DISSERTATION

TOOLS AND TECHNIQUES FOR THE STUDY AND EVALUATION OF MALARIA CONTROL MEASURES IN WEST AFRICA

Submitted by

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ABSTRACT

TOOLS AND TECHNIQUES FOR THE STUDY AND EVALUATION OF MALARIA CONTROL MEASURES IN WEST AFRICA

Malarial disease caused by *Plasmodium* genus parasites remains as one of the most pressing public health matters of our time. Malaria still kills roughly 500,000 people a year, predominately children under 5 years of age, mostly in sub-Saharan Africa [1]. However, this number represents a stark decrease from the 1,000,000+ deaths per year in the early 2000s. This reduction has been achieved through increased use of insecticide treated bednets (ITNs), spraying of insecticides (indoor residual spraying – IRS), and treatment of infections with artemisinin combinatorial therapies (ACTs). Many of these distribution programs have been undertaken by local governments, allowing for greater coverage in areas that may have otherwise been missed through other campaigns. This work has led to a 2.56 fold increase of people living in "elimination or pre-elimination settings" worldwide [2]. However, as more populations and countries are in pre-elimination stages, it has been found that complete eradication of the parasites is difficult. Mosquito behavioral modifications [3], increasing insecticide resistance [4], and low parasite detection limits [5] have all emerged as challenges in moving from pre-elimination to elimination. This dissertation addresses elements of these problems, and how the mass drug administration of ivermectin in West Africa may help to alieve or address gaps in current malaria control.

To develop outdoor-biting mosquito collection methods that minimize sampling bias and risk to collectors, Chapters 2 and 3 describe the design and testing of an active, no-exposure,

human-baited tent trapping system (Infoscitex Tent – IST). We compare capture efficiencies of this new method to that of the "gold-standard" approaches of Human Landing Catch (HLC) and a CDC Light Trap placed next to a bed net (LTC). In the laboratory, we found the IST could catch both *Aedes* and *Anopheles* spp. mosquitoes, though at a rate lower than that of HLC. Field comparisons of the IST to LTC in Southwestern Senegal showed that the IST was more efficient that LTC in the collection of wild *Anopheles gambiae* s.l. and *Culex* group V mosquitoes. Comparisons of the IST to HLC in Liberia and two villages in Burkina Faso showed that the IST caught mosquitoes at a decreased rate, though this difference was only statistically significant in 2 of 3 locations. Importantly, risk of mosquito exposure measured via the proportion of mosquitoes caught that had appreciable bloodmeals in their abdomens was significantly lower with the IST than HLC in all locations. These bloodmeals most likely came from bites acquired during the HLC process, which represents a risk as probing alone can be sufficient for pathogen transmission [6]. Finally, we performed parity dissections on mosquitoes captured via the IST during an ivermectin mass drug administration (MDA) and found that the parity levels in these mosquitoes were reduced by 25.65%, indicating a shift to a younger population structure post MDA

Chapter 4 of this dissertation has been to further the development of Near Infrared Spectroscopy (NIRS) for age-grading of wild *Anopheles* spp. to better understand how control measures may impact the age structure of wild mosquitoes. After initial laboratory validation of the approach, we collected and reared *An. gambiae* s.l. mosquitoes from natural larval habitat in Burkina Faso; scanned age-matched, anesthetized adult mosquitoes; developed Partial Least Squares based calibration models; and determined accuracy of these models against both validation and independent test sets. We generated four calibration models from two laboratory

strains of *An. gambiae*, and two collections of wild larvae from two rainy seasons in Burkina Faso. Validation set accuracy when discriminating young (< 7.5 day old) from old (> 7.5) mosquitoes was higher in colony strains, likely because genetic variation in these strains was lower. Overall prediction accuracies of laboratory reared *An. gambiae* G3; recently colonized "CSU-IRSS" strain *An. gambiae* s.s.; wild-reared larvae from Soumousso, Burkina Faso 2013; and Kodeni, Burkina Faso 2014 were 82.5%, 77.5%, 74.4%, and 67.0%, respectively. Prediction of an independent test set of varying age field mosquitoes was poor due to over prediction of young mosquitoes. However, models generated from multiple sources (larvae from four locations or five locations plus CSU-IRSS) were able to correctly predict nulliparous (young) mosquitoes as being younger than both parous (middle to old age) and sporozoite positive (old) mosquitoes by 1.7 and 3.8 days, respectively. The best of these models was then tested on mosquitoes collecting during an ivermectin MDA, and we found a 20.8% drop in the mean age of mosquitoes in the treatment village in the first week post MDA. While this result is preliminary due to the error found with other test sets, it does mimic the 25.7% drop in parity previously reported for ivermectin MDA [7].

Finally, Chapter 5 of this dissertation is the development of several tools for better molecular detection and epidemiological study of the "human-to-mosquito" transmission of *Plasmodium falciparum* parasites. This was done through detection of *Plasmodium* RNA from the blood meals of recently fed *Anopheles* mosquitoes. We were able to reliably detect transcripts up to 48 hours post ingestion, and down to 10-100 gametocytes/mL with the established quantitative nucleic acid sequence based amplification (QT-NASBA), and a newly designed multiplex reverse transcriptase polymerase chain reaction (RT-PCR). Using a ratio of transcripts from genes currently unused in detection methodology, we found we could determine

the parasite stage in the mosquito, the composition of parasites in the bitten human, and whether the bloodmeal infected the mosquito, all from a non-invasive sample. We demonstrated this approach on a subset of wild-caught *Anopheles* from Liberia, and found using mosquitoes as epidemiological sampling tools is highly amenable to study malaria in a natural state. We avoid biases present in other strategies that sample the human population directly as mosquitoes bite with heterogeneity across the population. We also do not have to hold mosquitoes artificially waiting for parasite development, and we avoid the use of laboratory mosquitoes or parasites. This technique should help to illuminate which individuals or classes of individuals are contributing most to the maintenance of the disease cycle, and how interventions could best target these populations.

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Chapter 1: Literature Review

Malarial disease caused by five species of *Plasmodium* genus parasites represents one of the most pressing public health issues of our time. In 2015, there were an estimated 214 million cases and 438,000 deaths due to malaria, with 88% of the cases and 90% of the deaths occurring in sub-Saharan Africa [1]. The vast majority of deaths are among children 0-5 years of age, and these deaths are predominantly caused by *P. falciparum* malaria parasites [1]. It is estimated that there were 65,493,100 disability adjusted life years (DALYs) lost due to malaria in 2013 [8]. These are the number of years lost across the world's population due to illness, disability, or early death, and malaria represents by far the biggest burden of the neglected tropical diseases [8]. It has been estimated previously that economic growth is reduced by 1.3% in those countries with the highest malaria burdens, and that $12 billion USD are lost each year from Africa's gross domestic product [9,10]. However, funding towards malaria control has increased greatly over the last decade, from $960 million in 2005 to $2.5 billion in 2014 [1], and intervention coverage has reached unprecedented levels across sub-Saharan Africa [2]. This literature review will detail the past and current state of control programs, where gaps exist in these endeavors, and how work in this dissertation has tried to solve some aspects of these problems.

Lifecycle of *Plasmodium falciparum*:

Plasmodium falciparum parasites that cause malarial disease have a diverse lifecycle spanning both the arthropod vector, and the human target. The development of the parasite in the vector is dependent on a range of factors. Through this description of the parasite's lifecycle in humans and mosquitoes, genes of interest and time to development will be listed due to their implications in this work. The first event of the *Plasmodium* lifecycle discussed here is the transmission of the parasite from the *Anopheles* mosquito to the human. The human-infectious

stage of the parasite is the sporozoite. This highly motile form of the parasite is injected from the salivary gland of the mosquito when it cannulates a subcutaneous blood vessel in the human's dermis during a bite [11,12]. Once in the bloodstream, the sporozoite travels to the liver through the hepatic arteriole or the portal venule where it infects a hepatocyte [11]. For ~7 days, the parasite replicates and produces tens of thousands of merozoites, which re-invade the blood system, infecting red blood cells (RBCs) and beginning the asexual, disease-causing 48 hour cycle [13]. During this cycle, erythrocytic schizonts form in red blood cells, the cells rupture, and release more merozoites into the blood stream to infect new red blood cells. Large amounts of heme are produced through the bi-products of this infection and degradation of hemoglobin in the blood by the parasite. This heme is crystallized into hemozoin, the "malaria pigment," by the parasite to deal with the toxicity caused by free radicals [14,15]. Histidine-Rich Protein II, which is used in parasite detection with rapid diagnostic tests (RDTs), has been shown to be important to this crystallization mechanism [16]. A small number of the merozoites differentiate into gametocytes, the mosquito-infectious stage, in the bone marrow of the infected human, reaching maturation in 10-12 days [17]. These gametocytes circulate for 3.4-6.5 days [13,17,18], during which they can be ingested by a mosquito during a bloodmeal to continue the cycle. In less than a minute upon ingestion by a mosquito, gametocytes undergo gametogenesis into micro- (male) and macrogametes (female), and the microgamete exflagellates from its erythrocyte (~10 minutes) in response to temperature, pH, and xanthanuric acid in the gut of the mosquito [19–23]. The exflagellated microgamete fuses with a macrogamete to produce a diploid zygote, which undergoes meiosis to form a tetraploid ookinete in the midgut (12-36 hours) [24]. The major role of the ookinete is to cross the chitin-rich peritrophic matrix that surrounds the bloodmeal (mediated by the gene chitinase) [25], traverse the midgut epithelium (assisted by

circumsporozoite and TRAP-related protein (CTRP)) [26], and survive the midgut immune response until development to the oocyst stage (using a range of antioxidant genes including Thioredoxin-like protein 1 (TrxL1)) [27–30]. Once the ookinete traverses the midgut they become lodged in the extracellular space between the epithelium and basal lamina, and develop into oocysts over ~9 days [21]. This oocyst undergoes many rounds of sporogony, producing thousands of sporozoites over this period [24]. After maturation, the oocyst ruptures, releasing the motile sporozoites into the hemocoel, some of which enter the distal lateral and median lobes of the salivary glands to be transmitted to a new host in the next bite [21]. Sporozoite development is contingent on circumsporozoite protein (CSP), a major surface protein which is often detected to determine if a mosquito is infectious [31]. The development time from gametocyte ingestion to the sporozoite presence in the salivary gland is referred to as the extrinsic incubation period (EIP), and is a critical metric in understanding the proportion of the mosquito population that is infectious (see Age grading section below).

A Brief History of Malarial Disease and Control:

Plasmodium spp. parasites vectored by anopheline mosquitoes have likely co-evolved with primates, and followed human migrations around the world [32]. The most lethal malarial species, *Plasmodium falciparum,* is thought to have evolved from a common ancestor with the parasites that cause avian malaria, at some point undergoing a host switch from birds to humans [33,34]. Apparent *Plasmodium*-like parasites have been found in the midgut of a *Culex*-like mosquito in amber from the tertiary period (14-45 million years ago) [35]. The parasite and the vectors that spread it have demonstrated their resilience, plasticity in host choice and feeding preferences, and adaptability to changing environments countless times. This variation and

adaptability of both the parasite and the vector are clear from the history of the disease over the last ~70 years.

In the 1950s the World Health Organization (WHO) undertook a Global Malaria Eradication Campaign (GMEC) to eradicate the disease outside of sub-Saharan Africa [36]. A major portion of this campaign was the spraying of dichloro-diphenyl-trichloroethane (DDT), which is known for its persistence and long-lasting mosquitocidal effect through agonism of neuronal sodium channels [37,38]. Predominantly, this insecticide was sprayed on the inside walls of houses, a technique known as insecticide residual spraying (IRS). IRS has a biphasic benefit as an approach in that it can 1) kill mosquitoes that rest on walls after entering the home or after blood feeding, and 2) limit mosquito entrance into the home due to repellency effects. DDT's high efficiency against arthropods as a contact poison was first discovered by Paul Herman Müller in 1939, for which he was awarded the Nobel Prize in Physiology and Medicine in 1948 [39,40]. During World War II, DDT was used extensively to control typhus and limit mosquito-borne disease in the South Pacific [41], and its use was maintained at high levels prior to the GMEC. Due to this use prior to the campaign, it was well known that there was danger posed by the development of resistance in anophelines that was posed to limit efficacy of the program [36]. However, the program went forward, with the primary goal to eradicate malaria in Europe, the Eastern Mediterranean, South-east Asia, and the Western Pacific [36]. No plan was made for sub-Saharan Africa due to infrastructure limitations, and no successful demonstration of areas cleared of malaria by residual spraying [36]. The campaign went through 1969, and was largely a success, though with flaws [42]. Of the 143 countries where malaria was endemic in the 1950s, 37 had successfully eradicated the disease by 1978, in large part due to the GMEC program [42,43]. Additionally, in some countries where malaria was not eradicated, the disease

burden was limited significantly. In India, the estimated number of cases in 1968 was reduced to less than one million, down from 110 million in 1955 [44]. The overall drop in mortality following GMEC is shown in Figure 1.1 below.

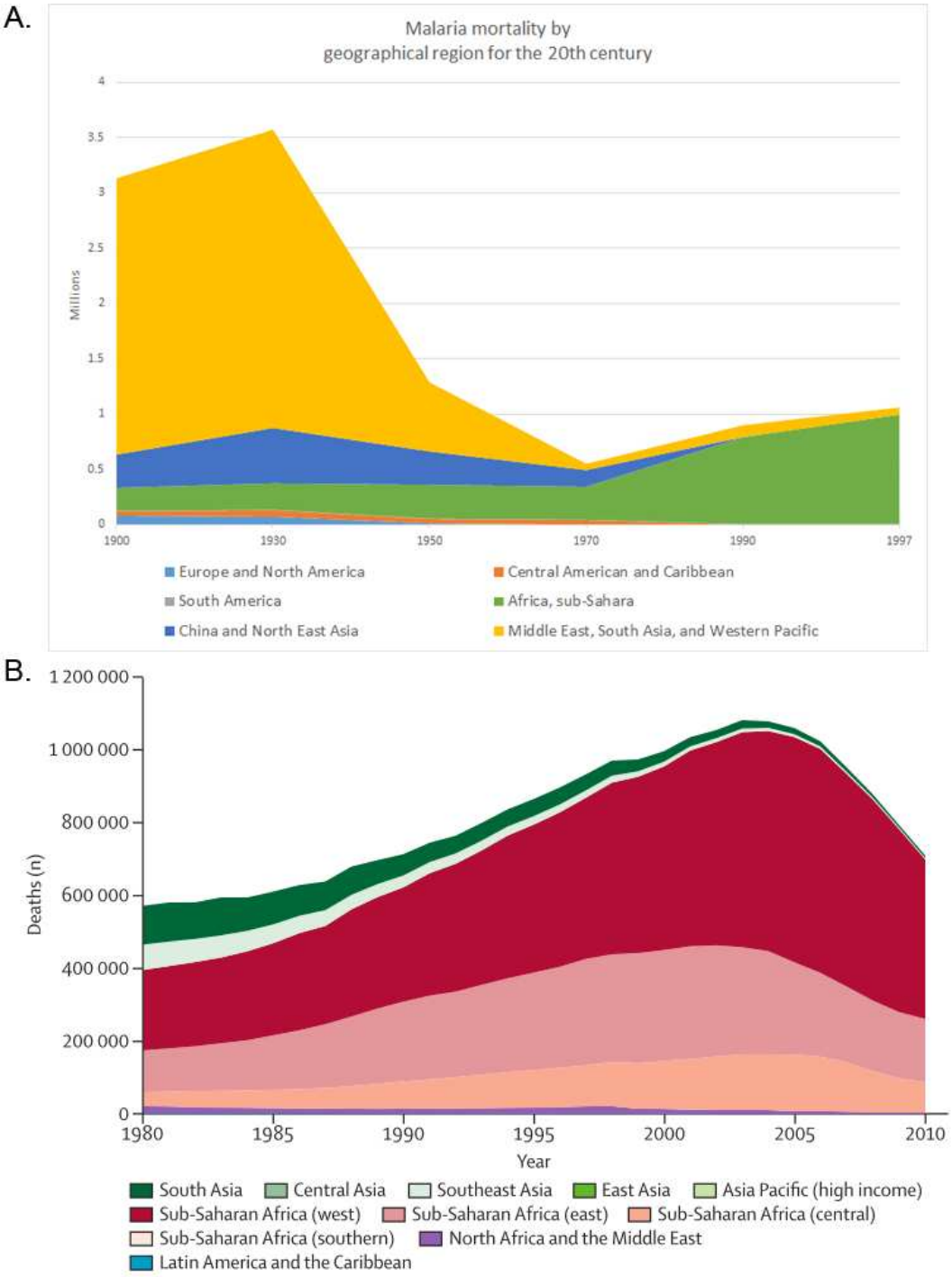

Figure 1.1: (A) Global malaria burden in the 1900s, adapted from Carter and Mendis, 2002 [44], (B) Malaria mortality from 1980-2010, modified from Murray *et al.* 2012 [45]

However, after this early success, issues with increasing resistance and failure to maintain high levels of control led to a cessation of the program in 1969 [46]. After this discontinuation, funding decreased as agencies like UNICEF withdrew support in favor of general health programs [37]. This caused malaria prevention programs under decreased budgets to shift from the problem-solving nature of the earlier approaches, and move to a "fire-fighting" strategy of malaria control, trying to target areas of greatest need [37]. However, this approach led to most of the control being concentrated to areas with past success as they had the necessary infrastructure [37]. In absence of concerted control efforts, malaria re-emerged through the 1980s into the 2000s (Fig. 1B) [45]. Coupled with reductions in spending, the emergence of parasites resistant to the frontline treatments of Chloroquine and Sulphadoxine-pyrimethamine in sub-Saharan Africa [47,48], the HIV/AIDS epidemic causing co-morbidity/mortality [49], and a high degree of emerging pyrethroid insecticide resistance [4,50], deaths reached their highest levels in over 50 years [45]. Much of this re-emergence occurred in sub-Saharan Africa, where control measures are limited, and the health infrastructure is least equipped to deal with disease burden [51].

In the post-DDT/WHO mass eradication era, malarial control has been more integrative, looking to utilize a variety of control tools for success. The predominant technique that has contributed most greatly to control measures since its initial randomized control trials in the late 1980s to mid-1990s is the insecticide impregnated bed net (ITN) [52–55]. This is a mesh netting that is hung over people as they sleep, preventing biting through physical separation, and causing an excito-repellency effect on mosquitoes due to the insecticide in the netting (usually a pyrethroid like permethrin) [56]. It has been shown that the use of ITNs reduces populations of vectors resting indoors [56,57], and a meta-analysis has shown that nets reduce the "incidence of

uncomplicated malaria episodes in areas of stable transmission by 50% compared to no nets, and 39% compared to untreated nets" [58]. Additionally, the use of IRS with pyrethroids has been evaluated recently both individually and in concert with ITN usage. In areas of stable transmission in Tanzania (entomological inoculation rate > 1 infectious bite per day), IRS was found to reduce re-infection with malaria parasites of children 0-5, with a protective efficacy ($(PE = (1 - risk\ ratio) * 100)$ of 54% [59]. In a study in Mozambique, the malaria prevalence was reduced from 60-65% to 4-8% after 7 years of IRS with a PE of 74% [59,60]. In a study in Tanzania with both IRS and ITN together, Okumu *et al.* found that all IRS/ITN combinations they investigated (with various net types) had increased vector mortality over IRS alone [61].

With the success of IRS/ITN programs, there has been a large push to increase coverage of these interventions, especially in children under 5 years of age. Through a number of governmental and non-governmental anti-malaria programs like the Roll Back Malaria Initiative, President's Malaria Initiative, and the greater United Nations Millennium Development Goals, the percentage of <5 year-old children who sleep under an ITN has increased from <2% in 2000 to ~68% in 2015 [1,2,62,63]. Additionally, the global funding towards malaria control has increased from 51 million annually in 2003 to 2.5 billion annually in 2015 [1,62]. These interventions have decreased the DALYs lost from malaria by 31.3% from 2005 to 2013 [8], a 48% reduction in the number of deaths from 2000-2015 [1], and a 255.93% increase in the number of individuals living in a pre-elimination or elimination setting (*P. falciparum* parasitemia rate in children 2-10 < 1%) [2,64]. In the midst of this success, the goals of the WHO's ambitious 1950s agenda seem attainable, but critical issues remain to be solved.

Challenges to elimination:

As the world attempts to move to greater control and possible eradication of malarial disease, there are several unique challenges that must be addressed. Some of these issues are: the behavioral modification of mosquito vectors in response to ITNs, insecticide resistance and the need to better understand how insecticides impact vector populations, and the detection of low level parasitemias that maintain the disease cycle. In this section, I will describe each of these issues in detail, what gaps remain in our understanding, and finally how the work of my dissertation tries to address these gaps.

The first issue is behavioral modification of *Anopheles* species in response to ITNs, insecticide residual spraying (IRS), and human behavior. The principle behavioral modification that has been seen with *Anopheles* vectors in response to wide-spread net usage is the change from predominantly indoor, middle-of-the-night biting to outdoor biting in the evening and morning. This behavior has been reported with *An. funestus* in Tanzania [3], western Kenya [65,66], southern coastal Kenya [67], and Senegal [68]. In Papua New Guinea, the biting of *An. farauti* s.s. and *An. punctalatus* also shifted slightly earlier in the night [69], and again in western Kenya *An. arabiensis* also had this phenotype [66]. For *An. gambiae,* these behavioral modifications are seemingly more sporadic. Mathenge *et al.* found this shift with *An. gambiae*, but not *An. funestus* [70], Mbogo found a shift in *An. gambiae* s.l. in coastal Kenya [71], and Russell *et al.* found it with *An. funestus*, but not *An. gambiae* [3].

This phenotype is not a new one, and had been seen previously with IRS campaigns involving the spraying of DDT and other insecticides. The Garki project, a large-scale control project which sprayed large amounts of propoxur, a carbamate insecticide, successfully reduced vectorial capacity by 90%, but only reduced prevalence of the parasite by 25% [72,73]. It was

suggested that residual, outdoor biting was maintaining the transmission cycle even during this time of intense spraying, and ultimately the project failed to achieve local elimination. After 40 years of the use of DDT as IRS in northern Ethiopia, *An. arabiensis* biting was mostly in the evenings from 18:00 to 22:00, with sporadic night-time host-seeking [74]. Some data from Sudan may suggest an increase in *An. arabiensis* biting in the mornings after DDT IRS, peaking at 6 A.M. [75], and a study of *Anopheles farauti* in the Solomon Islands in 1979 showed a clear relationship in both indoor and outdoor biting times after DDT IRS, with both shifting to evening biting, and a loss of the night-time indoor peak [72,76]. Similar behavioral modification may explain the finding of higher numbers of *An. arabiensis* in houses with televisions in Yemen [77]. They postulate this was due to the tendency of these houses to have evening congregations of people who sit with windows and doors open though this was only based on collections from 18 houses with televisions and 41 without ($P < 0.001$) [77].

The other change seen in response to ITNs/IRS has been a replacement of the dominant vector species of the area. The classic example of this is the replacement of *An. gambiae* s.s with *An. arabiensis,* seen with ITN introduction in western/southern Kenya and Tanzania [3,67,78]. This is due to both *An. gambiae* being predominantly endophilic/endophagic (indoor resting/biting) and very anthropophilic (only bites humans), whereas *An. arabiensis* is exophilic/exophagic and bites more catholically on humans and cattle [79]. Other examples of dominant species replacement have been fostered by IRS use in South Africa, Kenya and Tanzania, with *An. funestus* being replaced by *An. rivulorum/parensis* [80,81]; and with IRS in Guiana with zoophilic *An. aquasalis, An. albitarsis,* and *An. triannulatus* replacing the human-biting *An. darlingi* [82]. The net effect of these behavioral/biting preference changes is that the best control measures available, ITNs/IRS, are limited in their ability to control malaria. Control

measures must account for this outdoor, catholically biting population, or risk the maintenance of the transmission cycle, and the driving of resistance alleles in vectors. Methods to investigate these exophagic populations are discussed in the "Collection of exophilic/exophagic vectors" section below, and a novel approach towards their collection is discussed in chapters 1 and 2 of this dissertation.

