Pinkerton, A. C., K. Michel, D. A. O'Brochta, and P. W. Atkinson. 2000. Green flouresent protien as a marker in transgenic Aedes aegypti. Insect Molecular Biology 9: 1-10.

Reisen, W. K. 2002. Lessons from the past: an overview of studies by the University of Maryland and the University of California, Berkeley, pp. 25-32. *In* W. Takken and T. W. Scott [eds.], Ecological aspects for application of genetically modified mosquitoes. Kluwer Academic Publishers, Dordrecht.

Ribeiro, J., and M. G. Kidwell. 1994. Transpoable elements as population drive mechanisms: specification of critical parameter values. Journal of Medical Entomology 31: 10-16.

Robert, V., J. P. Verhave, and P. Carnevale. 1990. Plasmodium falciparum infection does not increase the precocious mortality rate of Anopheles gambiae. Transactions of the Royal Society of Tropical Medicine and Hygiene 84: 346-7.

Roberts, L. 2002. Mosquitoes and disease. Science 298: 82-3.

Sachs, J. D. 2002. A new global effort to control malaria. Science 298: 122-4.

Schluter, D., T. D. Price, and L. Rowe. 1991. Conflicting selection pressures and life history trade-offs. Proceedings of the Royal Society of London Series B-Biological Sciences 246: 11-17.

Schwartz, A., and J. C. Koella. 2001. Trade-offs, conflicts of interest and manipulation in Plasmodium- mosquito interactions. Trends in Parasitology 17: 189-194.

Scott, T. W., and A. C. Morrison. 2002. Aedes aegypti density and the risk of dengue-virus transmission, pp. 187-206. *In* W. Takken and T. W. Scott [eds.], Ecological aspects for application of genetically modified mosquitoes. Kluwer Academic Publishers, Dordrecht.

Scott, T. W., W. Takken, B. G. Knols, and C. Boete. 2002. The ecology of genetically modified mosquitoes. Science 298: 117-9.

Sidhu, A. B., D. Verdier_Pinard, and D. A. Fidock. 2002. Chloroquine resistance in Plasmodium falciparum malaria parasites conferred by pfcrt mutations. Science 298: 210-3.

Smith, T. A., L. Regula, and C. Lengeler. 2001. Child mortality and malaria transmission intensity in Africa. Trends in Parasitology 17: 145-149.

Spielman, A. 2002. Release ratios employed for genetically modifying populations of mosquitoes, pp. 209-215. *In* W. Takken and T. W. Scott [eds.], Ecological aspects for application of genetically modified mosquitoes. Kluwer Academic Publishers, Dordrecht.

Taylor, C. E., and N. C. Manoukis. 2002. Effective population size in relation to genetic modification of Anopheles gambiae sensu stricto, pp. 133-146. *In* W. Takken and T. W. Scott [eds.], Ecological aspects for application of genetically modified mosquitoes. Kluwer Academic Publishers, Dordrecht.

Taylor, C. E., Y. T. Touré, J. Carnahan, G. Dolo, S. F. Traoré, F. E. Edillo, and G. C. Lanzaro. 2001. Gene flow amoung populations of the malaria vector, *Anopheles gambiae*, in Mali, West Africa. Genetics 157: 743-750.

Tiedje, J. M., R. K. Colwell, Y. L. Grossman, R. E. Hodson, R. E. Lenski, R. N. Mack, and P. J. Regal. 1989. The planned introduction of genetically engineered organisms: ecological considerations and recomendations. Ecology 70: 298-315.

Touré, Y. T., A. M. J. Oduola, J. Sommerfeld, and C. M. Morel. 2002. Biosafety and risk assessment in the use of genetically modified mosquitoes for disease control, pp. 217-222. *In* W. Takken and T. W. Scott [eds.], Ecological aspects for application of genetically modified mosquitoes. Kluwer Academic Publishers, Dordrecht.

Vizioli, J., P. Bulet, M. Charlet, C. Lowenberger, C. Blass, H. M. Muller, G. Dimopoulos, J. Hoffman, F. C. Kafatos, and A. M. Richman. 2000. Cloning and analysis of a cecropin gene from the malaria vector mosquito, Anopheles gambiae. Insect Molecular Biology 9: 75-84.

Vogel, G. 2002. An elegant but imperfect tool. Science 298: 94-5.

Wellems, T. E. 2002. Plasmodium chloroquine resistance and the search for a replacement antimalarial drug. Science 298: 124-6.

Woodruff, R. C. 1992. Transposable DNA elements and life history traits. I. Transposition of P DNA elements in somatic cells reduces the lifespan of Drosophila melanogaster. Genetica 86: 143-54.

Yan, G., D. W. Severson, and B. M. Christensen. 1997. Costs and benifits of mosquito refractoriness to malaria parasites: implications for genetic variablity of mosquitoes and genetic control of malaria. Evolution 51: 441-450.