The spreading of insecticide resistance through *Anopheles* species is another major challenge to the control of malarial disease. This is an issue that has been reviewed many times in the literature, and will be touched on briefly here [4]. Since the World Health Organization's GMEC project in the 1950s with DDT, we have understood the cost of insecticide resistance towards mosquito control. This campaign failed in its goals because the organizers did not account for the data showing that resistance was widespread, and could not be overcome by volume [37]. We face much the same issue today when it comes to resistance to pyrethroids like permethrin and deltramethrin [4]. Through the massive scale-up in malaria control measures with long-lasting insecticide treated nets and insecticide residual spraying, reviewed above, the malaria control community is largely pursuing the same path as that of the failed WHO campaign. There is significant evidence across sub-Saharan Africa that resistance alleles such as *kdr-w* (knockdown resistance-west due to leucine to phenylalanine substitution at position 1014 in the sodium gated chloride channel, target of DDT/pyrethroids) [83,84], *kdr-e* (east: leucine to serine at position 1014) [85], mutations to acetylcholinesterase (*ace-1*, the target of organophosphate/carbamate insecticides) [86], and the presence of specific cytochrome P450 enzymes (capable of metabolizing both insecticide classes) [87], have led to mosquito populations largely immune to frontline insecticides. Novel compounds that can affect these highly resistant populations are urgently needed, as are compounds or strategies that are resilient

against current resistance mechanisms. One such strategy is the development of "evolution-proof insecticides" [88]. This idea is that if a class of insecticides could preferentially kill only the oldest mosquitoes that are transmitting disease (due to the delay from the extrinsic incubation period), then there would be limited selective pressure to develop resistance against the insecticide as most mosquitoes would have already reproduced [88]. This would suppress the population to be predominantly young, and thus less capable to disease transmission. This differential effectiveness has been seen to some degree with other insecticides, such as DDT, that has 5% mortality against newly-emerged, resistant *An. gambiae*, but 90% mortality in those 12-14 days of age [89]. However, there currently exists a deficit in the ability to test a possible insecticide or control strategy to see its effects on the age structure of mosquito populations. Thus, new tools and techniques for age-classification are needed. The current methodologies for age-grading, and the new methodology pursued in this dissertation are outlined in the "Age grading of *Culicidae*" section below, and in Chapter 4.

Finally, the third major issue in the current control methodology is the presence of sub-microscopic *Plasmodium* infections maintaining the transmission cycle. Most campaigns that have attempted to control or eradicate malaria in modern times have utilized mass screen and treat (MSAT) methodology which is the screening of entire populations for parasitemias with microscopy or RDTs, and then treating them with ACTs [5]. However, as is shown in Figure 1.2A below, the detection limits of RDT (200 parasites/µl) and microscopy (20 parasites/µl) miss large swaths of the infectious reservoir, meaning they would not be treated under MSAT guidelines.

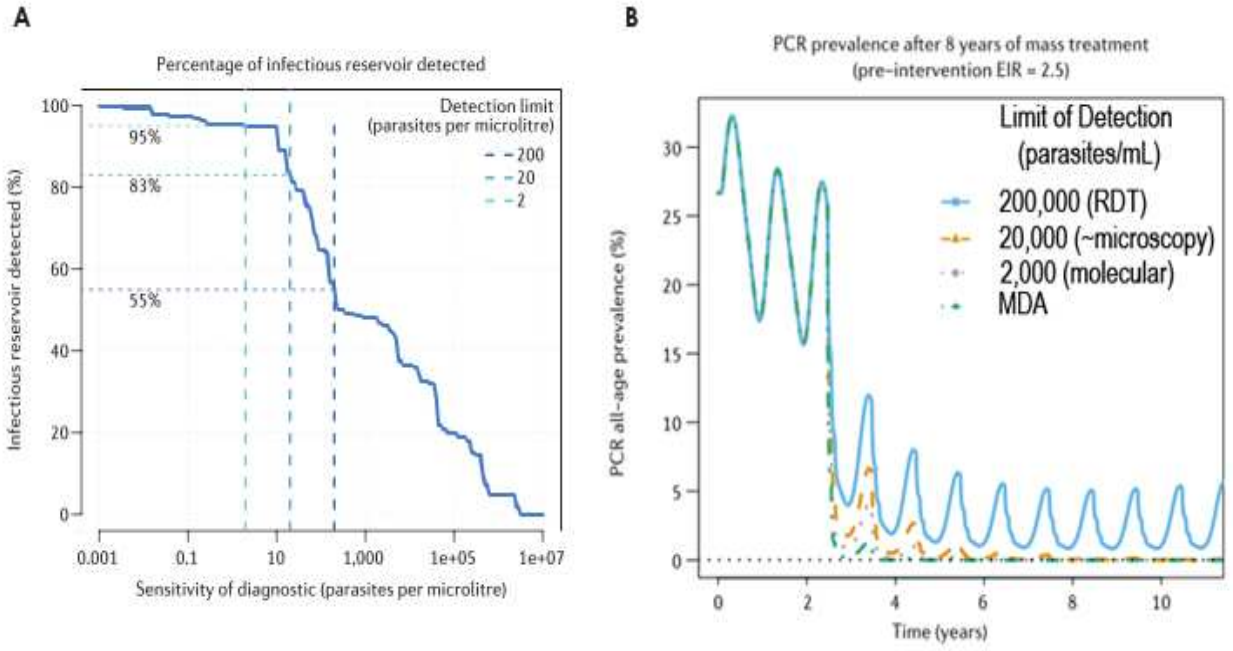

Figure 1.2: (A) Percentage of infectious reservoir detected at differing limits of detection (RDT, Microscopy, Molecular), and **(B)** effectiveness of mass screen and treatment programs at these limits. Modified from Slater *et al.* [5]

The net effect of these missed infections is that by failing to control parasites in 17-45% of the infectious population, intervention may lack the efficacy to control disease (Figure 1.2B) [5]. Failures in MSAT programs have been documented in Kenya with a two-year trial studying school-age cohorts of children that failed to show a decrease in parasite prevalence or prevalence of anemia [90], and in a one year study in Burkina Faso in which 4 MSAT days failed to reduce clinical malaria incidence in 9 treatment clusters (villages) [91]. Mosha *et al.* found in Tanzania that 80% of infections in high-prevalence households are submicroscopic, and that even with treatment of entire households if anyone was found to be parasite positive by RDT, that 45% of infections would still be missed [13,92]. This would indicate that even increased ACT treatment surrounding infected individuals would still be insufficient for control of these asymptomatic

populations. These detection methods are also limited by needing access to the individuals to sample them, a challenge in an increasingly mobile populous [93].

In addition, while the mosquito-infectious parasite stage (gametocytes) tend to make up a small portion of the total parasite numbers during infection (1 gametocyte is produced per 156 parasites on average) [17], these parasites can persist for months in the absence of asexual parasites or symptoms, even across the dry season [18,94]. This asymptomatic persistence at low concentrations can still infect mosquitoes, and may function as a parasite reservoir in times of limited vector prevalence [95–97]. Additionally, gametocytes may not always be cleared with ACT treatment [98], further increasing the mosquito-infectious reservoir of parasites in humans. New methodologies that could find these individuals with low-level parasite infections would be very beneficial to directing control interventions. There are some data that suggests that mosquitoes are more attracted to individuals with malaria parasites [99,100], and it has also been found that *Plasmodium* secrete volatile mosquito-attractant terpenes which may increase the ingestion of the parasite [101]. Chapter 5 of this dissertation will discuss work towards the development of new methodologies utilizing mosquitoes as epidemiological sampling tools in effort to address some of the above issues.

Collection of exophilic/exophagic vectors:

The gold-standard approach to the collection of outdoor biting mosquito vectors is the technique called human landing catch (HLC). This technique is where collectors use themselves as bait, collecting mosquitoes from their legs when they land to bite [102]. This approach has been utilized likely for as long as mosquitoes have been studied, but has become less favorable due to safety concerns regarding the transmission of many diseases possible by probing alone [6,103]. The risks of malaria transmission are limited as collectors can be given prophylaxis such

as malarone or doxycycline [104,105], but many arboviruses either have no vaccine or no treatment [106]. Additionally, informed consent may be difficult to achieve when using local collectors, some of whom may be young [102]. HLC, however, has been very important in developing understanding of the capacity of vectors to transmit disease in an area. A benchmark metric utilizing knowledge of the human biting rate through HLC is the entomological inoculation rate (EIR) [107,108]. This is a measure of the number of infectious bites per person per night, or the human biting rate times the sporozoite positive mosquito count. This is also related to the calculation of the vectorial capacity equation, below, which is a measure of the daily rate of infectious bites arising from a single infectious human, a metric similar to R_0 [107,109,110].

$$C = \frac{ma^2vp^n}{-\log_e p}$$

In this equation, *m* is the density of female mosquitoes per person, *a* is the mosquito biting rate, *v* is vector competence (proportion of bitten humans infected * proportion of biting mosquitoes infected), *p* is the daily probability of survivorship, and *n* is the length of the sporogonic cycle in days [107,109]. EIR and vectorial capacity have been utilized to map malaria risk across sub-Saharan Africa, data which can be utilized to best direct control interventions [108,111–114]. Thus the importance of accurate quantification of human biting rate is evident, so ways to quantify this metric without the risks presented by HLC are needed.

The first alternative to HLC is the use of a Centers for Disease Control Light Trap (LTC), hung near the feet of a person sleeping under a bed net [115–117]. The LTC is a small, battery powered light source and fan to which mosquitoes are attracted. Once near the fan, the vectors are sucked into a collection container below the trap. In general, this methodology samples *Anopheles* vectors successfully, and its catch numbers tend to correlate with those caught by

HLC, though at a lower overall rate [118,119]. Due to the light in this trap, the numbers of non-vector bycatch can be quite high [120,121]. Also, as vectors pass through a fan, mosquitoes can be damaged. Both of these factors make sorting, and species identification of vectors a challenge. Furthermore, depending on location, the general reliability of LTCs can be poor. On Bioko Island, Overgaard *et al.* saw that HLC and LTC numbers did not correlate on a nightly basis [122]. Additionally, the ratio of LTC caught mosquitoes to HLC varies considerably based on location from 1.86:1 LTC:HLC in Lwanda, Kenya [123] to 0.33:1 in the Kilombero Valley of Tanzania [124]. In urban Dar es Salaam, Tanzania, the LTC caught essentially no mosquitoes, likely due to the high amount of external light sources [125]. To alleviate some of these concerns, and to utilize collection methods without light sources, several alternative, human-baited traps have been created.

The first alternative tent trap designed was the Mbita trap [123,126–128]. This passive trap design is a modified bed net that has a passive funnel in which mosquitoes fly in one direction and then become caught as they attempt to exit. This trap, named for the location where it was tested in in Kenya, was found to catch 4.1 times the number of mosquitoes of an LTC, and 43.2±10% the number of HLC [128]. However, when the trap was subsequently tested against HLC in Madagascar, the Mbita fared very poorly compared to HLC [126]. Overall the Mbita trap only caught 85 mosquitoes (of all genera) versus 6,899 caught via HLC, with no correlation in catch numbers between methods per night. It was postulated that this was due to the strong exophagic/zoophilic behavior present in the mosquitoes of the area that had been reported previously [126,129].

The next passive tent designs were the Furvela, Ifakara A, and Ifakara B [130]. Charlwood developed the Furvela trap to be a simple design in which a CDC Light Trap (without

its light) was suspended near a small opening in the zipper of a Eureka camping tent that would collect vectors as they try to enter the opening. The Ifakara A and B designs are passive collection canvas tents that have window-like funnel openings above a sleeping individual. The collector is protected by a separate mesh layer in between these openings and where they sleep. In Govella *et al.* these three designs were compared against one another, and the Ifakara A and B designs were tested against HLC [130]. The best design in this study appeared to be the Ifakara B due to its correlation in catch numbers with HLC, though the relative catch sensitivity was lower with the Ifakara B compared to HLC (0.32 and 0.65 in two experiments). The predominant issues with the passive Ifakara designs are the exposure to vectors when removing them from the trap area, the loss of vectors due to the passive design, and the lack of comfort due to the heavy canvas material used [120,121,127,130].

There has been some recent work on the use of mosquito electrocution traps (MET) for vector collection. As the name suggests, these are traps that utilize battery-powered grids surrounding the feet of a sitting collector (the rest of the body is surrounded by a bed net), to which the mosquitoes are attracted [131]. Vectors are killed via electrocution and collected as they come to bite the feet of the collector. This methodology was based on previous use of these grids with odor "tubes" in which an indoor collector was respiring through a one-way valve to disperse his breath into the trap for bait [132]. The MET was successful in collecting a range of vector species, though at a rate lower than that of HLC for vectors *An. gambiae, An. coustani,* and *An. ziemani* (51.9%, 7.4%, and 9.1% of total, respectively) [131,133]. The rate of capture of *An. funestus* was not statistically different between MET and HLC (84.2% of total, P=0.18). The approach appears to fair better outdoors than indoors, and "may misrepresent […] proportion of human exposure to biting that occurs indoors" [131]. Additionally, there were some issues with

the stability of the power through the trap during the night which may have limited its effectiveness.

These traps are beneficial overall in that they reduce exposure and risk to collectors over that of human landing catch. The traps in which you can sleep through the night (Furvela, Ifakara, Mbita, LTC+Bed net) vastly decrease the labor required for the sampling, and allow for trapping on sequential nights. The passive traps likely suffer from vector escape, which may falsely represent the biting pressure of the sampling region. Overall, the trap designs catch fewer mosquitoes than HLC, likely due to the limitations in thermal or other host-seeking cues that the mosquitoes use for feeding [134,135]. To address some of these concerns, the first aim of my dissertation was to test a newly designed active, human-baited tent trap for the exposure free collection of human host-seeking vectors in West Africa. This work is discussed in its entirety in Chapters 2 and 3.

Age grading of Culicidae (with a focus on *An. gambiae*):

The extrinsic incubation period (EIP) is the time that it takes for a pathogen, in this case *Plasmodium* parasites, to develop from the ingested mosquito-infectious parasite stage (gametocyte) to the transmissible human-infectious stage (sporozoite), and is an important component of the transmissibility of the parasite and maintenance of the disease [136]. Only mosquitoes that have progressed through the EIP are capable of transmitting *Plasmodium* parasites. Thus, an important metric towards understanding the transmission cycle of *Plasmodium* is to see what proportion of the mosquito population has lived this minimum period of time. Additionally, as described above in the "Challenges to elimination" section, insecticide resistance has been a consistent challenge to control, and the development of insecticides that would preferentially target only the oldest or *Plasmodium* infected mosquitoes would be highly

beneficial. Critical to this goal is the development of tools to see if existing or novel control measures affect these old mosquitoes in field tests. The sections below outline the existing methodologies for the age-grading of *Anopheles* vectors, the shortcomings of these methods, and how they can best be applied to age-classification of wild mosquitoes.

The approaches that have been used previously to age-classify *Anopheles* (and other) mosquito species predominantly involve investigating changes in the ovaries after egg development. The first use of the ovaries for age grading was described by Mer in 1932 with *Anopheles elutus* from Israel [137]. He found that changes in the diameter of the ampullae of the oviducts could be utilized to distinguish between mosquitoes that have underwent a gonotrophic cycle. Detinova in 1945 expanded upon this approach, looking at ovarian tracheoles [138]. These tracheoles are in tightly coiled skeins in mosquitoes that have not gone through a gonotrophic cycle (nulliparous), while they are distended in those that have successfully developed an egg clutch (parous) [139]. This approach has persisted through time due to the relative ease and speed of the dissection, though there are limits to the amount of knowledge you can glean as this is a very coarse, binary measure of age. To address this on a finer scale, Polovodova developed a new dissection approach looking at the number of ovarian dilatations, which are follicular relics of past egg clutches seen on the distal portion of the ovariole [140]. This approach is complicated, technically demanding, and laborious, requiring injection of Paraffin oil into the ovaries through the common oviduct using a glass micropipette, removing the ovary from the dorsal side without damage, and counting dilatations under magnification [141]. It is easily possible to break these dilatations off when isolating the ovaries, falsely indicating the mosquito is uniparous or nulliparous [141–143]. Additionally, not all developing ovarioles are diagnostic, meaning indicative of a successful gonatrophic cycle, and ovariole relics have been seen in

known nulliparous mosquitoes [144]. The occurrence of non-diagnostic, rogue ovarioles has also been found to increase as the mosquito ages, likely due to decreasing fecundity [144,145]. A minor detraction from this approach as it relates to a general understanding of the *Anopheles* age structure is that these ovary-targeted approaches cannot be used to age-grade male mosquitoes. To address this with dissection, Huho *et al.* developed an approach using the number of spermatocysts, relative size of sperm reservoir, and presence/absence of a clear area around male accessory gland that could distinguish between young (≤ 4 day) and old (> 4 day) mosquitoes [146]. An alternative dissection approach to looking at sexual organs is to count daily growth bands on the thoracic apodemes, the indentations present in arthropod exoskeletons which support organ and muscle tissue (Moore et al., 1986; Schlein, 1979). However, this approach was found to be impractical for use due to the variability in their formation, and the time-consuming nature of the staining procedure [147].

With the limitations in dissection approaches in mind, many alternatives have been pursued to increase the throughput, reproducibility, accuracy, or ease of age-classification of mosquitoes. The first of these methods was developed by Wu *et al.* with reverse-phase high pressure liquid chromatography (HPLC) to detect fluorescent pteridines [149]. These pigments are found in the body of the insect [150], and decrease in fluorescence over their lifespan. This method was able to successfully discriminate between 5 day age intervals until 30-35 days [149]. The major issue with pteridine fluorescence is that these compounds vary based upon size, sex, and temperature which complicates translatability to broader sample collections [151]. Following this work, several papers used mass spectrometry approaches to detect changes in the ratio of cuticular hydrocarbons. First, Desena *et al.* utilized gas chromatography/mass spectrometry on *Aedes aegypti*, which was subsequently expanded to *An. stephensi* and *An. gambiae* [152–154].

In *Aedes*, they found that two hydrocarbons, nonacosane (C_{29}) and pentacosane (C_{25}) increased and decreased, respectively, in a linear fashion based on degree day (temperature in Celsius * days lived) [152]. This approach could be utilized to discriminate ages up to 12 days of age, at which point pentacosane was undetectable. For *Anopheles stephensi*, a similar relationship was found with nonacosane (C_{29}) and hetriacontane (C_{31}), with accuracy out to 15 days [153]. Caputo *et al.*'s work with *An. gambiae* found the ratio of C_{31} to C_{27} to be most accurate, though with a high degree of standard error (± 71.68 hours) [154]. The limitations to these methodologies largely are due to the destructive nature preventing downstream applications, and the cost (~$10/sample) [155]. Also, the modulation of these hydrocarbons in relation to varying temperatures, and dietary conditions is a concern [149].

The second alternative to dissection approaches for age grading has been transcriptional profiling via microarray to look for markers indicative of age [156,157]. Through screening of 2714 genes from *An. gambiae,* Cook & Sinkins found that the RNA from 4 genes in *An. gambiae* was differentially abundant, and could be used to distinguish with a mean 95% confidence interval of ± 4.2 days across 0, 5, 10, 15, 20, 25, and 30 day time points [157]. Similar to the approaches above, these are both destructive, and have yet to be validated on field samples.

The third alternative to dissection has been proteomic analysis. This was first done with matrix-assisted laser desorption ionization-mass spectrometry time of flight (MALDI-TOF) with *Ae. aegypti*, and then with an HPLC ion-trap mass spectrometer used after 2-D difference gel electrophoresis in *An. gambiae* [158–160]. These are two ways of separating proteins via their mass/charge ratio, coupled with a fragmentation method that allows for identification of the protein. With *An. gambiae,* they found three proteins that increase with age, and six that decreased [159]. These proteins were also investigated via western blot, and their intensities

were compared between time points. With western blot, only two proteins were shown to significantly decrease between 1 and 9 day time points (at which point 9 day samples were indistinguishable from 17 and 34 day samples) [159]. This approach then seems to have limited benefits over dissection as it is a binary measure only discriminating very young from other ages, and is also destructive.

The fourth alternative to dissection is near-infrared spectroscopy (NIRS). NIRS is a fast and non-destructive technique in which changes in the near-infrared spectrum (780-2526 nm) are analyzed [161]. Overtones of the loss in energy due to absorption, rotation, stretching and bending of C-H, N-H, O-H and other bonds are present in this range. Through the use of chemometric modeling, and carefully developed calibration sets, predictions about the analyzed material can be made. NIRS was first utilized in its modern form in the 1960s by Karl Norris from the U.S. Department of Agriculture to look at moisture content of various grain species [161,162]. This approach was first utilized for the age-classification of insects with house flies, and compared directly to pteridine fluorescence measurements which varied significantly in accuracy with changes in temperature, head size, or sex [151]. NIRS was found to have better accuracy than pteridine fluorescence, with smaller variation in age predictions. Accuracy of NIRS calibration models increased with the inclusion of more variety in size and temperature, but temperature variation was not necessary to achieve reasonable accuracy for independent test sets [151].

This technology was first applied to mosquitoes (*An. gambiae*) by Mayagaya *et al.* [163]. They found that NIRS could speciate *An. gambiae* s.s from *An. arabiensis* with 80-100% accuracy with field-caught/laboratory reared specimens, respectively, and age (≤ 7, >7) of laboratory-reared strains was predicted with 80% accuracy on average. There was poor

discrimination between ages >10 days, and on between-strain female predictions, ages 7, 10, 13, and 19 were indistinguishable from one another at $p < 0.05$. Accuracy increased for within-strain predictions, showing that 1, 4, 7-10, 13, 16-19 day groups were able to be distinguished from one another. Additional work from this group found that mosquitoes preserved in RNA*later* could be accurately age classified with 90% accuracy [164]. NIRS was also applied to populations held in a semi-field system at the Ifakara Health Institute in Tanzania [165]. With semi-field populations, overall accuracy was 78% for *An. gambiae* s.s. and 89% for *An. arabiensis*. They attempted to link "Christopher's stage > IIm" wild mosquitoes (having fully developed ovaries) to predicted age value via NIRS. Limited numbers were investigated, and results were lacking, but there appeared to be no predicted age difference between fully developed nulliparous and parous wild-caught mosquitoes. Work by Aw *et al.* with *Drosophila* species indicated the role of composite models that increase accuracy in these insects through varying scanning temperature, insect strain, and diets as being important to applying this technology to wild samples [166,167]. Diet was modulated through reduction in the standard amounts of treacle (molasses), semolina, yeast and nipagen (a preservative). For increasing genetic variability, they utilized 20 wild-caught lines of *Drosophila simulans* for inclusion in the model. They found a higher accuracy of NIRS prediction with inclusion of all temperatures and diets tested in the calibration model. This approach has also been applied to *Ae. aegypti* with variations in larval diets (infant cereal + yeast or fish food) and adult food sources (sugar or sugar + blood), and also found that increasing variation increased test set prediction accuracy [168]. Finally, Sikulu *et al.* utilized NIRS to predict the age of *An. gambiae* and *An. arabiensis* reared from wild-caught larvae and after exposure to pyrethroid insecticides [169]. Overall accuracy of the models was similar to that previously reported (78-82%). To see how their wild-larvae models work on natural populations,

a group of wild *Anopheles* were collected via pit traps. Pit traps are simply pits dug into the ground that are covered by coconut and banana leaves, and provide attractive resting habitat for mosquitoes. They reported an age distribution of susceptible and resistant populations collected via this trap, but found no difference in these populations. Most mosquitoes were predicted to be between 5-9 days old, with very few (<5%) under 3 days old. Importantly, there were no external validators of age in these mosquitoes, such as parity dissections or the presence of *Plasmodium* sporozoites that take a minimum amount of time to develop [136,170]. Thus it is unknown what degree of accuracy the presented age distribution has.

The predominant gap in the use of NIRS as an age-grading approach is that it has yet to be utilized for the prediction of wild-caught mosquitoes with external validators. The comparison of nulliparous mosquitoes assumed to be relatively young (3-4 days of age) against *Plasmodium* sporozoite positive mosquitoes (minimum ~12 days of age), would be the best candidates for these external validators [136,171]. The major goal of my work towards the study of Near-infrared spectroscopy for the age-grading of wild *Anopheles gambiae* was to develop models using wild larval sources, and to test the accuracy of these models on wild-caught mosquitoes that are either nulliparous (young) or sporozoite positive (old).

Approaches for the detection of *Plasmodium* parasites:

Though 130 years have passed since Alphonse Laveran's discovery of malaria-causing parasites in human blood, and 120 years have passed since Ronald Ross determined that mosquitoes spread the parasite, surprisingly little has changed in terms of parasite detection methodology [172,173]. While Laveran's initial discovery was performed without the differential eosin Y/methylene blue staining of parasites developed by Romanowsky in 1891 [174], he was able to discern trophozoite asexual stages, schizonts, and male/female gametocytes

under simple microscopy [172]. The basics of this detection of the parasite stages are still in place today, though microscope and staining technology has improved considerably. Thick and thin blood smears from human samples stained with Giemsa allow for the reliable detection down to concentrations of ~10,000-20,000 parasites/mL in the laboratory, though this limit of detection may be lower (50,000-100,000 parasites/mL) in field settings [5,175–177]. This microscopic approach is still in many ways the "gold standard" for diagnosis, as it can be performed cheaply, can identify different *Plasmodium* species and stages, and is amenable to field and/or low resource settings [175,178]. However, this approach does need highly trained microscopists, and can be slow and/or difficult to perform as a point-of-care (POC) type analysis. To complement microscopy, the use of rapid diagnostic tests has become popular in the field. RDTs are small cassettes to which a blood sample is added followed by a buffer to a wicking membrane. Antigens in the blood (predominantly Histidine Rich Protein II (HRPII) or Plasmodium Lactate Dehydrogenase (pLDH)) bind to a strip of antibodies present on the wick, and are subsequently bound by another antibody for detection [179]. The biggest benefits to RDTs are their ease of use and speed of detection (15-20 minutes) [179,180]. These tests can identify different *Plasmodium* species (usually discerning *falciparum* from other *Plasmodium* species), though the detection limit is lower than that of microscopy (100,000-200,000 parasites/mL) [181]. Additionally, even after treatment with ACT, the antigens can persist in the bloodstream causing a false-positive RDT in 98.2%, 94.6%, 92.0%, and 73.5% of children at days 14, 21, 28, and 35, respectively [180]. This complicates accurate diagnosis, and may cause overtreatment in uninfected individuals or a missed re-infection.

The alternative to protein detection via RDT is the detection of nucleic acids in the blood specific to the parasite. The first approach developed was polymerase chain reaction (PCR) to

detect a repeat region (pPF14) in parasite DNA [182,183]. Since this initial PCR approach, many alternative gene targets have been used, predominantly targeting 18S ribosomal sequence in conventional and nested-PCR methodologies [181,184–186]. These methods can achieve detection limits of 100-500 parasites/mL [187], but are less sensitive than the newer quantitative PCR (qPCR) reactions that have reached as low as 30-150 parasites/mL detection limits [187]. Additionally, a range of technologies for the detection of parasite RNA from human blood have been developed. These methods are: quantitative nucleic sequence based amplification (QT-NASBA) [188,189], reverse transcriptase -loop mediated isothermal amplification (RT-LAMP) [190–193], and reverse transcriptase PCR (RT-PCR) [194–196]. These approaches have a range of detection limits from 20 to 10,000 parasites/mL, but all tend to be more sensitive than microscopy [181,187]. QT-NASBA and RT-LAMP are both isothermal amplification approaches that allow for amplification without expensive thermocycling machines. RT-LAMP also has been utilized with colorimetric or turbidimetric assays that allow for determination of positivity by the naked eye [193,197]. Furthermore, a major benefit to RNA-based approaches is the ability to detect specific transcripts that are only expressed by certain stages of parasite. This has been utilized with QT-NASBA for the detection of *Pfs*25 and *Pfs*230, surface antigens that are specific to macro and microgametocytes, respectively [198]. These stage specific approaches are important in quantifying the infectious reservoir of humans in a population, many of whom may be asymptomatic [13,199].

The major issue with all of these approaches lies in that they fail to account for the heterogeneity in mosquito biting, and how often the bites on these individuals will infect the mosquito. There have been some xenodiagnostic and membrane feeding studies using laboratory reared mosquitoes to look at the efficiency of transmission of naturally infected people [200–

202]. Normally, the end point measure of these assays is oocyst detection. However, there are several biases with this approach. The first is that mosquitoes are assumed to be biting that individual, even though there is heterogeneity in how mosquitoes bite across a population [203,204]. In Graves *et al.*'s study in Papua New Guinea, they found that it was mostly children 0-4 years of age who were contributing to the infectious human reservoir of the parasite [201]. Bonnet *et al.* also showed that children under 10 years made up the bulk in the infectious reservoir in Cameroon [205]. Though in Drakeley *et al.*'s studies in The Gambia and Tanzania it was mostly older individuals >20 years of age contributing to these infections [200]. This sort of nuance can easily be missed or skewed based on whose blood is used for membrane feeding of mosquitoes, or who can come into the lab and is willing to provide blood (or their arm) for direct feeds. Additionally, as oocysts are the endpoint measure, the mosquitoes have to be held artificially with water and sugar *ad libitum* through the EIP. This biases the survivorship of the mosquito as the parasite develops, and may not reflect end-point infectivity [206]. Finally, as the use of wild-caught mosquitoes poses a risk towards anyone performing direct feeding assays, the use of laboratory-reared mosquitoes is necessary [202]. These strains would lack the heterogeneity in genetic background [207], microbiome infections [208,209], and a host of other factors that may influence successful infection [210]. Thus a major goal of the work in Aim 3, discussed in Chapter 5 of this dissertation, is to develop molecular tools that avoid many of these biases inherent to current methodology, and apply these novel techniques directly to natural systems.

The role of ivermectin for Malaria Control:

In the late 1990s, Bockarie *et al.* reported a unique finding in Papua New Guinea with *Anopheles punctulatus* mosquito survivorship [211]. They found that after a mass drug treatment

of ivermectin (IVM) and diethylcarbamazine (DEC) to clear the parasites that cause lymphatic filariasis [211], all blood-fed mosquitoes that were collected fewer than 4 days after the MDA died within 9 days, whereas 67% of those that had been collected in the pre-MDA period survived. After feeding the mosquitoes DEC and finding no loss in survivorship, and finding that mosquitoes that were collected 28 days post MDA survived the same as pre-treatment, they concluded that IVM was inducing this mortality. This finding was repeated in the laboratory with *An. farauti* mosquitoes shortly after [212], and then largely left unpursued. Recently, the Foy lab has again been exploring the effects of IVM on *An. gambiae* in West Africa [7,213–216]. They found that *An. gambiae* caught in Senegal surrounding IVM MDA had significantly less 5-day survivorship in treatment villages than controls [213], and that the proportion of infectious mosquitoes (those with *P. falciparum* sporozoites) dropped by 79% over a 2 week period post MDA [217]. Subsequent work from Kobylinski *et al.* showed also that co-feeding of a sub-lethal dosage of IVM (LC_{25}) with *P. falciparum* parasites reduced the proportion of *An. gambiae* that had development of oocysts and sporozoites [214].

After these findings, the remaining questions surrounding the use of IVM MDA for malaria control were 1) what is the length of reduction of mosquito survivorship and infectiousness, 2) what effect does it have on exophagic biting populations, 3) what effect does it have on the age structure of the mosquito population, 4) what are the effects of multiple-MDAs on these measures, and 5) what is the net benefit of IVM MDA on human incidence of malaria? As I joined the lab, I have been a part of three mass drug administrations in three different countries of Senegal, Liberia, and Burkina Faso to address aspects of these questions. Though not directly within the chapters of my dissertation work, the figure below is the culmination of some of this work as it directly relates to IVM MDA.

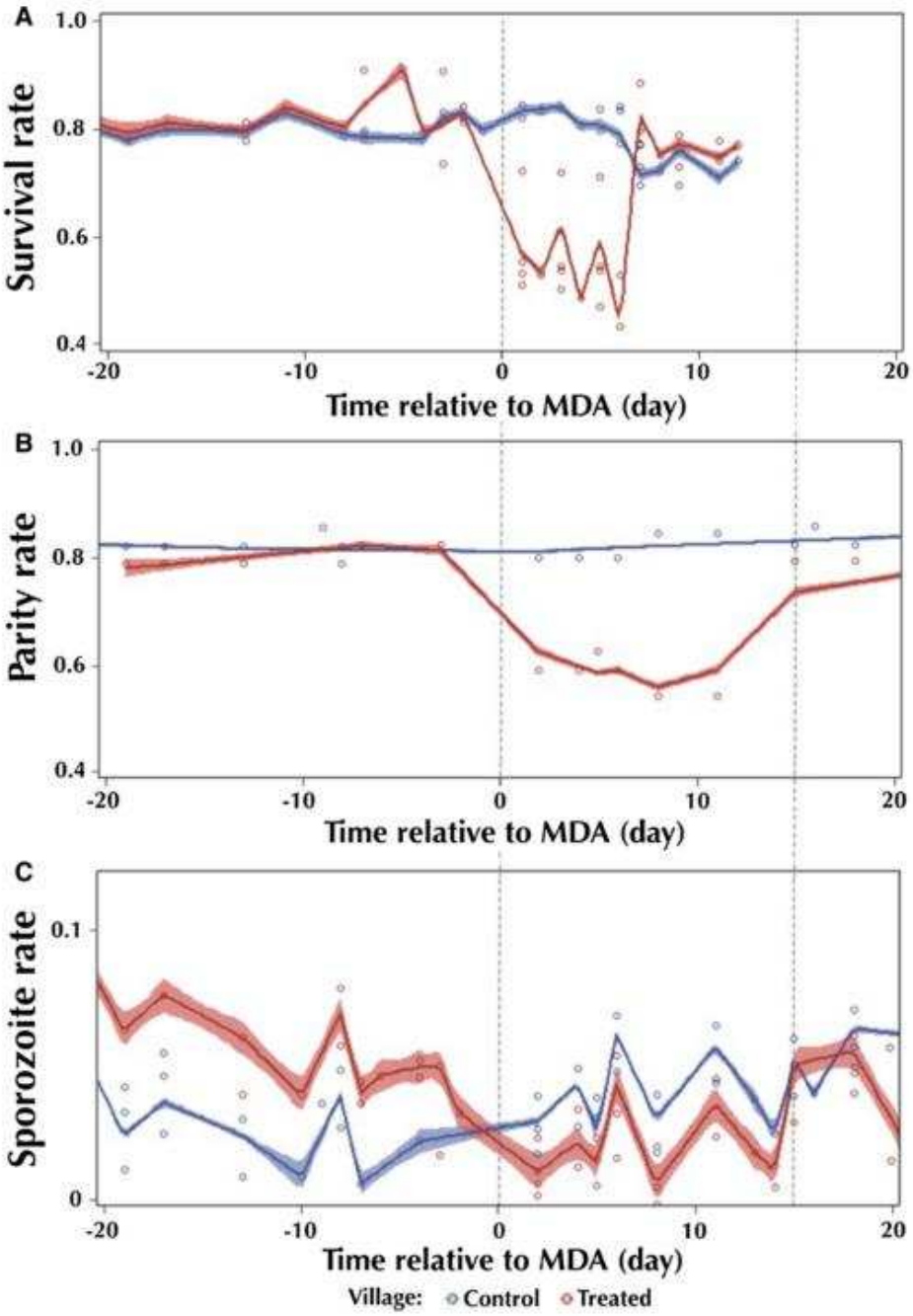

Figure 1.3: The effects of ivermectin MDA on the (a) 5-day survival, (b) parity rate, determined via dissection, and (c) sporozoite rate determined by qPCR on *An. gambiae* s.l. mosquitoes. From Alout *et al.* 2014 [7].

In this paper, we showed that the drop in 5-day mosquito survival persisted for ~1 week post-MDA (Figure 1.3a), with a drop in survivorship of 33.9% [7]. Additionally, as a measure of the age-structure change due to IVM, we found that parity (described above in the Age Grading

of *Culicidae* section) dropped in treatment villages by 25% for ~2 weeks (Figure 1.3b), with still significant decreases in parity rate by week 3. Importantly to my dissertation work, many of the mosquitoes used for parity analysis were collected during testing of a novel tent trap (Chapters 2 and 3 of this dissertation). Additionally, to try to quantify this drop with a finer scale metric of age, Chapter 4 of this dissertation describes the use of near infrared spectroscopy for age-grading wild mosquitoes. Finally, Chapter 5 of this dissertation describes the development of novel techniques for the detection of *Plasmodium falciparum* infection events in *An. gambiae*. This methodology could be utilized in the future for study of early sporogonic inhibition as the parasites develop in the mosquito midgut in the presence of IVM.

Chapter 2: Design and Testing of a Novel, Protective Human-baited Tent Trap for the Collection of Anthropophilic Disease Vectors. [1]

Introduction:

The development of an effective and safe sampling method for the collection of host-seeking anthropophagic mosquito vectors has long been a goal for medical entomologists. Human landing catches (HLC) are considered the gold-standard for sampling host-seeking anthropophagic mosquito populations and estimating the human biting rate (HBR), which is needed to measure the entomological inoculation rate (EIR). HLC consists of trained collectors luring host-seeking mosquitoes from the environment with his or her own cocktail of volatiles, gases, body heat and humidity, and collecting the vectors that land on and attempt to bite an exposed part of their body, usually their legs. There are inherent risks of contracting mosquito-borne pathogens with this technique, since mosquitoes transmit nematodes, arboviruses, and *Plasmodium* through probing alone, prior to imbibing any blood [6,103,218,219], and it is very difficult to only capture landing mosquitoes over a sampling interval without having any of them probe. HLC for arbovirus mosquito vectors can put non-immune collectors at particular risk because only supportive therapies are available for arboviral diseases. In malaria vector research, curative and prophylaxis drug regimens for *Plasmodium* infections lower the risk to the collectors, and so HLC are used more routinely, but it is not widely acknowledged that *Anopheles* can also transmit a variety of arboviruses in many areas of the world, such as

[1] Adapted from: **Krajacich B.J.,** Slade J.R., Mulligan R.F., LaBrecque B., Kobylinski K.C., Gray M., Kuklinski W.S., Burton T.A., Seaman J.A., Sylla M., Foy B.D. (2014). Design and Testing of a Novel, Protective Human-Baited Tent Trap for the Collection of Anthropophilic Disease Vectors. *Journal of Medical Entomology*. 51(1):253-263. [120].

O'nyong-nyong virus and Bwamba virus [220–222]. Because of these risks, some ethical review boards have deemed HLC unethical and will not approve them, while others have put constraints on how they are conducted, including requiring all collectors to take malaria prophylaxis medication and to undergo routine blood smear examinations during their work. The WHO recommends not performing HLC in malaria vector research when safer methods are available to estimate the HBR [223].

The alternatives to HLC are using various designs of nets or bed nets that both surround and protect the human bait while passively or actively capturing the host-seeking mosquitoes that come to bite. These human-occupied net traps have been used since the early 1900s [224]. Passive or semi-passive trap designs have been the most common, whereby a person rests or sleeps under a bed net, while host-seeking mosquitoes pass through a window, funnel, or under a gap of an outer entrapment net [224,225]. Passive designs may have a disadvantage in that some species are highly capable of exiting even small gaps and funnel-holes from which they entered the trap [226,227]. The Mbita trap is a passive trap that uses a funnel trap attached on top of the bed net [128]. It has been successful in estimating HLC in some studies [123,228], but unsuccessful in other studies [126,127]. In semi-passive designs, the collector drops a flap over the open window or drops the outer net to close the gap and entrap the host-seeking mosquitoes after the collecting interval is completed [224]. Most of these passive and semi-passive designs require the collectors to then spend their time aspirating mosquitoes from the relatively large holding chamber, which can be laborious and lead to risk of being bitten [130]. Trap collection counts can also be substantially reduced when mosquitoes need to navigate through a window, gap or slit in the entrapment net [229]. There has been recent success utilizing the Ifakara tent designs for purely passive mosquito collection [125,130,230]. These designs can be quite

effective, meeting or exceeding capture levels to that of HLC for *Anopheles* species, though *Culex* spp. are caught at a decreased rate [230].

Active trapping system alternatives to HLC most often attach a fan trap to the entrapment net, and usually augment the attractiveness with a light. Charlwood *et al*. attached an inverted CDC light trap over the outer entrapment net, and the design was successful in capturing host-seeking *Anopheles farauti* [226]. The odor-baited entry trap (OBET) was designed for anemotactic behavioral studies in the laboratory, but modified for successful field capture of African *Anopheles* malaria vectors [231]. The OBET separates the host-holding tent from the capture device, and connects the two with a hose through which a fan system blows odors from the host-holding tent through the capture device. In Senegal, the OBET accurately reflected outdoor HLC, but not indoor HLC [232]. Mutero *et. al.* used a homemade updraft trap suspended over a bed net to successfully capture host-seeking African malaria vectors [233]. Many investigators now utilize a similar design, but for consistency most use commercially-purchased CDC mini-light traps (LTC) hung next to the feet of a human resting under a bed net [122,234,235]. Several investigators have observed no differences in *Anopheles* capture rates if long-lasting permethrin-treated bed nets are used in LTC rather than untreated bed nets, thus improving the safety for the collector [118,119,125]. In some studies, LTC accurately reflected the species composition, capture rates, or vector bionomics of the HLC [123,130], while in other studies, consistent associations failed [122]. When LTC accurately reflects HLC, a conversion factor is typically needed to adjust the raw numbers because the LTC tends to underestimate the number of mosquitoes captured by HLC [122].

Missing so far from the panoply of trapping systems is a standardized active trapping system for use with human hosts that accurately samples certain host-seeking vectors, is simple to use,

easily adjustable to attract different vectors, comfortable for the human bait, and that makes use of modern camping tent technology. Modern camping tents have been developed for decades so they are now lightweight and contained for easy transport by a single user. They are also efficiently designed to require minimal time to set-up and tear-down, and they can be extremely rugged and protective in diverse weather situations. Lastly, they protect the human inside from the majority of biting vectors while still maintaining airflow by utilizing micromesh fabric panels, thus they offer both comfort and protection for the user. Here we describe the development and initial testing of a modern collection technique utilizing the benefits of standardized tent technology coupled with a novel, safe active-trapping system.

Materials and Methods:

Tent Trap Design and Construction:

The tent trap was designed by engineers at Infoscitex Corp (Waltham, MA) by modifying a three-person Losi™ tent (Nemo Equipment Inc, Dover, NH).The final design consisted of a standard rectangle shaped footprint measuring 91" x 79" (50 sq. ft.) with zippered doors on the two longer sides (Figure 2.1a). The tent trap walls consist of lightweight breathable 70 denier nylon rip stop fabric. The floor was made of 70D waterproof nylon taffeta fabric. The rain fly cover is made out of 70D waterproof nylon rip stop fabric. Air venting material was composed of 2010 “no-see-um” nylon mesh with grid sizes approximately ~0.25 mm^2 and situated at the top of the fabric wall panels on all four sides. The supports are made from DAC feather-lite anodized aluminum poles. Suction ports for vector capture were placed immediately above the air venting panels on the four walls to aspirate vectors following the source of the odor plume. Plastic suction ports had a 20.5 cm wide and 2.5 cm high intake opening, and were designed with a lip that faced down into the

odor plume (Figure 2.2b). Air intake tubes connect the suction ports to the manifold integrated into the center top of the tent and run along the outer top of the tent (Figure 2.1a); the long axis tubes were 95.5 cm long and the short axis tubes 52 cm.

Figure 2.1: Picture of tent at an angle with rainfly off showing ports attached to center manifold (A) and with rainfly on (B). Arrows indicate vector entry point at ports (A) or through gaps in rainfly leading to ports (B).

The manifold was designed to fit into gasketed fabric on the tent ceiling, and only be open to incoming vectors when the vector trap was fully installed below it. The cone-shaped vector trap (Figure 2.4a-f) with ~0.8 mm steel mesh was designed to be screwed into the

manifold, to open when the fan was closed underneath it, and to close when the fan attachment was opened underneath (Figure 2.4a).

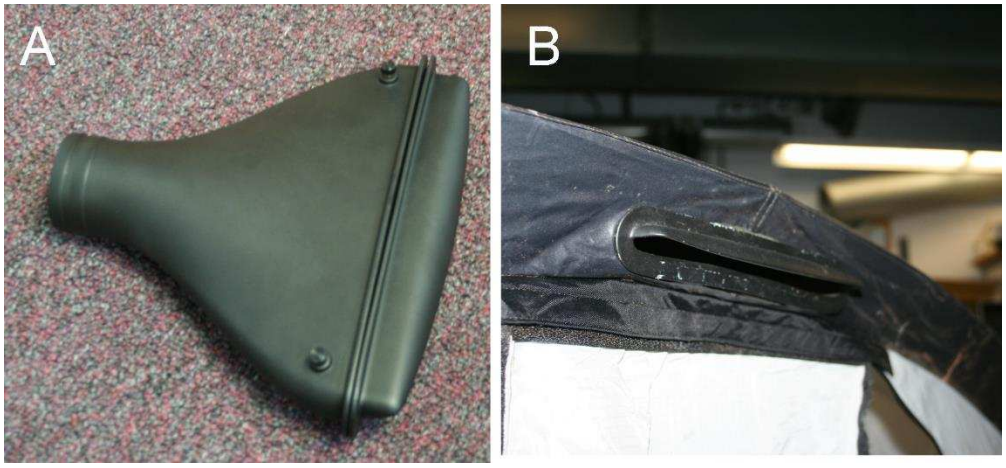

Figure 2.2: Overview of tent suction port showing: top-down view (A), installed port showing opening design (B).

The fan pulls vectors into the trap from the bottom of the manifold-trap apparatus. The fan is powered by 12V sealed lead acid gel cell batteries (108 Watt hours) and controlled by a power conditioning and control system developed by IST. The tent trap was designed to work with or without a rain fly to facilitate its use in all climates and weather conditions. The rain fly covers the entire tent and has two overhanging gaps on each long side and a one ground gap on each short side to allow vectors to fly into the space between the fly and the tent (Figure 2.1b, indicated by arrows).

Mosquitoes:

Aedes aegypti Chetumal (L.) and *Anopheles gambiae* sensu stricto (s.s) (Giles) were reared at 28^0C ± 2^0C, 80% humidity under a photoperiod of 14:10 (light:dark). *Aedes* larvae were reared in 28 L containers filled with approximately 15 L of tap water and fed a diet of ground Tetramin® fish food mixed with ground mouse food. *Culex quinquefasciatus* (Say) larvae were reared in a similar fashion. *Anopheles* larvae were reared in 44 L bins with 15 L

of tap water, and fed a diet of ground Tetramin® fish food. All adult mosquitoes were provided with water and raisins or 10% sucrose as a sugar source *ad libitum*. Adult mosquitoes were separated by aspiration into release containers at least 24 hours prior to testing and provided with sugar and water to imbibe freely. Twelve hours before testing, the sugar source was removed. They were kept in a separated, humidified, and temperature-controlled insectary on a 14:10 light:dark cycle until used in experiments.

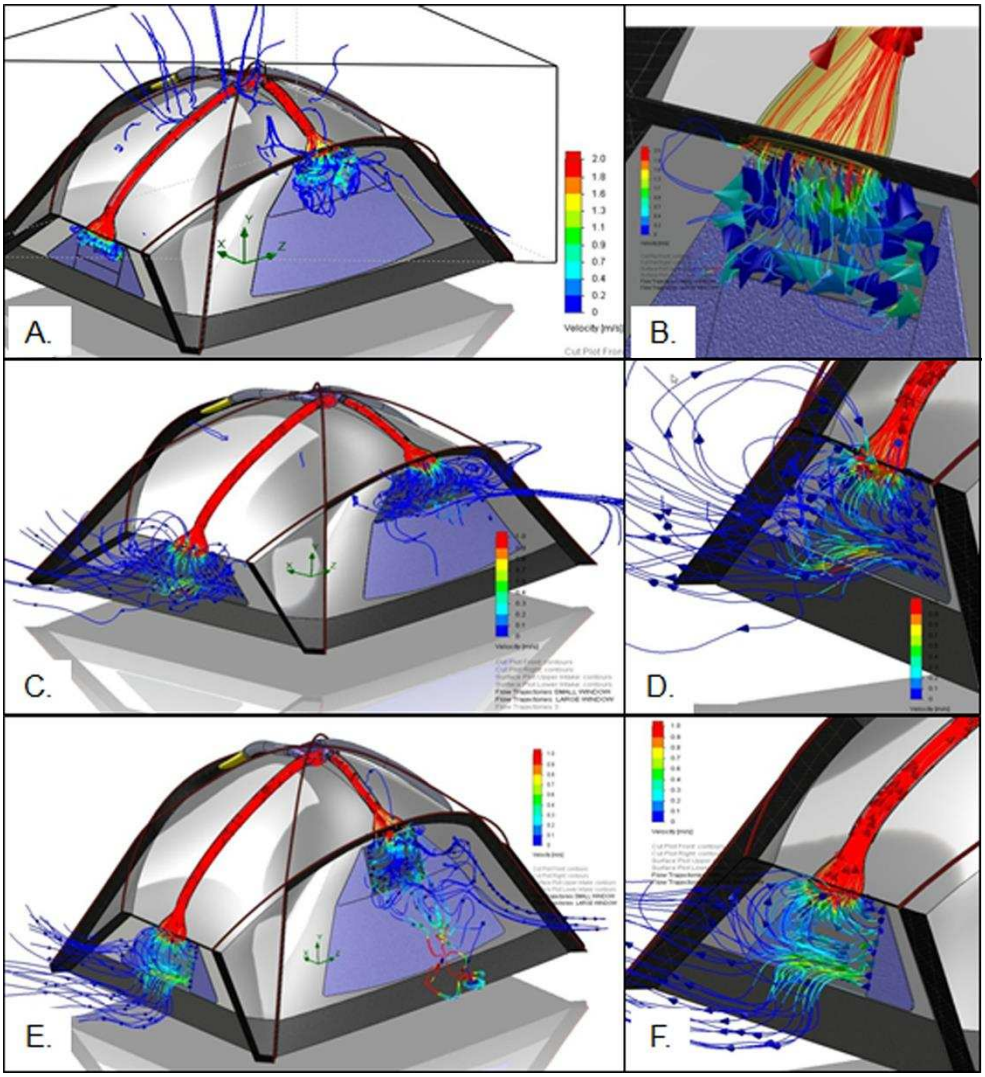

Figure 2.3: Computational fluid dynamics visualization demonstrating volatile distribution and recirculation in all possible patch configurations.

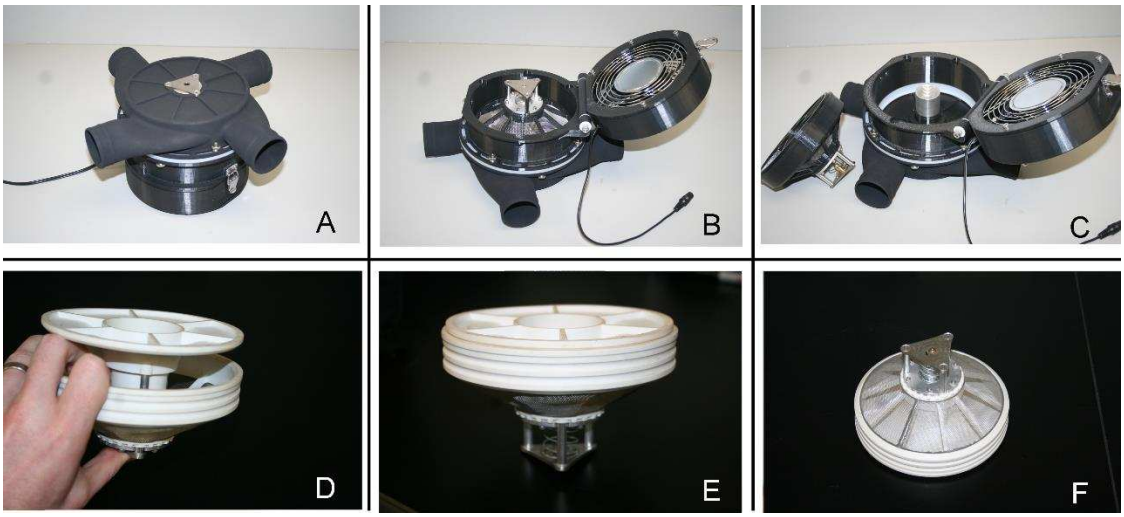

Figure 2.4A-F. Pictures of whole manifold (A), vector trap screwed in manifold with fan attachment open (flipped upside-down) (B), manifold with vector trap removed (flipped upside-down) (C), vector trap open from side (D), closed collection chamber from above (E), and below (F). Note: D-F are the newer version of the collection chamber. This design added the spokes to limit vector movement, and utilized white plastic for easier vector viewing/removal.

Assaying the prototype tent materials:

A spatial repellency assay was used with swatches of the tent trap construction material to first determine whether or not the tent trap construction materials were repellent or attractive to *Aedes aegypti*, Chetumal strain. The assay chamber was similar in design to the high throughput screening chamber described by Grieco et al., 2005, except it was modified to increase the proportion of mosquitoes responding in the assay (moving from the center cylinder to either end of the chamber) [236]. This was done by introducing human breath from the researcher that was gently blown into each end of the cylinder from a bifurcating tube originating near the operator's mouth, as well as from body heat emanating from the researcher's hands being placed over each end cap, for the duration of each test. The assays were performed in a fume hood, with temperature maintained between 21-27ºC and 20-22% relative humidity. For each test, a fabric swatch was placed in the holding chamber of one end, and nothing was placed in the opposite (control) end; the fabric was moved to the

opposite side on each consecutive test and 6 replicates were performed for each fabric swatch. The Spatial activity index was calculated from 20 female mosquitoes per replicate assaying movement from the center chamber into either end of the assay chamber.

Prototype tent trap efficacy:

To assess the mosquito-trapping ability of the initially-conceived tent design, a prototype tent trap was deployed in a 2.5 m x 4.6 m insectary at Colorado State University for capturing colonized mosquito vectors released into the room with or without a human in the tent. Note: the prototype design was different from the final design in that it consisted of air-venting nylon mesh on the ceiling of the tent, in addition to the side wall panels. Additionally, the vector capture trap was not yet designed and installed, and instead a modified capture net was fitted over the fan housing. Twenty, 5-7 day post-emergence *Aedes aegypti* (Chetumal strain) and *Anopheles gambiae* (G3 strain) females were used in each test. Insectary humidity was maintained between 70-85% for each test and temperature was maintained between 27-31ºC. Replicate tests were performed sequentially in random order determined by flip of a coin. Mosquitoes were released from a cage in the back of the room at the start of each test, following the tent fans being activated and the tent door being sealed (with or without human bait inside). After releasing mosquitoes, the operator immediately left the room and allowed the mosquitoes to host seek in the room for 20 minutes during each test with the lights on. Following each test, the operator re-entered the room and used a back pack aspirator to aspirate any uncaught mosquitoes from the room. The insectary was vented with a fan blowing from the insectary into the hallway for 2-5 min. between each test.

Computational fluid dynamics:

Following fabric and prototype testing, a powerful computational fluid dynamics (CFD) tool named SolidWorks® Flow Simulation (SolidWorks Corp.) was used at IST to model airflow patterns on the finalized tent trap design and its critical components. This design effort sought to maximize the output of human generated attractants from the tent and to maximize the effect of the suction trap. Parameters that were modeled included: a) inlet and outlet port locations, b) inlet and outlet port size and shape, c) fan mass flow, and d) inlet tube diameter. This tool allowed for design down-selection for rapid product development, and enabled an evaluation of numerous tent traps in a virtual environment that would otherwise be too costly to achieve. In parallel with the design effort various trap containers, lightweight fans, solar charging panels, and other hardware were identified for integration.

Second generation tent trap insectary testing:

A CFD re-engineered tent trap was deployed in a larger 4.5 m x 3.6 m insectary at Colorado State University for comparative trials against HLC using colonized mosquito vectors released into the insectary. The fabric window panels below each port were rolled down to expose a two inch gap of mesh fabric for bait-scented air exhaust (see Results). For each trial, forty, 5-7 day post-emergence female mosquitoes (*Aedes aegypti*, HWE strain; *Anopheles gambiae,* G3 strain; or *Culex quinquefasciatus* Wadsworth strain) that had been sugar-starved, but not water deprived, for 12-16 hours prior, were released into one corner of the insectary. One researcher stayed in the operational tent trap while the other performed a HLC while seated in a chair in one corner of the insectary. The researcher performing the HLC was fully covered with clothing except for their left foot and leg, which was exposed to the knee. Any mosquitoes landing on the exposed skin were aspirated by a handheld electric

aspirator. To control for differential attractiveness, each trial always consisted of paired successive experiments whereby the two researchers switched places in a Latin squares rotational design. Following each 30 minute test, the room was aspirated for any non-host seeking or uncaught mosquitoes. The insectary was opened and air evacuated into the hallway with a fan to remove conflicting volatiles between experiments.

Field tests in Senegal:

Preliminary sampling with the tent trap was performed in the town of Kedougou and the outlying villages of Damboucouye and Nathia in Southeastern Senegal. The anthropophilic mosquito vectors *Aedes aegypti*, *Aedes vittatus*, *Anopheles gambiae*, and *Anopheles funestus* are prevalent in this area during the rainy season, and the region is endemic for the transmission of malaria parasites and many arboviruses [106]. Comparative testing between the tent trap (without protective rain fly) and HLC was performed by two researchers placed 10-15m apart in a semi-urban portion of Kedougou during evening crepuscular periods (18:00-20:00) when *Aedes aegypti* and *Aedes vittatus* are actively host seeking. The property was flanked by thatched roof huts with open eaves, and several concrete housing structures. Sampling of human overnight host-seeking vectors was performed in Kedougou and the two outlying villages from 22:00-6:00. Due to time and resource limitations, HLC were not performed in Damboucoye and Nathia. All captured mosquitoes were frozen until dead in a -20C freezer, then identified to species or possible lowest taxa group using taxonomic keys [237,238]. Species discrimination between members of the *Anopheles gambiae* s.l. complex was done via multiplex polymerase chain reaction (PCR) [239]. Species discrimination for mosquitoes keyed to *Culex* Group V (CGV) was done on a subsample of mosquitoes in a separate multiplex PCR [240].

Statistical Analysis:

Comparisons of proportions captured by the prototype tent were performed with contingency tables and a two-tailed Fisher's exact test. To assess difference among the trapping methods (tent vs. HLC), two-tailed Paired t tests were performed with log(x+1) transformed data. Data were analyzed with GraphPad Prism version 5 (GraphPad Software, La Jolla California USA, www.graphpad.com). Tests during the crepuscular biting period from Kedougou, Senegal were analyzed using the Wilcoxon matched pairs test due to the unknown level of normality and small sample sizes of this portion of the data set.

Human subjects:

All HLC experiments were performed by trained volunteers who had read and signed required informed consent documentation. HLCs were performed following human subjects research protocols approved by the Institutional Review Board at Colorado State University, and in compliance with the Helsinki Declaration. HLC in Senegal was performed as described for laboratory testing, but with the addition of a head net. Collectors were provided with Atovaquone + Proguanil for the duration of the testing.

Results:

System design and prototype testing:

The overall system design was to mount a battery-powered fan in the center tent ceiling that would blow air on the human laying inside, and push their volatiles and odors primarily out of the venting patches on each side of the tent. This creates odor plums that would attract flying insect vectors from any of the four sides. Simultaneously, air-intake suction ports leading to the vector trap housed immediately above the fan would be positioned over the venting patches to actively capture vectors flying towards the odor source. With

proper engineering, it was hypothesized that a portion of the odor plume would be recirculated by the fan trap while some would exit the venting patches and create an odor plume. Air intake tubes connect the suction ports to the manifold integrated into the center top of the tent. Spatial assays confirmed that the tent materials were not significantly attractive, nor repellent to *Aedes aegypti* mosquitoes (Table 2.1).

Table 2.1: Neutral repellence/attractance of *Aedes aegypti* to tent materials.

Test	Attractant used	Replicates (Total # Mosq. Tested)	Average % Responding (SE)	Mean SAI (SE)	*p*-value
1CSMO	Breath+Hand	6(120)	35 (±0.05)	0.33 (±0.2)	0.144
Fly Fabric	Breath+Hand	6(120)	12 (±0.02)	-0.05(±0.3)	0.715
Ultralight Fly Fabric	Breath+Hand	6(120)	16 (±0.02)	0.15 (±0.266)	0.715
Mesh	Breath+Hand	6(120)	10 (±0.015)	0.1 (±0.33)	0.285
Floor Fabric	Breath+Hand	4(80)	9 (±0.02)	0.09 (±0.318)	0.593

Approximately half of the released *Aedes aegypti* mosquitoes were captured by the prototype tent in initial testing, but the proportions were irrespective of whether the tent was occupied by a person or not ($P = 0.24$) (Table 2.2). The human-occupied prototype tent trap successfully captured more colonized *Anopheles gambiae* mosquitoes than an unoccupied prototype tent ($P = 0.01$) (Table 2.2). It was observed by the tent operator that the open mesh tent ceiling of the prototype caused many mosquitoes to host seek on the top rather than being directed to the suction ports over the side walls where they could be captured. Likewise, it was noted that the modified capture net fitted over the fan housing on the prototype seemed to stifle proper air circulation, and the quality of the captured mosquitoes was poor because they had to pass through the fan. Upon re-engineering, the ceiling mesh was replaced with tent fabric and the vector trap was designed to fit in-between the manifold and the fan, so that the

fan pulled specimens into the trap and they would not pass through the fan blade in fashion similar to CDC-light traps.

Table 2.2: Proportions of mosquitoes captured by prototype tent

Species tested	Tent status	% captured [95% CI]
Aedes aegypti	human-occupied	58 [44.7, 71.3]
	empty	46 [32.5, 59.6]
Anopheles gambiae	human-occupied	38 [24.3, 51.1]
	empty	14 [4.3, 23.7]

Computational fluid dynamics modeling.

Initial modeling efforts at IST focused on the diameter of the air intake tubes and its impact on airflow velocity and volumetric flow rates. Preliminary design of the lengths of these tubes measured 20 inches for the front and back tubes, and 40 inches long for the side tubes. The tube diameters investigated were 3", 2", 1.5" and 1". For simplicity the tubes were all assumed to be straight, and the inlet/suction port was assumed to be conical. Several reference planes were inserted along the flow paths in the model to aide in the post processing of the flow results (Figure 2.5A). Figures 2.5A and B show typical air velocity intensity images for two different tube diameters. Figure 2.6 graphs the velocity of the air at the different reference plane locations detailed in Figure 2.5. As expected, the 1.5" and 1.0" diameter tubes produce significantly faster air flow than the 2.0" and 3.0" diameter tubes. The tent trap was designed to mimic the performance of the CDC updraft trap that had a reported air velocity between 19 and 39 in/s at the trap opening [241]. The simulated velocities at the intake entrance for all four tube diameters are less than 52 in/s, but the drastic increase in air velocity seen as the air enters the 1.0" and 1.5" diameter tubes could potentially cause damage to vector specimens.

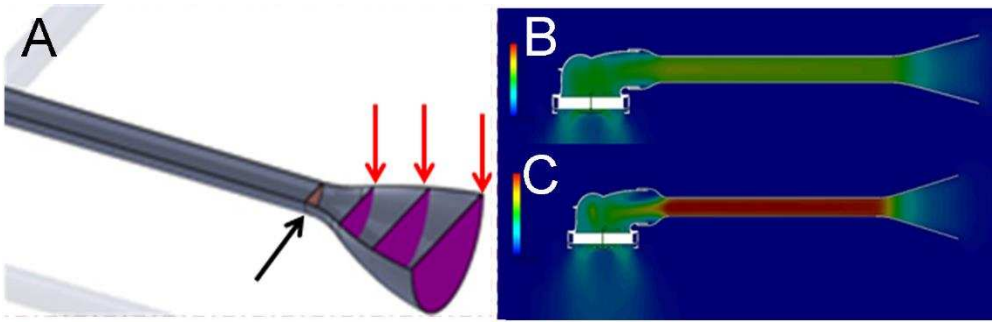

Figure 2.5: (A): Layout of the reference planes (red arrows) in CFD simulation models. Black arrow indicates final tube (pipe) diameter. (B and C): Cross section cut plot of air velocity in of 2" diameter x 20" Long tube and a 1.5" x 20" Long Tube, respectively.

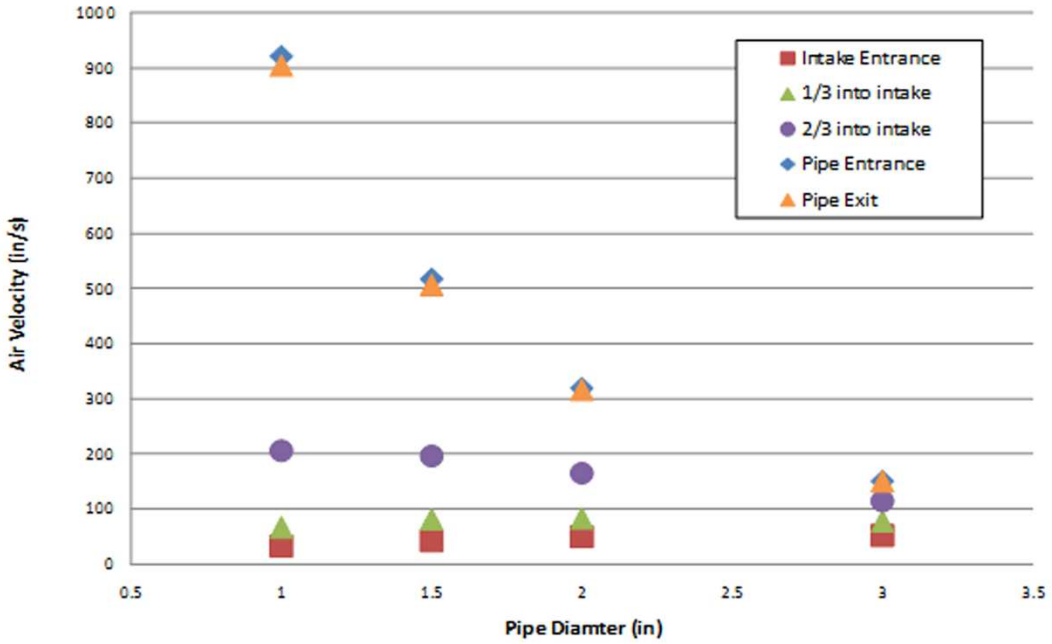

Figure 2.6: Relative airspeed velocities at various depths of the port compared to pipe diameter.

In addition to the air velocity, the volumetric flow rate in cubic feet per minute (CFM) for each tube diameter and length was calculated (Table 2.3). These data showed relatively small reductions in flow rates between the two lengths of tube for a given diameter, but much larger (~38%) reductions in flow rates as tube diameter decreased from 3" to 1". Based on these results, the diameter of the tubes were set at 2.0" in order achieve a balance between airflow

velocity and volumetric flowrate. This dimension also provided a gap between the tubes and the rain fly which helped to reduce the chance of water leaking through the rain fly material.

Table 2.3: Volumetric Flow Rate (in CFM) in both the 20" Length and 40" Length Pipes

	3" diameter pipe	2" diameter pipe	1.5" diameter pipe	1" diameter pipe
20" Length	39.23	35.59	31.82	24.39
40" Length	37.2	31.6	33.75	23.5

The relationship between the size and location of the exhaust air windows and the quantity of bait-laden air that was recirculated back into the suction ports was also examined using CFD. The overall goal was to determine which exhaust-window and suction-port configuration would give some amount of recirculated air so that there is bait-scented air at the collection port, drawing the vectors in, but still a sufficient amount of bait-scented air being blown into the area surrounding the tent to create an odor plume along which vectors can orient and follow into the suction port. It was assumed that all four mesh windows, one on each side of the tent, were the same size and shape and could be covered with a set of five patches of rip-stop tent fabric; three rectangular patches in the center (top, middle and bottom) and one triangular patch on either side. This configuration provided more control over the location and velocity at which the exhaust plumes exit the tent. In the model, each of these regions could be made permeable (mesh covered) or impermeable (tent fabric covered) as needed. The actual air permeability of the mesh windows located below each inlet port and the rip-stop fabric that was selected for the main body of the tent were determined based on empirical measurements of the pressure drop across these materials, and this was incorporated into the models. The suction ports were also modeled to create a lower profile that would make them easier to integrate with the tent structure. The results from three of these simulations are shown in Table 2.4 and Fig 2.3.

Table 2.4: Degree of volatile recirculation of all tent configurations calculated via computational fluid dynamics modeling.

		Recirculation (%)			
Config. #	Configuration Description	Lower Intake	Higher intake	Total	Comment
F02-003(A-B)	Top-center patch open	99	100	100	Nearly all exiting air recirculated back into tent
F02-002(C-D)	All patches open	57	39	48	Reduced recirculation at higher air intake
F02-004(E-F)	All center patches open	69	25	47	

Modeled results for 7.5" wide air suction ports and 2" diameter intake pipes.

For the first simulation (Fig 2.3A and 2.3B; F02-003), having only the top-center patch open created conditions where almost all of the bait-scented air coming from the tent was immediately recirculated back into the tent via the inlet port. For the simulations where all of the patches were open (Figs. 2.3C and 2.3D; F02-002) or where only the center patches were open (Figs. 2.3E and 2.3F; F02-004), a more balanced scenario appeared where some of the exhaust air is recirculated but some is expelled from each side of the tent to generate an odor plume. However, the air recirculation percentages for the upper and lower intake ports were different for the latter two simulations (Table 2.4). Based on these simulations it was determined that a balance of air recirculation and odor plume generation could be best achieved at each port by reconfiguring and manually manipulating the window panels while holding the fan speed steady.

Second generation tent trap modification and insectary testing.

The CFD re-designed tent trap was employed in laboratory tests whereby a two-inch gap of mesh was exposed in the center panel below each suction port to exhaust bait-scented air and maximize catch rates. The average air intake velocity was measured with an anemometer having

a 1" diameter impeller at the center of the lower and upper suction ports, and shown to be consistent with measurements from six other vectors traps (Table 2.5) [241].

Table 2.5: Airspeed at trap intake of IST tent trap against other vector trapping methods.

Test Parameter	IST tent	Standard CDC (under lid)	Inverted CDC (no intake bowl)	CDC updraft (with intake bowl)	CDC updraft (bowl and ball)	MMX Trap
Airspeed at edge of trap intake $(m/s)^2$	0.35-0.65	1.5-1.7[1]	2.2-2.6[1]	0.5-1.0[1]	1.7-1.9[1]	4.2-4.6[1]

[1] From Hoel, Kline and Allan. 2009. [241]

The recirculation of air exiting the mesh exhaust windows in this final design was also visibly confirmed using CO_2 fumes (generated by dropping dry ice into beakers of water) emanating from within the tent. With the re-designed trap, mosquito attraction to non-port locations was not observed. Comparison between HLC and the tent across rotational trap sessions in the laboratory showed that the tent trap caught, on average, 58.7% of colony *A. gambiae* caught by HLC ($P = 0.0068$) and 12.4% of colony *A. aegypti* caught by HLC ($P = 0.0085$) (Table 2.6). The colony *Culex quinquefasciatus* were not successfully caught by HLC or tent trap when released into the insectary.

Table 2.6: Number of colony-raised *Culicidae* captured in direct HLC vs. Tent, 30-minute trials in closed insectary.

Mosquito Species (release #)	Released	Exited holding container	HLC caught	Tent caught	*p*-value
Ae. aegypti (n=4)	160	142	97	12	0.0085
An. gambiae (n=20)	769	701	259	152	0.0068
Cx. quinquefasciatus (n=4)	160	104	0	3	0.391

p-value based on difference in trapping efficiency via two-tailed Paired t-test.

Field tests in Senegal.

In overnight tests in Senegal, the IST tent caught a range of 8.91-31.00 *Anopheles gambiae* s.l. per trap per night across three locations (Table 2.7). Only one other anopheline species was captured, which was a single female *An. coustani*. A subsample of tent-caught *Anopheles gambiae* s.l. from overnights were tested for species discrimination, of which 262 of 263 were identified as *An. gambiae* s.s. and one was identified as *An. arabiensis*.

Table 2.7: Number of *Anopheles gambiae* s.l. and *Culex* caught per tent per night in overnight testing in three locations in Senegal.

	An. gambiae s.l. Damboucoye, Senegal	*An. gambiae* s.l. Nathia, Senegal	*An. gambiae* s.l. Kedougou, Senegal	*Culex* group V Kedougou, Senegal
Trap Nights	11	8	23	23
Mean (95% CI)	31.00 (16.31-45.69)	23.13 (7.75 – 38.50)	8.91 (5.86 – 11.97)	42.57 (29.09-56.04)
Max Caught	81	50	25	138

In Kedougou overnight sampling, the tent caught 42.57 *Culex* Group V mosquitoes per trap per night (Table 2.7). In evening crepuscular period captures in the town of Kedougou, the IST captured 59.4% of the mean *Culex* Group V, 40% of *Ae. vittatus*, and 5% of *Ae. aegypti* relative to the HLC (Table 2.8). This capture efficiency difference between trapping methods was only statistically different for *Ae. aegypti*. *Culex pipiens* complex discrimination via PCR identified 10 of 22 in a randomly selected sample as *Cx. quinquefasciatus.* The remaining were likely to be one of the other morphologically and molecularly-indistinguishable *Culex* Group V known to be in the area.

Table 2.8: Number of *Culicidae* from crepuscular sampling in Kedougou, Senegal over 6 comparative 2 hour trap sessions. *p*-value based on difference in trapping efficacy via Wilcoxson's two-tailed matched pairs test with Gaussian or Exact value as noted.

	Crepuscular Sampling – # Tent caught	Crepuscular Sampling – # HLC Caught	*p*-value
Mean *Cx.* group V caught (95% CI)	7.33 (0.57-14.09)	12.33 (7.25-17.42)	0.4004*
Mean *Ae. aegypti* caught (95% CI)	0.50 (-0.37-1.38)	9.00 (0.89-17.10)	0.0355*
Mean *Ae.vittatus* caught (95% CI)	0.40 (-0.28-1.08)	0.83 (0.040-1.62)	1

p-value based on difference in trapping efficacy via Wilcoxson's two-tailed matched pairs test with Gaussian* or Exact value as noted

Discussion:

Mosquitoes and other vectors host seek from a distance by using the rapidly changing CO_2 concentrations found along the edge of the odor plume [242]. Nearer the host, mosquitoes tend to follow body odor plumes, body heat and water vapor to land on the host [135]. The IST trap utilizes these host-seeking cues by maximizing gas and volatile dispersion by a battery-powered active trapping system. This system uses a fan that creates suction at the intake ports to capture vectors following the odor plume; while simultaneously blowing air on the tent operator. This creates positive pressure inside the tent, pushing human bait-scented odor plumes that exhaust from each side of the tent.

Operator comfort and protection from weather and biting insects was ensured during tent use due to the utilization of modern camping tent materials. The benefit of having a durable, insect-proof and weather-proof tent that was lightweight and quickly and easily deployable was apparent in our field trapping. Southeastern Senegal is a vector-rich area that historically receives an average of 1,256 mm^3 of rain in the May-October rainy season [243]. It rained in the early evening or night approximately half of the time we performed these tests, and the

rainfly prevented any rain from entering the tent. Furthermore, the base was water-resistant and prevented pooling water from soaking into the inner tent chamber. Over the duration of our sampling (8/1/12-9/10/12), the average ambient temperature was 29.3ºC (25.7-34.6 ºC) and average humidity was 74.0% (55.5-85.4). The breathable tent fabric combined with the blowing fan made sleeping outdoors in the tent cooler and more comfortable than sleeping in our concrete field house. This is in contrast to a previous tent design made of plastic sheeting that was described as "very uncomfortable to sleep under" in the high ambient temperatures common in mosquito-prevalent areas [244]. The tent can be easily deployed, required less than ten minutes for set up and tear down. When the vector trap is properly screwed into the manifold and the door zippers are sealed, vector exposure risk to the human operator is essentially zero. This is in comparison to HLC, whereby even after 30 min. laboratory trials with the lights on, we aspirated partially blood fed mosquitoes from the insectary at the end of several tests. It can be expected that crepuscular or all-night HLC in field conditions and stealthier wild vectors results in significant human exposure to vector bites and risk of infection with vector-borne pathogens.

The air flow simulations were performed to optimize the air intake speed and determine the amount of bait-scented air that would recycled into the suction ports or leave the tent in odor plumes when various sections of the window mesh were covered or left open. Though the control box was designed to activate the fan at 3 different speeds, it was determined that the proper balance of bait-scented air recycled:exhausted was most easily adjusted by repositioning the tent fabric patch just under the suction port (Table 2.4), and all tests were performed at 9V fan speed. Nevertheless, the "optimal" tent configuration between fan speed and area of exhaust mesh exposed will highly depend on ambient conditions in the field.

Anecdotally, we noticed that mosquito captures were poor over evenings and nights when the weather was windy or consisted of continuous heavy rains.

In examining the comparison testing, it is of foremost importance to recognize that all sampling methods of biting vectors are biased, and only estimate the biting pressure and force of infection. The HLC is considered the gold standard, but it almost certainly overestimates true biting pressure because humans rarely sit still over hours with parts of their bodies exposed to biting vectors. The insectary data suggested that the tent may adequately capture wild host-seeking *Anopheles gambiae*, but might poorly to capture *A. aegypti* or *Cx. quinquefasciatus*. However, these experiments were performed in a closed insectary not much bigger than the tent itself, which likely interfered with odor plume formation outside the tent. Secondly, our colonized mosquitoes might have an altered or diminished capacity to host-seek like wild vectors. Indeed, the strain of *Cx. quinquefasciatus* we used in the lab tests failed to be attracted to humans either performing the HLC or operating the tent.

Crepuscular comparison sampling in Kedougou showed that, in opposition to our laboratory studies, the occupied tent does indeed attract anthropophilic *Culex* Group V (including *Culex quinquefaciatus*) mosquitoes, and all-night catches were dominated by these species (Table 2.7). This semi-urban environment was highly conducive to the container-breeding *Culex*, with overnight catch numbers ranging from 11 to 131 per tent per night. In comparison, the villages of Dambocouye and Nathia averaged 0-1 *Culex* spp. per tent per night. While overall *Culex* numbers at these villages are likely much lower based on numbers from aspiration catches, there may be a component of density dependence affecting the catch rate of the tent, as has been mentioned with the Ifakara tents [125,130].

Aedes activity in Kedougou followed the classic description of a strong evening crepuscular or dusk-biting period [243,245]. During our two hour sampling time, most *Aedes aegypti* were caught in a 30 minute time window of intense exophagic biting just as the sun was setting, and HLC was much more efficient at capturing this species. One hypothesis is that the narrow feeding interval may force the vectors into biting a more available target of the HLC operator. Alternatively, this species may resist being aspirated by the suction ports or may follow near host cues in different ways. During the HLC it was observed that *Aedes aegypti* were very small, flew in fast zig-zag patterns around one's exposed leg, and were reluctant to land, in contrast to larger and directly-landing *Cx. quinquefasciatus*. All-night captures in outlying villages demonstrated the tent's efficacy in capturing anthropophilic *Anopheles* species (31.0 HBR). Though we did not compare these capture rates to HLC in this study, the catch numbers/trap/night are in the same range of both indoor and outdoor HBRs reported from HLC and LTC in villages around Kedougou during the rainy season [232,235,246].

Interestingly, 99.8% of the identified tent captures in the villages were *An. gambiae* s.l. These data are in contrast to 94.4% *An. gambiae* s.l. that were aspirated, blood fed and resting in houses the morning after the night catch, and suggests that some vectors are preferentially trapped by the tent, while others might avoid it. The second most abundant aspiration-caught vector was *An. funestus* Giles (3.0% of aspiration collections), and based on this we would have expected around 20 of this species to captured in the tent while host-seeking during the nights prior to morning aspirations. However, *An. funestus* were never caught by the tent. This species is primarily described as having highly endophagic/endophilic biting/resting

patterns [3]. Its absence from tent collections may mean this species avoid the tent, it host-seeks differently than *An. gambiae*, or the tent is biased towards more exophagic biters [247].

Finally, the vector trap design limited the in-trap movement and desiccation of the captured vectors, and they did not pass through the fan to enter the trap. Taxonomically relevant morphological characteristics of 98.7% (*n*=674) of tent caught *Anopheles* were maintained, and the majority of mosquitoes survived and were flying in the trap container when it was removed from the manifold in the morning. The high specimen quality provides the ability to utilize these vectors in bioassays, for colonization, or for other techniques in which live, intact mosquitoes are necessary. Though nightly captures of anthropophilic mosquitoes were in line with those reported from the same region, future direct comparative testing against HLC (both indoor and outdoor) and testing of the proportion of pathogen-infected captured vectors will be necessary to properly evaluate this new system's relative efficiency and ability to estimate pathogen transmission. However in conclusion, the design and preliminary evaluation of the tent demonstrated how the combination of modern camping materials and CFD can be used to construct trapping system for anthropophilic mosquitoes. The implementation, comfort, and general safety of the trapping system was significantly higher than HLC. Thus, this IST trap provides a needed entry into the realm of safe, effective mosquito trapping, and represents a significant vector surveillance breakthrough which has the potential to dramatically enhance the capability of health officials to more effectively survey, and thus control, vectors that may transmit malaria and other vector-borne diseases.

Chapter 3: Sampling host-seeking anthropophilic mosquito vectors in West Africa: comparisons of an active human-baited tent-trap against gold standard methods. [2]

Introduction:

The current 'gold-standard' approach for the collection of anthropophilic vectors is the Human Landing Catch (HLC) technique. This approach involves the use of human participants as bait to attract arthropod vectors which can be collected as they attempt to bite. It has been well-documented that this technique, while effective and the benchmark for calculation of a variety of entomological measures, is laborious, skill-dependent, difficult to manage, and puts participants at direct risk of vector-borne disease [120,122,123,130,248]. Due to these concerns, development of alternatives to HLC for the capture of host-seeking vectors is important. Currently utilized alternative approaches include the Centers for Disease Control Light Trap [122], baited odor boxes [249], vector-electrocuting grids [248], and a variety of human-baited tent trap designs including the Mbita, Ifakara B and C, and Furvela tent traps [123,130,250]. These techniques have found varying degrees of success and applicability, though unreliability in biting estimation [122,130], failure to distinguish between endophagic/exophagic populations [248], and possible exposure to vectors have all been reported [130]. Furthermore, most of the previously-described tent traps have been passive rather than active designs, and did not make use of modern camping tent materials for increased durability, collector comfort, and ease of use and set up.

[2] Adapted from: **Krajacich BJ**, Slade JR, Mulligan RF, LaBrecque B, Alout H, Grubaugh ND, Meyers JI, Fakoli LS, Bolay FK, Brackney DE, Burton T A., Seaman J A., Diclaro JW, Dabire RK, Foy BD. (2015). Sampling Host-Seeking Anthropophilic Mosquito Vectors in West Africa: Comparisons of an Active Human-Baited Tent-Trap Against Gold Standard Methods. *Am J Trop Med Hyg*. 2015;92(2):415–421. doi:10.4269/ajtmh.14-0303. [121].

Previously, we described the design, laboratory testing, and initial field testing of the Infoscitex Tent, which features an active, battery-powered trapping design and is constructed with modern camping tent materials [120]. The IST tent meets the initial critical requirement of essentially zero exposure of the person in the tent to biting vectors, while still attracting and collecting vectors. It successfully sampled *Anopheles gambiae* s.l. and *Culex* group V but these initial comparative trials only investigated the crepuscular biting period in which the primary African malaria vector, *Anopheles gambiae,* rarely feeds [251]. Here, we directly evaluated the IST tent trap in overnight sampling against either LTC or HLC to analyze its safety and to compare the sampling efficiency of all three approaches in three regions of West Africa that are hyper or holoendemic for mosquito-borne diseases.

Materials and methods:

Operation, trapping, locations and sampling design.

The design and operation of the IST tent trap has been previously described [120]. For all experiments, a human collector rested or slept in the custom designed camping tent for the duration of trapping each night, with the doors zipped and the airflow panels on all four sides opened to make a 5 cm gap of exposed micro-mesh fabric. All tests were performed outside, and the tent was always deployed with its rainfly attached to protect the collector from rainfall that would often occur during the nights of testing. The IST tent is pictured in Figure 3.1 deployed in Liberia during testing, with arrows marking mosquito entry gaps through the rainfly. A battery-operated fan attached to the inside tent ceiling was activated through the control box in the tent by the collector at the start of each night's sampling period. The fan speed on the control box was set at 9V in all sampling. The fan blows air onto the resting collector and forces the collector's odors and volatiles out through the open micro-mesh panels, creating odor plumes

emanating from all four sides of the tent. This same fan creates negative suction to capture vectors that navigate up the odor plumes to the micro-mesh panel. Suction ports positioned immediately above each open mesh panel aspirate the host-seeking vectors into a trap, which is accessible to the collector inside the tent. We tested the tent from July to September, 2012 in southeastern Senegal, from June 7-30, 2013 in northeastern Liberia, and from August to October, 2013 in southwestern Burkina Faso. In all of these tests, the IST tent was directly compared to LTC or HLC. These trapping sessions were during portions of the rainy season in each location, when mosquito numbers and malaria transmission are high [106].

Figure 3.1: Infoscitex Tent in "location 1" of Bolahun, Liberia. Three openings for mosquito entry on this side are marked.

In Senegal, sampling was performed overnight (10:00 PM – 6:00 AM) over 16 nights in the semi-urban town of Kedougou which has been described previously [120]. Testing was performed with a rotation of two collectors that would sleep underneath either a deltamethrin-treated Permanet 2.0 (Vestergaard Frandsen SA, Denmark) with a CDC Light trap hung near

their feet, or sleeping in the IST that was placed >10m away. A treated net was used to prevent the sleeping human from being bitten if they inadvertently moved in the night and rested an exposed body part against the net; it was also shown in another study that the use of insecticide-coated nets had no effect on the ability of the CDC Light trap to catch *Anopheles gambiae* s.l. compared to untreated nets [119].

In Liberia, we compared IST tent trap to HLC in a 2x2 rotational design utilizing four local collectors over eight nights in the town of Bolahun. This area of Liberia is tropical rainforest, with an average yearly rainfall of 2346.6 mm (Climatic Research Unity of University of East Anglia). The area is holoendemic for malaria, with the Bolahun Health Care Center reporting year round cases of malaria (diagnosed via *Plasmodium falciparum* Histidine Rich Protein-2 specific rapid diagnostic test) with peak transmission during rains from May-October (Henry Jallah, Bolahun Health Center, personal communication). Two IST tents were deployed with HLC stations placed roughly 20 meters away. The collector performing the HLC was next to a canopy under which he could work if it rained. Two individuals would sleep in the IST tents and two individuals would perform HLC overnight (10:00 PM – 6:00 AM). Over this period, HLC collectors would sample 45 minutes with their legs exposed to biting vectors, which they would collect either via mouth aspirator (Model 412; John W. Hock Company, Gainesville, FL) or 15 ml conical tube (Thermo-Fisher Scientific, Lafayette, CO) and then place in a paper container. The final fifteen minutes of every hour the HLC collector was given a break in which they could rest. Due to sampling for only 45 minutes of each hour with the HLC, the collection numbers with the tent were multiplied by 0.75 so sampling periods were equivalent. Each collector sampled in each of the four locations (Tent #1, Tent #2, HLC #1 and HLC #2) twice.

In Burkina Faso, sampling was performed in the rural villages of Bougouriba and Diarkadougou. These are Sudan savannah zones that are hyperendemic for malaria transmission, with peak rainfall in July and August, and a yearly average rainfall of 829.29 mm (Climatic Research Unity of University of East Anglia) [252]. These villages are separated by ~10km, the Nabere forest, and the Bougouriba River which borders the fishing village of Bougouriba. Eight collections were completed in Bougouriba, and nine collections were completed in Diarkadougou using local collectors. Each night of collection involved an individual collecting by HLC (as described above) or with the IST tent for the first half of the night (9:00 PM – 1:30 AM), and then switching techniques for the second half of the night (1:30 AM – 6:00 AM) for a total of nine hours of collection. Collectors also rotated which technique they began with, and which village they sampled in throughout the duration of testing. This approach allowed for sampling to be continuous throughout the night for HLC, while not forcing the collector to attempt the laborious HLC process for nine hours consecutively, and allowed for slightly earlier sampling than Liberia to account for any earlier feeding. Additional aspiration sampling of indoor-resting blood fed mosquitoes was performed in both villages on the morning following overnight trapping utilizing InsectaZooka field aspirators (BioQuip Products, Rancho Dominguez, CA, USA).

Mosquito Identification and Processing.

After collection, mosquitoes were frozen until dead or killed with chloroform, and then identified to species or lowest taxa group using published keys [237,238,253]. Mosquito DNA was extracted from the head and thorax using the 96-well format DNeasy Blood & Tissue Kit following manufacturer instructions for insect tissue (Qiagen Sciences Inc. Germantown, MD, USA). *Anopheles gambiae* s.l. complex members were analyzed with multiplex polymerase

chain reaction (PCR) that distinguishes between *An. gambiae* sensu stricto (s.s.), *An. arabiensis*, *An.quadriannulatus,* and *An. merus/melas* [239]. *Plasmodium* sporozoites were detected from extracted DNA using a quantitative PCR (qPCR) protocol that distinguishes between *Plasmodium falciparum* and *Plasmodium ovale/vivax/malariae* [254].

Statistical Analysis.

Catch numbers for each method were compared on a nightly basis utilizing a Wilcoxon matched pairs test with Graphpad Prism Version 5 (GraphPad Software, La Jolla, CA, USA). Differences in *Plasmodium falciparum* infection rates and *Anopheles gambiae* s.l. complex species identities between methods were analyzed with 2x2 contingency tables and two-tailed Fisher's exact tests also using Graphpad Prism.

Ethical Considerations.

Sampling using human subjects was approved by the Institutional Review Board at Colorado State University (protocol #11-2874H), and by human subjects research reviews in each country (Senegal, 'Etude des vecteurs du paludisme en zone onchocerquienne au Sénégal; Liberia, EC/LIBR/012/033; Burkina Faso, 28-2013/CE-CM) in compliance with the Helsinki Declaration. Informed consent was obtained from all adult, paid mosquito collectors. Antimalarial prophylaxis drug regimens (either atovaquone-proguanil or doxycycline) were made available to all collectors, as well as diagnosis and treatment of any malaria infections, for the duration of testing. No collectors became ill over the course of the collections.

Results:

Capture efficiency of IST compared to reference methods.

In Senegal, the IST tent trap outperformed the LTC across 16 nights for *Culex* Group V (CGV) and *Anopheles gambiae* s.l. (Table 3.1, Table 3.3). Overnight mean catch numbers for *Culex* Group V and *An. gambiae* using LTC were 3.38 and 1.38, respectively. Mean catch numbers per night for the tent were 29.63 and 8.44 for CGV and *An. gambiae,* respectively, or 8.78 times the LTC catch rates for *Culex* and 6.14 times the rate for *Anopheles.* The difference in capture numbers were highly significant for both species (Wilcoxon signed rank, $p<0.0001$). Additionally, there were five nights where the LTC alone caught no mosquitoes. Due to this, it was most appropriate to analyze catch number correlation using a log(x+1) transformation as used elsewhere [122]. Collection numbers between methods correlated between LTC and IST tent for *An. gambiae* s.l. (Pearson r=0.5439, $p=0.0254$), but not for *Culex* spp. (Pearson r=0.3761, $p=0.1511$) (Figure 3.2).

Table 3.1: *An. gambiae* s.l. catch numbers per night and species composition of the Infoscitex Tent compared to reference methods

Location	Method	Trap Nights	Mean # *An. gambiae* s.l. caught (95% CI)	Total	% of Ref. Method	*p*-value[2]	Proportion *An. gambiae* s.s *(N)*	*p*-value[3]
Kedougou, Senegal	IST	16	8.44 (5.51-11.37)	135	613.67%	$p <$ 0.0001	1.000 (69)	1.000
	LTC	16	1.38 (0.51-2.24)	22	-		1.000 (16)	
Bolahun, Liberia	IST[1]	14	11.30 (7.32-15.29)	211	38.03%	$p =$ 0.0203	1.000 (86)	1.000
	HLC	14	29.71 (17.23-42.20)	416	-		1.000 (140)	
Bougouriba, Burkina Faso	IST	8	52.38 (21.23-83.52)	419	52.91%	$p =$ 0.0391	0.965 (141)	0.070
	HLC	8	99.00 (56.00-142.0)	792	-		1.000 (109)	
Diarkadougou, Burkina Faso	IST	9	57.56 (15.53-99.58)	518	71.56%	$p =$ 0.1289	1.000 (142)	0.228
	HLC	9	80.44 (30.89-130.0)	724	-		0.985 (130)	

[1]Mean IST catch values from Liberia were reduced by 0.25x to compare with the 45 minute HLC sampling duration for each hour of the testing. [2]*p*-values are calculated via Wilcoxon signed rank test comparing nightly catch numbers for each method. [3]*p*-values for proportion *An. gambiae* s.s. calculated via two-tailed Fisher's exact. "-" = Not applicable as it is the reference method

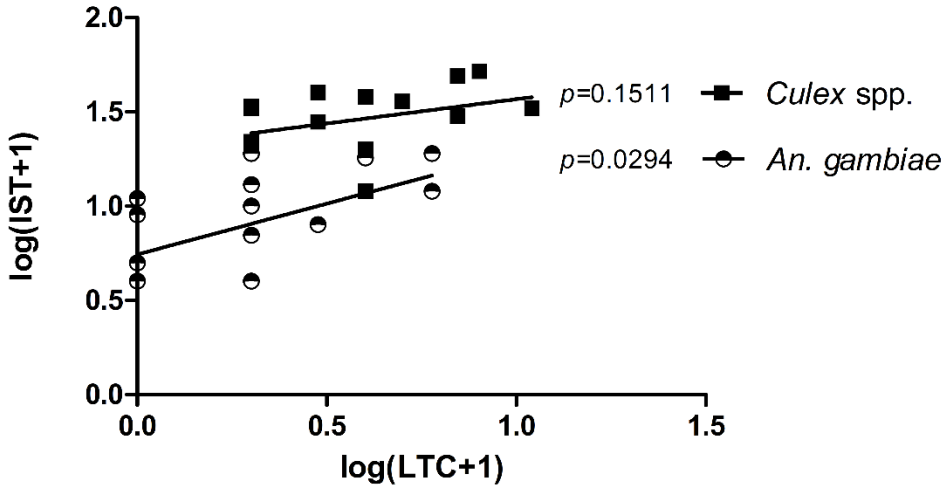

Figure 3.2: Correlation of log(x+1) transformed nightly catch numbers between the Infoscitex Tent and Light Trap Catch in Kedougou, Senegal

In Liberia, there were 14 successful pair-matched nights of sampling, with one night having battery failures in both tents at ~12:30 AM due to user error. Though the trap was restarted with new batteries, it is unknown how many mosquitoes were lost during this period, thus we removed these IST/HLC pair data from analysis. In this sampling, the dominant mosquito caught was *An. gambiae* s.l. (*n*=627). Mean nightly catch numbers for each method were 11.30 and 29.71 for IST and HLC, respectively. With IST tent data adjusted for 45 minutes sampling, the difference in median catch number between methods differed significantly (Wilcoxon signed rank test, p=0.0203). The IST tent trap caught no other species in Liberia, while the HLC caught a minimal number of *Culex* spp. (*n*=3) and *Mansonia uniformis* (*n*=1) (Table 3.3). Methods failed to correlate in this location (Pearson r=-0.2215, p=0.4466) (Figure 3.3). Additionally, collection numbers by HLC increased 1.8 or 4.1 mosquitoes per night of sampling on average (5.8 and 14.9%, respectively) depending on location (Figure 3.4, all nights included). In the same time period, IST efficiency increased by 0.29 or decreased by 0.56 mosquitoes per night of sampling on average (3.0% and -4.6%, respectively) over the two collection locations.

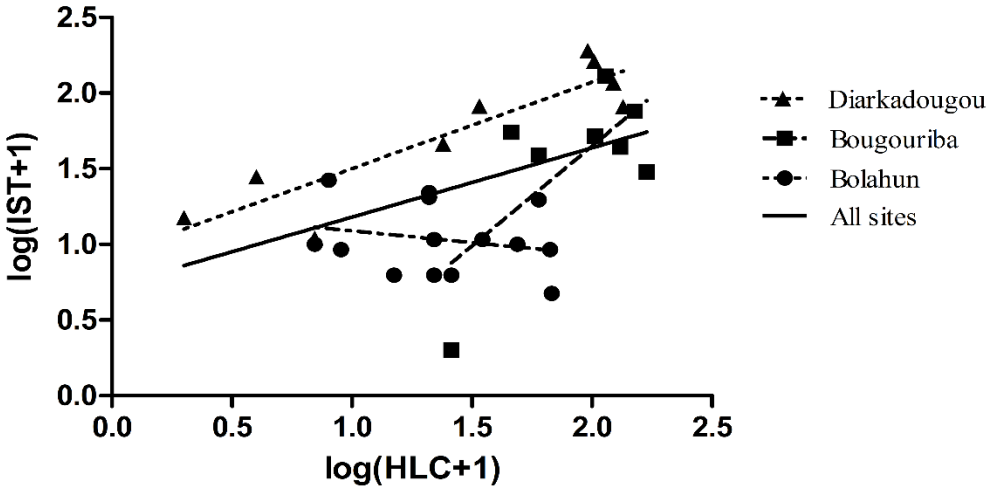

Figure 3.3: Correlation of log(x+1) transformed nightly catch numbers between the Infoscitex Tent and Human Landing Catch in one location in Liberia, and two locations in Burkina Faso. Dashed lines are linear regressions for each site individually, and the solid line is the regression from all sites combined.

Testing in Burkina Faso again analyzed the HLC against the IST tent. In this location, mosquito density and diversity was higher than in the other sites of this trial (Table 3.3). *An. gambiae* s.l. collection numbers for the tent were 52.38/night and 57.56/night in Bougouriba and Diarkadougou, respectively, and 99.00/night and 80.44/night for the HLC in these respective locations (Table 3.1). The difference in collection numbers between methods was significant in Bougouriba (p=0.0391), but not Diarkadougou (p=0.1289). Additionally, in Bougouriba *An. nili* were sampled more often by both HLC and the IST tent (48.50/night and 15.88/night, respectively) than in all other locations. The sampling efficiency difference between approaches was nearly significant (p=0.0547). The methods approached significant correlation in Bougouriba (Pearson r=0.6918, p=0.0573) and correlated significantly in Diarkadougou (Pearson r=0.8951, p=0.0011) (Figure 3.3). The increase in HLC sampling efficiency relative to the tent seen in Liberia was not seen in Burkina Faso, with equivalent increasing regression slopes in Bougouriba (HLC:9.4, IST:5.2), and an inverse relationship in Diarkadougou (HLC: -0.6, IST: 5.2) (Figure 3.4).

An. gambiae s.l. species discrimination, Plasmodium infection rates, and proportions with blood meals.

In Senegal, a proportion of captured *An. gambiae* s.l. were analyzed for species composition from each method, and of the 85 successfully amplified samples from the two methods, all were *An. gambiae* s.s. (Table 3.1). Due to low relative catch numbers, *Plasmodium* infection status was not analyzed at this location. Mosquitoes captured in Bolahun, Liberia were also analyzed by molecular sibling species discrimination (Table 3.1). Of the 226 successfully amplified specimens (86 IST, 140 HLC) all were found to be *Anopheles gambiae* s.s. Due to a limited presence of cattle and the early season sampling, it is unsurprising that *An. arabiensis* were not captured via either method at this location. Testing for *Plasmodium* sporozoites was performed on 82.3% of all HLC (n=386) and 94.5% of all IST tent (n=223) *An. gambiae* s.l. samples (Table 3.2). As sporozoite infection rates are low, the data were pooled for analysis by method. From this, no statistically significant difference was found in *Plasmodium* infection status of mosquitoes captured between the two methods (two-tailed Fisher's exact test, p=0.6782). The dominant *Plasmodium* species was *falciparum*, with only one mosquito testing positive for either *Plasmodium ovale/vivax/malariae*.

In both locations in Burkina Faso, proportions of *An. gambiae* s.s. and *An. arabiensis* were not significantly different with sampling via either method (Table 3.1). Unlike the other locations, *An. arabiensis* were collected with both methods, though in the tested sub-sample this species was still a small fraction of those caught (3.5% of tent caught *An. gambiae* s.l. in Bougouriba and 1.6% of *An. gambiae* s.l. in Diarkadougou). No significant differences in *Plasmodium* sporozoite infection status was observed from *An. gambiae* s.l. collected in either location (p=0.334 and p=1.000) (Table 3.2). *Plasmodium falciparum* was again the dominant

species of parasite, with only one IST-caught *An. funestus* testing positive for *P. ovale/vivax/malariae.*

Table 3.2: *An. gambiae* s.l. *P. falciparum* infection status, monthly entomological inoculation rate, and bloodedness based on collection method

Location	Method	Trap Nights	Proportion *Pf* sporozoite+	*p*-value	30-day EIR	Proportion with blood meal (# analyzed)	*p*-value
Kedougou, Senegal	IST	16	-	-	-	0.052 (135)	0.3662
	LTC	16	-	-	-	0.091 (22)	
Bolahun, Liberia	IST	14	0.036	0.678	16.219	0.005 (236)	< 0.0001
	HLC	14	0.047		41.563	0.214 (416)	
Bougouriba, Burkina Faso	IST	8	0.024	0.334	37.714	0.007 (282)	< 0.0001
	HLC	8	0.051		151.017	0.361 (288)	
Diarkadougou, Burkina Faso	IST	9	0.020	1.000	35.241	0.012 (258)	< 0.0001
	HLC	9	0.015		37.126	0.261 (230)	

"-" = Not tested due to low catch numbers, "*Pf*" = *Plasmodium falciparum*

Mosquitoes from the IST were significantly less likely to be blood fed than those caught via HLC in all locations ($p<0.0001$, two-tailed Fisher's exact) (Table 3.2). Also, many of the blood meals from HLC-captured mosquitoes were fresh (the blood meal was red), but this was less often true of the fewer blood meals observed in IST-captured mosquitoes. There was no statistical difference in the proportion of mosquitoes containing blood meals of those caught between LTC and the IST tent.

Discussion:

In this paper the efficiency and safety of a novel mosquito-trapping human-baited tent was examined against the 'gold standard' trapping methods in areas of West Africa with high endemicity for mosquito-borne disease. Overall, catch numbers for the IST tent trap were significantly higher than LTC in the one location tested (Kedougou, Senegal) and were not significantly different from catch numbers for HLC in 1 of 2 locations in Burkina Faso (Table

3.1). However, the mean number of *An. gambiae* s.l. captured per night was lower for the IST tent compared to HLC over the three sampling locations where these comparisons were performed (IST = 35.33 [20.49-50.17] *An. gambiae* s.l./night, HLC = 62.32 [42.63-82.01] /night), and in Bolahun, Liberia and Bougouriba, Burkina Faso the mean catch numbers were significantly different (Table 3.1). There are at least two reasons for the reduced sampling efficiency of the tent compared to HLC. First, the lack of visual and/or thermal cues from an individual in the tent could limit the mosquito's fine host-seeking ability after it has followed the odor plume to the open mesh panels, as has been suggested with the IST tent in crepuscular sampling of *Aedes* spp. and in other trapping methods for *An. gambiae* [120,232]. Secondly, it is possible that some mosquitoes or mosquito species are more able to escape from the suction at the ports positioned above the open-mesh panels. This possibility might be easily remedied by increasing the fan speed via the control box. Nevertheless, the reduced trapping efficiency of the IST tent could be fixed by having a collector sleep in the tent on sequential days, which would also alleviate temporal collecting bias (changes in daily catch numbers due to weather and other uncontrollable environmental and biological factors). Sequential sampling is not practical with HLC as it is difficult for collectors to be awake and focused many nights in a row.

One drawback to increased sampling is the necessity of maintaining charged batteries. Testing overnight in Burkina Faso lasted 9 hours as opposed to the 8 hour testing period in Liberia. Consequently there were several battery failures in Burkina Faso in last hour of sampling as this extended time drained the 12 volt, 14 amperage batteries that were available. Having larger batteries with higher amperage would likely have eliminated this problem. Previous descriptions of tent traps with passive designs highlighted the advantage of requiring no batteries in simplifying the tents' utility and lowering their cost. In contrast, the active design of

the IST mandates a power system that will increase its cost and the batteries need to be regularly charged after use. With cell phones and solar charging systems now often found in even the most remote areas, this latter critique may be less of a concern. Other than cost, the main trade-offs of a passive versus an active design may then be, a) the comfort of the collector in each respective tent, b) trapping efficiency when an odor plume passively diffuses from the tent versus when an odor plume is forced from the tent and c) the ability to trap exophagic mosquitoes. The forced air of the IST tent's fan blowing down on the user increases the comfort for the collector in the tent throughout the night; it remains to be seen if the resulting active odor plume is more successful for trapping mosquitoes than that from passive designs. Future experiments should be conducted to directly compare these two designs. To understand how the IST samples mosquitoes with exophagic or endophagic tendencies, it may be useful to examine the proportions of *Anopheles* species caught relative to their biting location preference as described by the literature [232]. While each field site has unique mosquito populations, in general *An. gambiae* s.s. have been observed biting indoors and outdoors in approximately equal proportions [81], while *An. arabiensis*, *An. funestus*, *An. nili* and *An. coustani* have been observed to bite outdoors more often than bite indoors [79,119,232,247,248]. Indoor resting aspiration collections in Bougouriba and Diarkadougou, respectively, conducted the morning immediately following overnight IST and HLC testing resulted in 95.3, 98.1% *An. gambiae* s.l. (98.5% of which are *An. gambiae* s.s.); but only 0.4, 4.1% *An. funestus*; 0.2, 0.4% *An. nili;* and 0% *An. coustani* (Table 3.3). Compared to both HLC and IST tent collections the night prior, significantly lower proportions of predominantly outdoor biting species were collected (Table 3.3). While some of these data could be explained by these secondary vector species having bitten indoors and then immediately exiting the house, they nevertheless highlight the importance of collecting outdoor biting

mosquitoes. Overall, the data show that the IST tent, while 29-62% less efficient than HLC, samples outdoor biting mosquito populations in approximately equal proportions to HLC, and the reduced efficiency could be addressed by sequential sampling. This is important because exophagic vectors are thought to be increasingly maintaining *Plasmodium* transmission in the face of wide-scale implementation of indoor vector control measures such as indoor-residual spraying, insecticide-treated bednets and use of indoor spatial repellents [3,255].

Finally, the *Plasmodium* sporozoite infection status was examined between *Anopheles* vectors captured between methods. No differences in Bolahun, Liberia nor in either location of Burkina Faso were observed (Table 3.2). Catch numbers in Kedougou, Senegal were too low for meaningful testing. These data were used to calculate the entomological inoculation rate (EIR), or the number of bites per person per day, expanded over the month of testing, and multiplied by the percentage of mosquitoes infected. The monthly EIR was high, and ranged from 16-151 infectious bites/person/month depending on the collection method and location (Table 3.2). At least three *P. falciparum* sporozoite positive mosquitoes caught via HLC contained blood in their abdomens. It is unknown if this blood was taken from the collector performing HLC, but as the overall percentage of blood fed *An. gambiae* s.l. was significantly higher in HLC approaches (27.1%) than tent (0.7%), it is likely that the blood was taken during HLC. Additionally, it was frequently noted that blooded mosquitoes from HLC often had fresh blood meals and many were blood fed to near repletion, which makes it unlikely that they were actively host-seeking after having bitten some other person in the village. These data clearly demonstrate the risk to HLC collectors, and it has been previously demonstrated that *Brugia* nematodes, *Plasmodium* protozoa, and arboviruses can be transmitted through probing alone [6,103,218,219]. HLC is also a skill-dependent approach (Table 3.3 and Figure 3.4). In Liberia, the trained collectors were

novices, while in Burkina Faso, the collectors had performed this method many times previously on other projects. Reflecting this, the sampling efficiency of HLC in Liberia increased over successive nights, but the efficiency did not correspondingly increase with the tent. This is compared to the efficiencies of the two methods in Burkina Faso which held relatively constant to each other. On the other hand, the Liberian collectors seemed slightly more diligent at preventing mosquito engorgement from their exposed legs. Compared to HLC, the IST tent affords a standardized approach that is not skill-dependent, has minimal risk, and removes the over-sampling bias associated with HLC (performing an unnatural behavior of sitting outdoors overnight, exposing their legs purposely to biting vectors).

In light of the dangers presented from Human Landing Catch, alternative methods of modern sampling are needed. In our testing, we found that the Infoscitex Tent was much safer than Human Landing Catch. This approach more closely mimics realistic overnight behavior of individuals in an area, having them sleep throughout the night, rather than intentionally expose themselves to mosquito biting. Additionally, it provides a standardized platform that removes the skill component present in HLC whereby previous experience, or a willingness to allow mosquitoes to probe fully can increase catch numbers. The primary disadvantages of the tent are likely to be cost relative to other methods (although it has not yet been marketed), and electric infrastructure must be present in the sampling area to adequately charge batteries for frequent sampling. However, long term costs may be minimized if it is decided that ethical considerations could be minimized and drug prophylaxis would not be needed, and there may be additional cost reductions associated with training fewer collectors. Ultimately the IST tent approach presented here catches fewer numbers of mosquitoes compared to HLC, but allows for a more robust

sampling design, may more accurately reflect true disease risk, and limits disease risk to human volunteers.

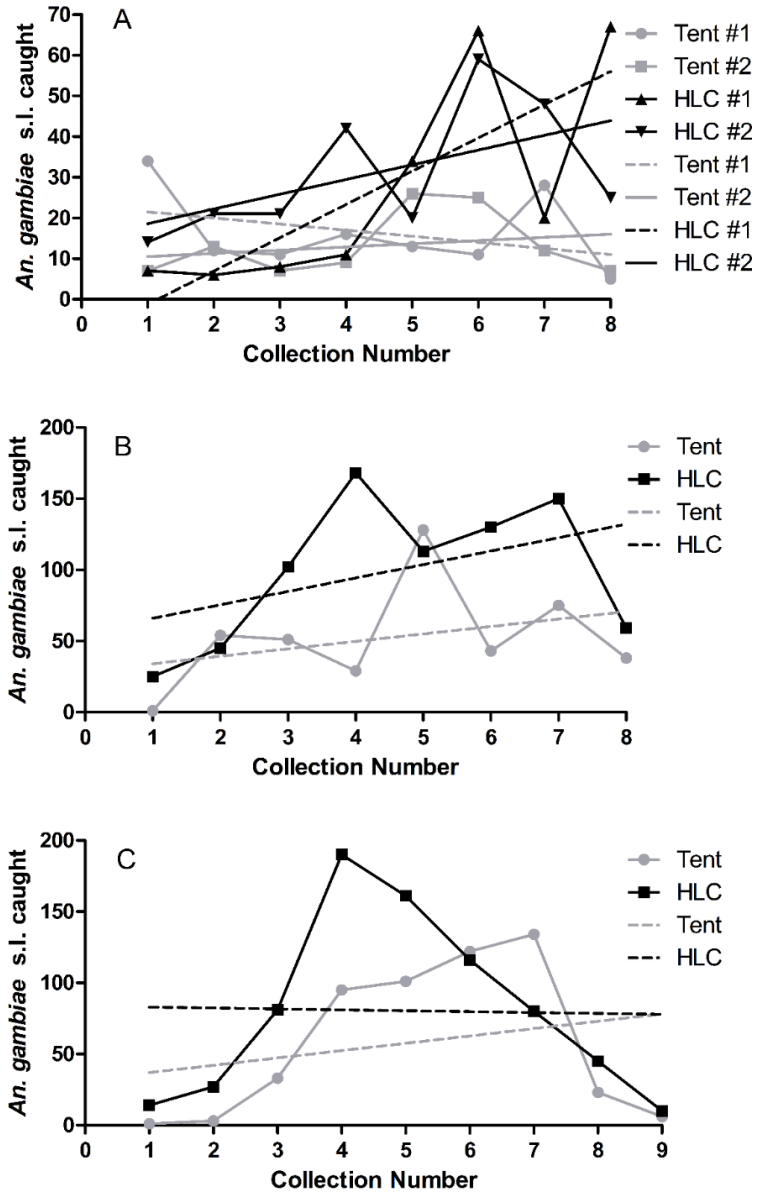

Figure 3.4: Bolahun, Liberia (A); Bougouriba, Burkina Faso (B); and Diarkadougou, Burkina Faso (C) nightly catch numbers with IST or HLC approaches. Dashed lines are linear regressions of each trapping method in that location. Slopes of Tent and HLC regressions differed significantly in Bolahun (Analysis of Covariance, p=0.0043), but not in Bougouriba (p=0.6702) or Diarkadougou (p=0.6229).

Table 3.3: Nightly catch numbers of all caught species over all locations/methods.

		Average/night [percentage of species in total catch] (*n* # total)									
Location	**Method**	***Anopheles gambiae* s.l.**	***An. nili***	***An. funestus***	***An. coustani***	***An. pharoensis***	**Other *Anopheles***	***Culex* spp.**	***Aedes* spp.**	***Mansonia uniformis***	**Total**
Kedougou, Senegal	IST	8.44 [22.1%] (135)	0.00 [0%] (0)	0.00 [0%] (0)	0.00 [0%] (0)	0.00 [0%] (0)	0.00 [0%] (0)	29.63 [77.6%] (474)	0.13 [0.3%] (2)	0.00 [0%] (0)	611
	LTC	1.38 [26.5%] (22)	0.00 [0%] (0)	0.063 [1.2%] (1)	0.00 [0%] (0)	0.00 [0] (0)	0.00 [0%] (0)	3.38 [65.1%] (54)	0.31 [6.0%] (5)	0.00 [0%] (0)	83
Bolahun, Liberia	IST	15.07 [100%] (211)	0.00 [0%] (0)	0.00 [0%] (0)	0.00 [0%] (0)	0.00 [0%] (0)	0.00 [0%] (0)	0.00 [0%] (0)	0.00 [0%] (0)	0.00 [0%] (0)	211
	HLC	29.71 [98.8%] (416)	0.00 [0%] (0)	0.00 [0%] (0)	0.00 [0%] (0)	0.00 [0%] (0)	0.00 [0%] (0)	0.21 [0.7%] (3)	0.00 [0] (0)	0.14 [0.5%] (2)	421
Bougouriba, Burkina Faso	IST	52.38 [67.9%] (419)	15.88 [20.6%] (127)	1.50 [1.9%] (12)	2.00 [2.6%] (16)	1.88 [2.4%] (15)	0.25 [0.3%] (2)	2.63 [3.4%] (21)	0.63 [0.8%] (5)	0.00 [0%] (0)	617
	HLC	99.00 [60.7%] (792)	48.50 [29.7%] (388)	11.00 [6.7%] (88)	1.13 [0.7%] (9)	0.25 [0.2%] (2)	0.25 [0.2%] (2)	1.00 [0.6%] (8)	2.00 [1.2%] (16)	0.00 [0%] (0)	1305
	Asp	N/A [98.1%] (790)	N/A [0.2%] (2)	N/A [0.4%] (4)	N/A [0%] (0)	N/A [0%] (0)	N/A [0.1%] (1)	N/A [1.0%] (8)	N/A [0%] (0)	N/A [0%] (0)	805
Diarkadougou, Burkina Faso	IST	57.56 [87.9%] (518)	1.11 [1.7%] (10)	4.78 [7.3%] (43)	0.22 [0.3%] (2)	0.00 [0%] (0)	0.67 [1.0%] (6)	1.00 [1.5%] (9)	0.11 [0.2%] (1)	0.00 [0%] (0)	589
	HLC	80.44 [89.6%] (724)	1.89 [2.1%] (17)	4.33 [4.8%] (39)	0.56 [0.6%] (5)	0.00 [0%] (0)	0.33 [0.4%] (3)	1.89 [2.1%] (17)	0.33 [0.4%] (3)	0.00 [0%] (0)	808
	Asp	N/A [95.3%] (988)	N/A [0.4%] (4)	N/A [4.1%] (43)	N/A [0%] (0)	N/A [0%] (0)	N/A [0.2%] (2)	N/A [0%] (0)	N/A [0%] (0)	N/A [0%] (0)	1037

Chapter 4: Validation of Near Infrared Spectroscopy for age-grading of wild *Anopheles gambiae* [3]

Introduction:

Knowledge of the age structure of mosquitoes is critical to understand the spread of vector-borne disease. *Anopheles gambiae,* the major vector of malaria-causing *Plasmodium* spp. parasites, must undergo a 10-12 day extrinsic incubation period (EIP) during which a parasite develops into the human-infectious sporozoite stage [136]. A large portion of the mosquito population is therefore unable to spread parasites, and very old mosquitoes are disproportionately important to the transmission cycle. Recent work has brought about the idea of "evolution-proof" insecticides that preferentially target older age classes of mosquitoes that have already exhausted most of their reproductive potential, but are at their peak of their disease-transmission potential [88]. Functionally, this approach can be performed through the use of existing insecticides in lower doses that would only be fatal to older, infection-stressed adults, or through fungal or biological control measures that shorten life or are disproportionately effective against older mosquitoes [89,256–258]. Rapidly assessing population-level age-structure is critical to evaluating the efficacy of these and other control endeavors, but there currently are limited tools available to do so [136].

The majority of our understanding of vector age-structure has relied on female ovary dissections, especially characterizing the status of ovarian tracheoles [138,139]. If a mosquito has not yet undergone a gonotrophic cycle (nulliparous), the tracheoles are in tightly coiled

[3] For submission to *Parasites and Vectors.* Krajacich BJ, Alout H, Meyers JI, Dowell FE, Foy BD. Validation of Near Infrared Spectroscopy for age-grading of wild *Anopheles gambiae*.

"skeins." However, if one or several of the tracheoles are distended, an egg clutch has likely successfully developed (parous). This methodology has been utilized widely as it is a relatively simple dissection procedure, though it results in a coarse metric of age as many mosquitoes become parous early in life. Thus, this method can only distinguish very young from all other age classes. A subsequent dissection technique was developed by Polovodova that counts the ovarian dilations (relics of past egg clutches) that can be found on the distal end of the ovariole [140,259]. This technique is highly technically demanding, requiring an injection of paraffin oil into the ovaries via the oviduct, and delicate removal of the ovary without damage [142]. Few researchers have successfully used this technique due to these limitations [141,260,261], and others have indicated that even when done successfully, the approach is flawed because of the presence of "rogue" ovarioles that indicate a gonotrophic cycle that did not occur [144,145]. These non-diagnostic ovarioles increase in their frequency as the mosquito ages, and can also be confounded by taking multiple blood meals between age clutches [262,263]. All dissection approaches are also limited by the speed of the dissection, making high throughput processing difficult [264].

A range of alternative chemical and molecular approaches have been considered to address these limitations, including detection of fluorescent pteridines, changes in the ratio of cuticular hydrocarbons, transcriptomic variation, proteomic analyses, and most recently the use of near-infrared spectroscopy (NIRS) [149,152,159,265]. NIRS is a fast and non-destructive technique that detects changes in the diffuse reflection of light due to the rotation, bending and stretching of C-H, N-H, O-H and other bonds within the near-infrared spectrum (780-2526 nm) [161]. This technique was first utilized for the study of moisture content of various grain species, but has recently been used with insects [151,161,162,266]. Mayagaya *et al.* applied this approach

to age *An. gambiae* s.l. as young (< 7 days old) and old (≥ 7 days), and to speciate them into *An. arabiensis* and *An. gambiae* s.s. [163]. Subsequently, this approach has been utilized with mosquitoes reared in semi-field enclosures and on some wild-caught adults [164,165,263,267]. NIRS age-grading has demonstrated some robustness, with accuracy remaining consistent with varying developmental status (i.e. oviposition) [263]. However, species diversity, diet, and rearing temperature may alter the accuracy of NIRS-based age grading techniques [166–168]. These studies have found that the inclusion of a higher number of these variables in calibration models increases overall prediction accuracy when applied to varied test sets.

Lacking, to date, is an evaluation of NIRS's age-grading ability with wild-caught vectors compared to classical measures of age-classification as external validators of age (parity status and the presence of sporozoites in the salivary gland). With a combination of these two independent measures, NIRS-predicted ages can be compared to known nulliparous (assumed young), parous (assumed mid-to-old), or sporozoite positive (known old) wild mosquitoes to validate and assess the accuracy of this methodology. In this study, we performed such an evaluation, using calibration models of *An. gambiae* s.l. generated from wild larvae collected in the field to predict age classes with the above external validators. Additionally, as a proof of concept for future studies, we explore the age structure changes resultant from a population level control measure with the mass drug administration (MDA) of the endectocide ivermectin.

Methods:

Mosquito rearing:

Two strains of laboratory-reared *An. gambiae* mosquitoes were utilized in this study: "CSU-IRSS" and "CSU-G3." *An. gambiae* s.s. strain "CSU-IRSS" mosquitoes were recently colonized from field-caught larvae collected in southwestern Burkina Faso by the Institut de

Recherche en Sciences de la Santé (IRSS), shipped to Colorado State University (CSU). *An. gambiae* strain "CSU-G3" mosquitoes were originally colonized in 1975, and have been in colony at CSU for hundreds of generations [268]. Both colony strains were reared at 28 ± 2°C and 80% humidity under a 14:10 light:dark photoperiod. Larvae were hatched in 15 liters of tap water with ground Tetramin fish food supplementation in 44-liter bins.

Field-caught larvae were collected in the rainy season of 2013 and 2014 in natural pools in southwestern Burkina Faso around Soumousso (11.01681, -4.052893), Kodeni (11°10'N; 4°15'W), Bougouriba (10.9313363, -3.6667348), and Diarkadougou (10.9014352, -3.5514027) with a mosquito dipper (#1132BQ, BioQuip Products, Rancho Dominguez, CA). Larvae and their collection water were placed in plastic water bottles and transferred to Bobo-Dioulasso, Burkina Faso (11.1727, -4.3304). They were placed into 44-liter bins stored under a shaded roof. Larvae were kept in water from the sources they were collected in for rearing, and kept outside under a shaded roof which exposed them to the natural variation in humidity and temperature present in the region.

Adult mosquito groups of roughly 100 per time point from both the laboratory and field were collected in 24 hour emergence periods (day 0). They were separated via aspiration (InsectaZooka field aspirator – BioQuip Products, Rancho Dominguez, CA), and placed in containers with a cotton ball soaked in 10% sucrose and water provided *ad libitum*. These mosquitoes were held for either 3, 6, 9, 12 or 15 days prior to scanning. Blood meals were provided via the arm of a human volunteer at day 2 post emergence (Colorado State University Institutional Review Board approval #09-1148H), and the evening prior to their designated scan day (12-18 hours later). Prior to scanning, all adult mosquitoes were classified under light microscopy to species by taxonomic key [238].

Near-infrared spectroscopy/scanning:

At days 3, 6, 9, 12, and 15, mosquitoes were killed with trimethylamine or chloroform before scanning (for all strains except CSU-G3). CSU-G3 were killed via freezing at -20°C for approximately 30 minutes, and then left for another 30 minutes to equilibrate to room temperature (~25°C). Our NIRS set-up and data processing largely follows previously published methodology [163]. Mosquitoes were placed on their dorsal side on a spectralon plate, and their head/thorax was scanned with a LabSpec4i spectrometer with a 3mm bifurcated reflectance probe at a height of 3mm (ASD Inc., Boulder, CO). The instrument takes 20 spectra from each mosquito which it stores as an average spectrum. Absorbance values are recorded from 300-2500nm across the near-infrared region.

Data analysis and model creation:

Spectra were converted from the .asd format to the .spc format for use with the GRAMS IQ v9.1 statistical software package (Thermo Scientific, Waltham, MA) using the ASD to SPC Version 6.0 software provided with the instrument (ASD Inc.), as wavelength vs. Log(1/R). Spectra were manually viewed using the IQ Predict software, and any spectral profiles that were flat (indicating a poor scanning position or a moving mosquito) or 'wavy' (indicating a poorly stabilized instrument) were discarded from analysis. Models from six different sample sets were created (Table 4.1) on mean-centered spectra using partial least squares (PLS) regression with a 'leave-one-out' cross validation approach in the GRAMS IQ software package. This technique improves models by leaving one spectrum out of model creation, generating a model with the remaining n-1 samples, predicting a value for the left out spectra, then repeating this process until all samples are included [166]. Predictions from this process are labeled here as "cross-validation." All models utilized the 700-2,350 nm spectral range to avoid the noise present on the

edges of the scanned range due to poor sensor sensitivity in these regions [168]. Sample sizes for each model were based on collection amounts for each variable. In general, sample sizes were held to 40 samples per time point in an effort to make the sample sizes equal across all days. In some instances this was impossible due to low sample numbers; in these cases all samples were held to a similar value (i.e. ~32 in M2).

For selection of the number of factors of the model (latent variables), a Durbin-Watson statistic was calculated based on the regression coefficient plots for factors 1-18 [269]. This statistic allows for quantification of the amount of noise in this plot, and it has been reported that large increases in this figure indicate over-fitting [269,270]. This metric with the Predicted Residual Sum of Squares (PRESS) and Standard Error of Cross-Validation (SECV) generated from the leave-one-out process allows identification of inflection points on the graph at which point over-fitting increases with added factors (Figure 4.1) [270,271]. Two different factor models were selected for each sample set, and evaluated empirically for optimal prediction based on accuracy in validation and test sets.

Table 4.1: Calibration Model sample size and collection locations of single (M1-M4), and multi-source (M5-M6) origin. BG: Bougouriba, Burkina Faso, DK: Diarkadougou, Burkina Faso, NP: Nulliparous, SP: Sporozoite Positive

Single Source Models:					**Age**			
Model Name	**Location/Year**	**Source**	*Day 3*	*Day 6*	*Day 9*	*Day 12*	*Day 15*	*Total*
"M1"	Soumousso 2013	Field	37	40	39	39	23	178
"M2"	Kodeni 2014	Field	31	32	30	32	31	156
"M3"	CSU-IRSS 2015	Laboratory	40	39	12	35	34	160
"M4"	CSU-G3 2012	Laboratory	40	40	40	40	40	200
Multi Source Models:					**Age**			
Model Name	**Location/Year**	**Source**	*Day 3*	*Day 6*	*Day 9*	*Day 12*	*Day 15*	*Total*
"M5"	Soumousso 2013	Field	37	40	39	39	23	178
	Kodeni 2014	Field	31	32	30	32	31	156
	BG 2013 NP	Field	19					19
	DK 2013 NP	Field	29					29
	BG 2013 SP	Field				10		10
	DK 2013 SP	Field				8		8
		Total	116	72	69	89	54	400
"M6"	Soumousso 2013	Field	37	40	39	39	23	178
	Kodeni 2014	Field	31	32	30	32	31	156
	BG 2013 NP	Field	19					19
	DK 2013 NP	Field	29					29
	BG 2013 SP	Field				10		10
	DK 2013 SP	Field				8		8
	DK 2014	Field			40			40
	CSU-IRSS 2015	Laboratory	40	39	12	35	34	160
		Total	156	111	121	124	88	600

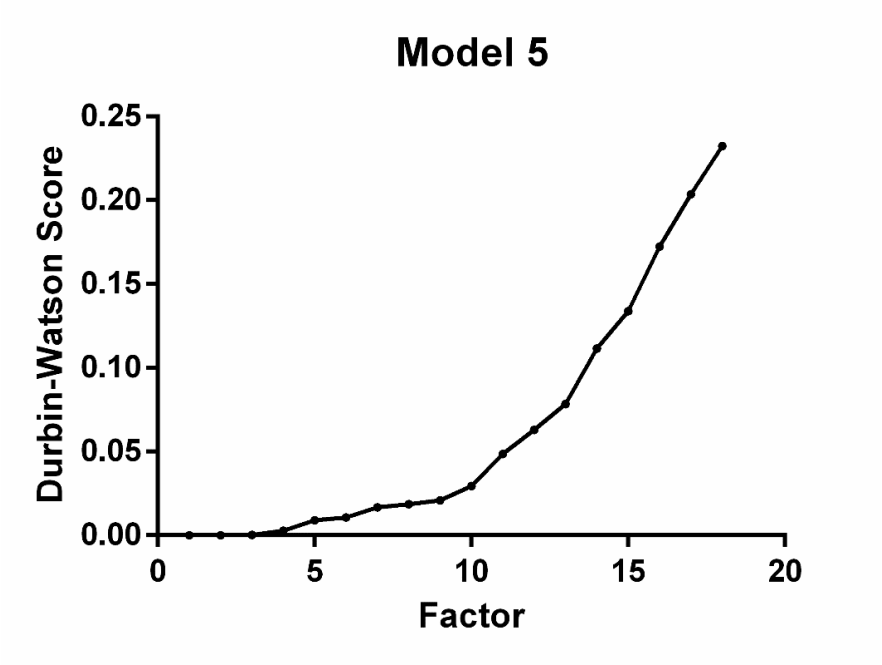

Figure 4.1: Example of Durbin-Watson scores across differing numbers of PLS factors. The first large increase in slope is after factor 10, thus this was one of the two models chosen for analysis. The second model was chosen at factor 7.

Validation and independent test sets:

Validation sets (Table 4.2) were created by leaving ~20% of the spectra out of the sample set prior to model creation. These samples were chosen at random using the "Pick me!" random file selection software (Matías Nahuel Carballo) [272]. As above, we attempted to hold sample sizes consistent across days. Means for each day were calculated and analyzed via an Analysis of Variance (ANOVA) test with Tukey's multiple comparison's adjustment with GraphPad Prism v6.1 (GraphPad Software, San Diego, CA).

Independent test sets (Table 4.3) were utilized to determine overall model accuracy on a non-biased sample set [163]. These samples were considered to be independent as they were reared separately, and were from unique collection days and/or locations relative to samples in

the model [168,273]. Test set 1 (TS1) was comprised of adults reared from various larval sources from Burkina Faso, collected in 2013 and 2014.

Table 4.2: Single and multi-source validation set sample number and locations.

Single source validation sets:			Age					
Validation Set Name	**Location/Year**	**Source**	*Day 3*	*Day 6*	*Day 9*	*Day 12*	*Day 15*	*Total*
"V1"	Soumousso 2013	Field	10	10	10	10	5	45
"V2"	Kodeni 2014	Field	8	8	7	8	8	39
"V3"	CSU-IRSS 2015	Lab	10	10	3	10	8	41
"V4"	CSU-G3 2012	Lab	10	10	10	10	10	50

Multi source validation sets:			Age					
Validation Set Name	**Location/Year**	**Source**	*Day 3*	*Day 6*	*Day 9*	*Day 12*	*Day 15*	*Total*
"V5"	Soumousso 2013	Field	10	10	9	10	5	44
	Kodeni 2014	Field	8	8	7	8	7	38
	Total		18	18	16	18	12	82
"V6"	Soumousso 2013	Field	10	10	9	10	5	44
	Kodeni 2014	Field	8	8	7	8	7	38
	Diarkadougou 2014	Field			9			9
	CSU-IRSS 2015	Lab	10	10	3	10	8	41
	Total		28	28	28	28	20	132

Test sets 2 and 3 (TS2, TS3) were comprised of adult mosquitoes caught via indoor aspiration or by a human baited tent-trap and were noted for blood fed status, scanned, parity dissected (see below), and stored in individual 1.5-ml tubes containing t.h.e. 100% indicating desiccant beads (#EM-DX0017-1, EMD Millipore, Billerica, MA) for sporozoite analysis (see below) [120,121]. TS3 consisted of the nulliparous and parous mosquitoes, while TS2 consisted of the nulliparous mosquitoes compared to the sporozoite positive mosquitoes.

Table 4.3: Independent test set sample numbers and locations. "Unknown 2014" were from either Kodeni, Soumousso, or Diarkadougou in 2014.

		Age				
Independent Set Name	**Location/Year**	*Day 3*	*Day 6*	*Day 9*	*Day 12*	*Total*
"TS1"	Soumousso 2013	7	0	5	0	12
	Kodeni 2014	1	27	3	10	41
	Soumousso 2014			2	8	10
	Diark 2014	1		8		9
	Unknown 2014				3	3
	Total	9	27	18	21	75

		Age		
Independent Set Name	**Location/Year**	*Day 3*	*Day 12*	*Total*
"TS2"	BG 2013 NP	21		21
	DK 2013 NP	29		29
	BG 2013 SP		10	10
	DK 2013 SP		8	8
	Total	50	18	68

		Age		
Independent Set Name	**Location/Year**	*Day 3*	*Day 5-15*	*Total*
"TS3"	BG 2013 NP	21		21
	BG 2013 P		175	175
	DK 2013 NP	29		29
	DK 2013 P		111	111
	Total	50	286	336

Parity dissection and Sporozoite analysis:

After scanning, a random batch of approximately 20 wild adult mosquitoes caught per collection day in the villages of Bougouriba and Diarkadougou, Burkina Faso in 2013 were dissected to assess parity status via Detinova's method under light microscopy [7,139]. From the saved head/thorax, DNA was extracted with the 96-well format DNeasy Blood and Tissue Kit (#69504, Qiagen, Hilden, Germany), and analyzed for the presence of *Plasmodium* spp. sporozoites via Taqman quantitative-Polymerase Chain Reaction (qPCR) [254].

Effects of ivermectin mass drug administration on age structure:

During the summer of 2013, we collected mosquitoes via aspiration and tent-trap surrounding the mass drug administration (MDA) of the anthelminthic ivermectin to humans in southwestern Burkina Faso to control lymphatic filariasis and onchocerciasis. The results of this study have been reported elsewhere [7]. During this trial parity dissections were performed as described above on mosquitoes collected in treatment and control villages two weeks prior to MDA, and for four weeks after. Results of NIRS age prediction were compared to the parity status of mosquitoes caught during this time period post intervention.

Results:

Accuracy of cross-validation and validation set predication:

In cross-validations, the age of younger mosquitoes (days 3 and 6) were over predicted (0.4-3.8 days), while middle age (day 9) mosquitoes tended to be slightly under predicted (0.6-1.4 days), and the oldest (days 12 and 15) were under predicted (1-4.1 days) (Figure 4.2, Tables 4.4 and 4.5). This follows what has been reported previously with age-classification of insects with NIRS [163,166,167]. Due to the over prediction in day 6 and the under prediction in day 9, these groups are difficult to distinguish from one another (Figure 4.2).

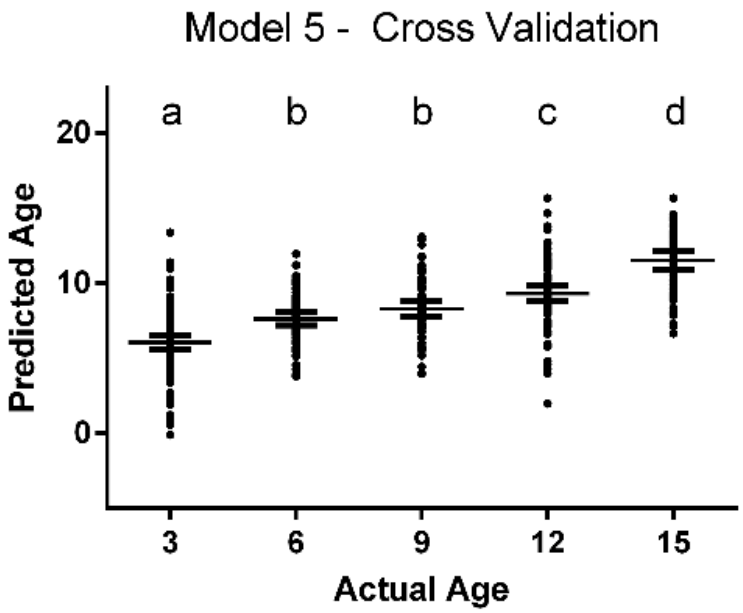

Figure 4.2: Example cross-validation prediction for a multisource model used in the study. Differing letters between groups indicate differing means at $P < 0.05$ (ANOVA, Tukey's multiple comparisons correction).

In general, old mosquitoes were distinguishable from young in both cross validation and validation sets. The highest overall accuracy for correctly classifying validation sets as young (< 7.5 days) or old (> 7.5 days) was with models from the two laboratory strains of mosquitoes, M4: CSU-G3 and M3: CSU-IRSS, with 82.5% and 77.5% accuracy (Tables 4.4, 4.6). This cutoff was based on those previously utilized (7 days), but equally between days 6 and 9 [163]. This validation set accuracy decreased as the models became more outbred (field caught populations) with M1: Soumousso, Burkina Faso 2013 predicting correctly 74.4% of the time, and M2: Kodeni, Burkina Faso 2014 predicting correctly 67.0% of the time. The multi-source models had poor overall accuracy on the validation sets (54.3 and 62.5% for M5 and M6, respectively) as they tended to more strongly over predict young spectra, pushing them over the 7.5 day threshold for comparing "young" vs. "old" (Figure 4.3, Panels M5, M6).

Table 4.4: Cross validation and validation set accuracy for the four single source models. "Over/under predicted" is the predicted minus actual age.

	M1 - Cross Validation			**M1 - Validation**	
Actual Age	Mean Predicted Age (95% CI)	Over/under predicted	Actual Age	Mean Predicted Age (95% CI)	Over/under predicted
3	5.3 (4.6-6.0)	2.3	3	6.8 (5.5-8.0)	3.8
6	7.7 (7.1-8.3)	1.7	6	7.3 (6.2-8.4)	1.3
9	8.4 (7.7-9.1)	-0.6	9	9.0 (7.7-10.4)	0
12	10.5 (9.8-11.2)	-1.5	12	11.3 (9.5-13.1)	-0.7
15	11.7 (10.6-12.9)	-3.3	15	12.2 (8.4-16.1)	-2.8
	M2 - Cross Validation			**M2 - Validation**	
Actual Age	Mean Predicted Age (95% CI)	Over/under predicted	Actual Age	Mean Predicted Age (95% CI)	Over/under predicted
3	5.8 (5.0-6.7)	2.8	3	7.1 (5.1-9.2)	4.1
6	6.4 (5.8-7.0)	0.4	6	6.8 (5.1-8.4)	0.8
9	7.8 (7.1-8.5)	-1.2	9	7.7 (5.9-9.4)	0
12	10.8 (10.0-11.6)	-1.2	12	11.5 (10.1-12.9)	-0.5
15	14.0 (13.5-14.6)	-1	15	14.7 (13.0-16.3)	-0.3
	M3 - Cross Validation			**M3 - Validation**	
Actual Age	Mean Predicted Age (95% CI)	Over/under predicted	Actual Age	Mean Predicted Age (95% CI)	Over/under predicted
3	5.8 (5.0-6.7)	2.8	3	6.8 (5.7-8.0)	3.8
6	6.4 (5.8-7.0)	0.4	6	6.9 (5.7-8.1)	0.9
9	7.8 (7.1-8.5)	-1.2	9	9.6 (7.9-11.2)	0.6
12	10.8 (10.0-11.6)	-1.2	12	11.9 (10.4-13.5)	-0.1
15	14.0 (13.5-14.6)	-1	15	10.5 (8.4-12.5)	-4.5
	M4 - Cross Validation			**M4 - Validation**	
Actual Age	Mean Predicted Age (95% CI)	Over/under predicted	Actual Age	Mean Predicted Age (95% CI)	Over/under predicted
3	4.4 (3.8-5.1)	1.4	3	3.5 (1.9-5.2)	0.5
6	7.5 (7.1-8.0)	1.5	6	7.5 (6.2-8.7)	1.5
9	9.9 (9.2-10.7)	0.9	9	10.2 (8.6-11.9)	1.2
12	10.0 (9.1-10.8)	-2	12	9.7 (8.1-11.3)	-2.3
15	13.1 (12.5-13.6)	-1.9	15	12.8 (11.6-14.0)	-2.2

Table 4.5: Cross validation and validation set accuracy for the two multi-source models.

M5 - Cross Validation			M5 - Validation		
Actual Age	Mean Predicted Age (95% CI)	Over/under predicted	Actual Age	Mean Predicted Age (95% CI)	Over/under predicted
3	6.1 (5.6-6.5)	3.1	3	7.6 (6.7-8.6)	4.6
6	7.6 (7.2-8.1)	1.6	6	8.0 (6.9-9.0)	2
9	8.3 (7.8-8.8)	-0.7	9	8.3 (7.3-9.3)	-0.7
12	9.3 (8.8-9.8)	-2.7	12	10.2 (9.4-11.0)	-1.8
15	11.5 (10.9-12.2)	-3.5	15	12.1 (11.9-13.3)	-2.9
M6 - Cross Validation			**M6 - Validation**		
Actual Age	Mean Predicted Age (95% CI)	Over/under predicted	Actual Age	Mean Predicted Age (95% CI)	Over/under predicted
3	6.8 (6.5-7.2)	3.8	3	7.3 (6.7-7.9)	4.3
6	7.9 (7.5-8.3)	1.9	6	8.1 (7.2-8.9)	2.1
9	7.6 (7.2-7.9)	-1.4	9	7.7 (7.0-8.4)	-1.3
12	9.7 (9.3-10.2)	-2.3	12	10.1 (9.2-11.1)	-1.9
15	10.9 (10.5-11.3)	-4.1	15	11.9 (11.2-12.7)	-3.1

Distinction between age classes with validation sets largely mirrors that of the cross-validation sets, with the mean predicted ages of days 3/6 and days 12/15 being indistinguishable at $P < 0.05$ for most models (Figure 4.3). The only model that was able to distinguish between the two youngest groups was the one created from CSU-G3 mosquitoes. The models created from the field (M1, M2, M5, M6) seem to best delineate between days less than 9 and days 12 or greater.

Table 4.6: % Correct of all models for validation sets and test set 1 (TS1). % Correct overall is based on normalized sample sizes to account for variation between days.

	Validation set			**TS1 - Independent**		
Calibration Model	% Correct < 7.5 days [a]	% Correct >7.5 days [b]	% Correct Overall	% Correct < 7.5 [a]	% Correct > 7.5 [b]	% Correct Overall
M1	65.0 (N=20)	87.5 (N=24)	74.4	11.4	100	58.1
M2	62.5 (N=16)	81.8 (N=22)	67.0	37.1	71.1	47.0
M3	55.0 (N=20)	95.2 (N=21)	77.5	88.6	26.3	58.0
M4	75.0 (N=20)	93.3 (N=30)	82.5	0	100	50.0
M5	33.3 (N=36)	82.6 (N=46)	54.3	45.7	84.2	59.0
M6	50.0 (N=56)	81.6 (N=76)	62.5	48.6	84.2	57.5

[a] Days 3 and 6, Sample numbers listed in Tables 4.2 and 4.3 for Validation and TS1 sets, respectively.
[b] Days 9 and 12.

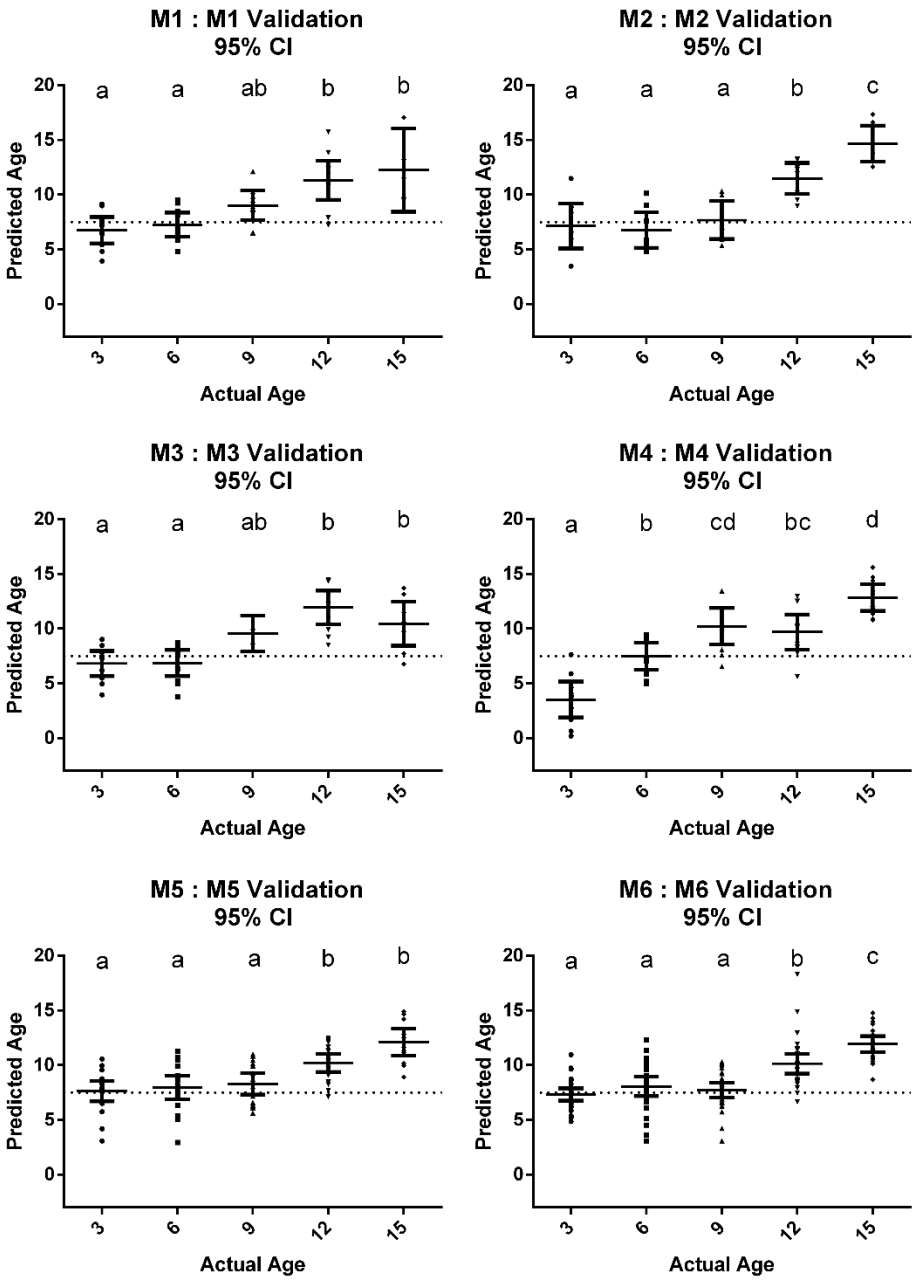

Figure 4.3: Validation set predicted age for each model. Groups with statistically different means ($P < 0.05$) via ANOVA with Tukey's Multiple Comparisons adjustment are marked with different letters. Dotted line is at 7.5 days, delineating correctness less than or greater than this value.

Accuracy on independent test set 1:

Accuracy for the three independent test sets varied considerably based on which set and model was used. Correctness < 7.5 or > 7.5 for TS1 is listed above (Table 4.6). With TS1, only

M1 was able to discriminate between 3 day old mosquitoes and the other sample groups. All other models, including the multisource models that include the calibration spectra from M1, failed to show this age class as "young." Most models over predicted 3 day old mosquitoes by over 5 days. Due to over prediction of all ages with M4 (CSU-G3), this model was only tested with TS1. The sample size and location of TS1 was limited due to poor availability of larval habitat during collection.

Table 4.7: Test set 1 accuracy for each model. Sample collection locations and numbers for this set are listed in Table 4.3. Overall accuracy of these models (< 7.5, > 7.5) is shown above in Table 4.6.

Single source:

M1 - Test Set 1

Actual Age	Mean Predicted Age (95% CI)[a]	Over/under predicted
3	8.5a (7.3-9.8)	5.5
6	10.8b (10.0-11.6)	4.8
9	11.7b (11.0-12.4)	2.7
12	11.7b (10.7-12.7)	-0.3

M2 - Test Set 1

Actual Age	Mean Predicted Age (95% CI)[a]	Over/under predicted
3	10.8a (9.4-12.1)	7.8
6	7.7b (7.2-8.2)	1.7
9	7.9b (6.4-9.4)	-1.1
12	9.2ab (8.0-10.4)	-2.8

M3 - Test Set 1

Actual Age	Mean Predicted Age (95% CI)[a]	Over/under predicted
3	5.8a (4.4-7.2)	2.8
6	5.3a (4.3-6.2)	-0.7
9	6.7a (5.2-8.1)	-2.3
12	5.6a (4.7-6.5)	-6.4

Multi-source:

M5 - Test Set 1

Actual Age	Mean Predicted Age (95% CI)[a]	Over/under predicted
3	9.4b (7.9-10.8)	6.4
6	7.0a (6.4-7.6)	1
9	8.8b (7.6-10.0)	-0.2
12	9.2b (8.0-10.4)	-2.8

M6 - Test Set 1

Actual Age	Mean Predicted Age (95% CI)[a]	Over/under predicted
3	9.4b (7.7-11.1)	6.4
6	6.8a (6.0-7.5)	0.8
9	8.5b (7.8-9.2)	-0.5
12	9.1b (8.2-10.1)	-2.9

[a] Differing means by an analysis of variance test marked by unique letters

Comparison to externally validated test sets (parity and sporozoite):

In total, 102 nulliparous, 286 parous, and 36 sporozoite positive (all *P. falciparum) An. gambiae* were used in either the model or independent data sets. Accuracy of the multisource

models (M5 and M6) for predicting both test sets 2 and 3 (TS2 and TS3) was higher than that of the single source models though overall accuracy was poor (Table 4.8).

Table 4.8: Accuracy of each model for test Sets 3 (nulliparous vs. parous) and test set 2 (nulliparous vs sporozoite positive). Accompanying figure (Figure 4.5) shows mean ages of each model in box and whisker plots. "% Correct" is accuracy of prediction greater than or less than 7.5 days.

	TS3 - Nulliparous vs Parous			TS2 -Nulliparous vs Sporozoite Positive		
Calibration Model	Nulliparous (N=48) % Correct < 7.5	Parous (N=268) % Correct >7.5	Total Correct (N=316) % Correct Overall	Nulliparous (N=48) % Correct < 7.5	Sporozoite+ (N=18) % Correct >7.5	Total (N=316) % Correct Overall
M1	54.2	56.3	55.3	54.2	72.2	63.2
M2	64.6	45.2	54.9	64.6	66.7	65.6
M3	43.8	52.6	48.2	43.8	44.4	44.1
M4	-	-	-	-	-	-
M5	79.2	38.8	59.0	79.2	72.2	75.7
M6	64.6	58.2	61.4	75.0	66.7	70.8

Importantly, the mean age prediction was only statistically significantly different for age predictions of nulliparous vs parous mosquitoes (test set 3) with the multi-source models (One-way ANOVA, Sidak's multiple comparisons test adjusted *P* value of 0.0025 and 0.0173 for M5 and M6, respectively). Nulliparous and parous mosquitoes had mean ages of 5.03 and 6.67 days with M5, and 6.4 and 7.8 days with M6 (Figure 4.5A). This differed from any of the single-source models which failed to predict a difference in age. With TS2, the mean age predictions again were only different with M5 and M6 (P = 0.0003, and 0.0018, respectively), with means of 5.03 to 8.78, and 6.42 to 9.7 days for nulliparous to sporozoite positive with models M5 and M6 (Figure 4.5B).

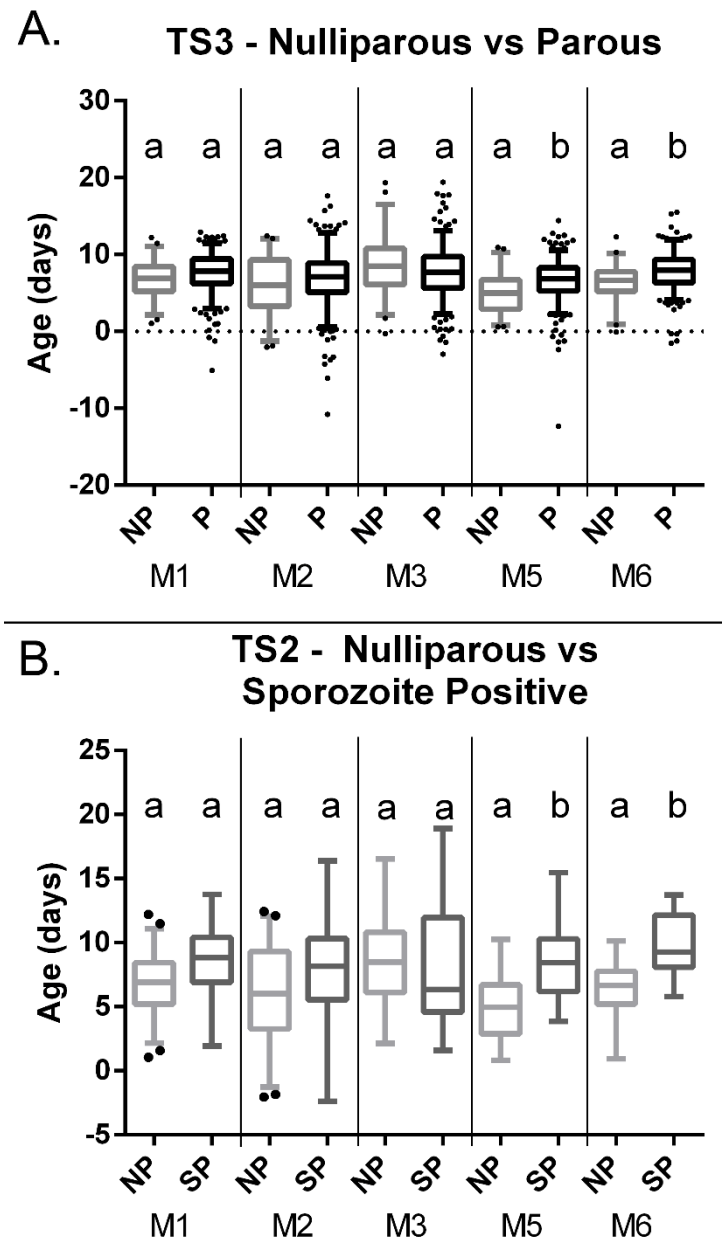

Figure 4.5: Mean age predictions for externally validated test sets, with 25-75% confidence (box) and 5-95% confidence intervals (whiskers). Significance between means is marked with unique letters above the box plots.

Possible age related effects of ivermectin mass drug administration:

Over the 6 weeks of sampling in two villages in 2013, 1788 *An. gambiae* s.l. were collected and scanned via NIRS (Table 4.9A). Mean ages were calculated using M5 for each of

these time periods as this model had the highest accuracy for TS2/TS3, and means were compared via one-way ANOVA with Tukey's multiple comparisons correction (Table 4.9B). After MDA, the mean age dropped 20.8% in the treatment village compared to 6.2% in the control (Table 4.9B). Mean ages were significantly different between post-MDA "Week 1" and time periods "Pre-MDA", "Week 2," and "Week 3" ($P < 0.0001$ for all comparisons), and between "Week 2" and "Week 4" ($P < 0.05$).

Table 4.9: (A) Sample numbers for treatment and control villages surrounding mass drug administration (MDA) of ivermectin. (B) Mean mosquito age for treatment and control villages (95% confidence interval). Different letters indicate differing means as described above.

(A)

Time Period	Treatment	Control	Total
Pre-MDA	296	311	607
Week 1	231	210	441
Week 2	98	190	288
Week 3	132	173	305
Week 4	59	88	147
Total	816	972	1788

(B)

Time Period	Treatment	Control
Pre-MDA	7.45b (7.09-7.82)	8.11a (7.78-8.44)
Week 1	5.90a (5.45-6.35)	7.61a (7.22-8.44)
Week 2	7.86b (7.22-8.51)	7.43a (7.00-7.86)
Week 3	7.71b (7.28-8.14)	7.41a (7.05-7.78)
Week 4	6.37ac (5.60-7.13)	7.27a (6.79-7.75)

We also compared the population structure during these time periods, grouping mosquitoes into 3 day age classes (< 3 days, >3 to < 6 days, > 6 to < 9 days, > 9 to < 12 days, and > 12 days), based on the ~3 day gonotrophic/feeding cycle [274]. The results of this analysis are shown in Figure 4.6. In the control village, the middle age class (> 6 to < 9 days) is the dominant group across time points, being larger than the next group by 1.45 to 2.61 times (Figure 4.6 left side). In the treatment village, in the 1 week post time period, group "< 3" and "> 3 to < 6" are 2.44 and 1.35 times higher than in pre-MDA respectively, while the older groups "> 9 to < 12" and "> 12" decrease in this time period by 1.73 and 2.86 times, respectively. As these population breakdowns are relative to one another, it appears that there is a shift to a younger population post-MDA. After week 2, there is a subsequent shift to higher proportions of

older mosquitoes. The age shift seen in week 1 mirrors the reported ~25% drop in parous mosquitoes previously reported for ivermectin MDA [7].

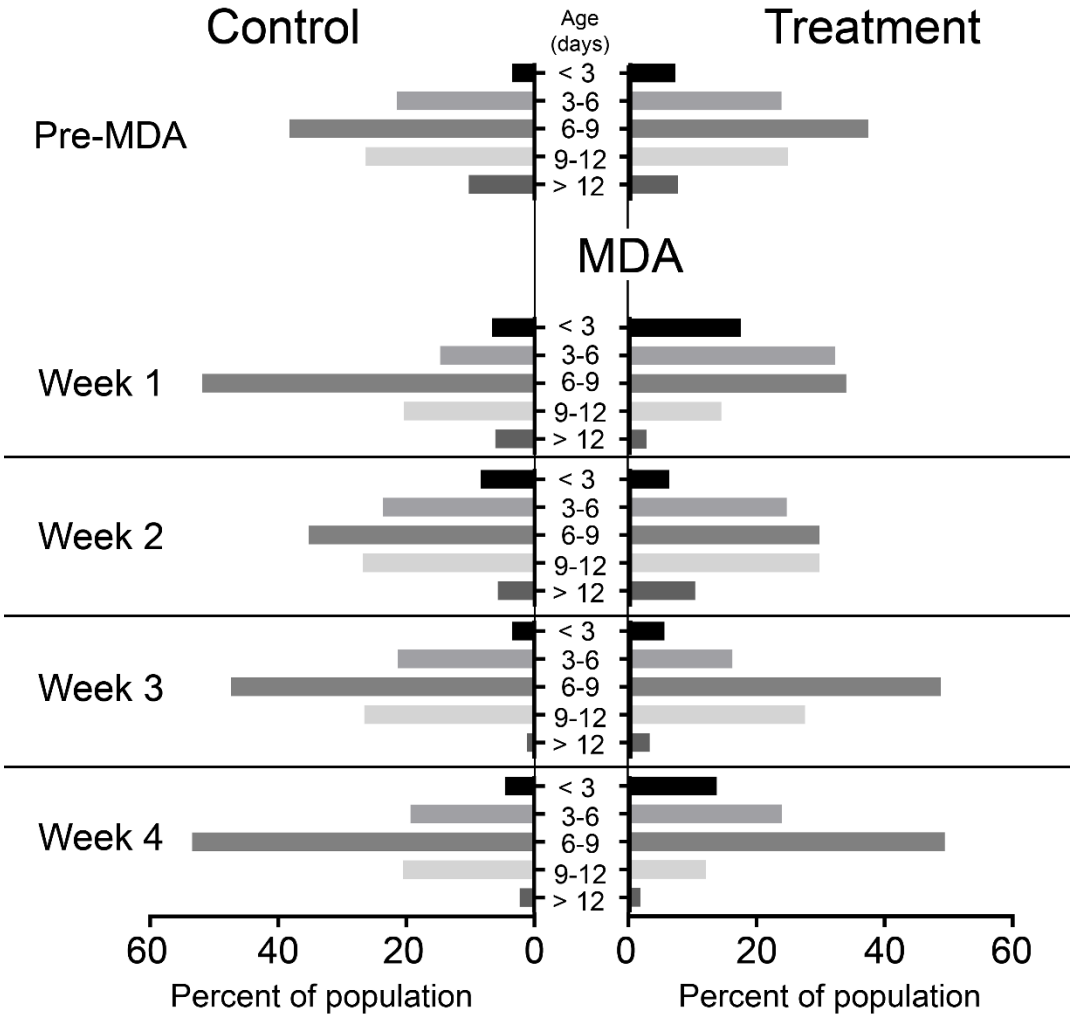

Figure 4.6: Population age structure of *An. gambiae* s.l. mosquitoes as assessed by NIRS before and after a mass drug administration of ivermectin.

Discussion:

In this study we assessed the use of NIRS for the age-grading of wild caught vectors whose age had been externally validated by existing approaches. While the overall accuracy of the models generated in this work was poor for age-classification of wild-caught mosquito

vectors (test set 1), some success was found in linking this approach to external validators of age (test sets 2 and 3). The best of the external validators (nulliparous vs. sporozoite positive) tested with NIRS in TS2 showed with the multi-source models a maximum difference in age of 3.75 days between classes, and differences between ages only seen in multi-source models (Figure 4.5B). This difference is likely larger in reality assuming most females feed, mate, and lay eggs within the first 5 days of their lives [275,276], and sporozoite positivity is at its earliest 10-12 days post emergence [277]. Though due to the over prediction of young mosquitoes, and the under prediction of old mosquitoes compressing the population structure to the middle, the observed difference is still promising. The difference in mean ages for nulliparous vs parous mosquitoes was smaller, being at most 1.67 days apart (Figure 4.5A). Parous mosquitoes would have a larger age range than sporozoite positive mosquitoes (i.e. over 5 days of age vs. 10+ days), so this difference being smaller is not unexpected.

Though the accuracy of prediction of test set 1 was poor, the numbers of the worst predicted time point (Day 3, Table 4.3) were low (9 mosquitoes), and were predominantly from one sampling location. The model that best predicted Day 3 in TS1 was M1, which was made solely from mosquitoes caught in the same location as 7 of the 9 samples for Day 3 (Tables 4.2 and 4.3). This indicates the need for sampling coverage of calibration spectra in areas in close geographic proximity to the desired testing location that would be subjected to similar variations in temperature, rainfall, and larval habitat conditions. Additionally, due to degradation in DNA quality in samples from 2014, we were unable to reliably speciate these populations between *An. arabiensis* and *An. gambiae* s.s. by PCR [239]. Mosquitoes from 2013's calibration model (M1) were able to be speciated, with 68.0% found to be *An. arabiensis* (data not shown). Due to insufficient sample numbers in these periods, we were unable to create two calibration models

for each identified species. Furthermore, attempts to create a predictive model to distinguish *Anopheles* species via NIRS, as had been reported by Mayagaya *et al.,* were unsuccessful. With this in mind, we believe that accounting for this variation in species, also in regards to the recent species delineation of *An. gambiae* s.s to *An. coluzzii,* would be beneficial to improving calibration accuracy [278].

Due to the slightly decreased accuracy of Model 6 compared to Model 5 for independent test sets (Table 4.8, TS2), it seems that the inclusion of colonized mosquitoes, even those recently colonized, may decrease calibration performance on wild vectors. Semi-field or true field vectors that have a higher degree of genetic variability are likely superior for increasing accuracy in wild sample prediction. It may be possible to increase predictive ability of these colonized vectors through supplementation of rearing water with natural water sources. This would also limit the need for specialized diets (caloric restriction/food source variation) that may not represent true field conditions. Towards NIRS analytical methodology, we found the inclusion of the Durbin-Watson statistic was found to be an easy to generate measure that allows for quantification of noise in regression coefficient plots due to overfitting, making the selection of the number of factors in the PLS model less subjective. This is helpful as overfitting can be difficult to determine, especially as overall accuracy on independent sets was marginal.

Finally, having external validators for calibration models is critical to their use on wild samples. Without some form of external validation, the values generated by NIRS should be questioned heavily due to the variation present in wild samples. Bearing in mind that all models are abstractions [279], our demonstration of population structure change in response to ivermectin MDA with NIRS is still promising when compared to the known changes in parity structure previously reported [7]. While this sort of analysis requires careful and robust

calibration of the machine, we believe that this sort of population-level view of mosquito age structure could influence the use of entomological control measures, and provide a novel and rapid way to quantify their effects. This has implications towards a variety of future policy and disease management applications, and could help to evaluate the next generation of insecticide technologies.

Chapter 5: New molecular methods using mosquito bloodmeals as epidemiological tools to study local *Plasmodium* transmission.[4]

Introduction:

The global rate of *Plasmodium*-caused disease has been decreasing steadily as funding and intervention programs have been increasing [280,64,45]. This has brought the most recent malaria mortality figures to roughly half those of 30 years ago [45], and increased the number of people living in "pre-elimination or eliminating" areas of sub-Saharan Africa by 2.5 times since 2000 [2]. However, complete elimination in these regions has been difficult due to the maintenance of low level parasitemias in asymptomatic individuals, who often are either not screened for parasites or have parasitemias too low to detect with standard screening methods [281]. This has been shown through the failure of "mass screen and treat" programs using Rapid Diagnostic Tests (RDTs) or microscopy for detection, and Artemisinin Combinatorial Therapy (ACTs) for treatment. These methods have sensitivities of ~200,000 and ~10,000 parasites/mL for RDTs and microscopy, respectively [90,5,282,91,283]. If an active infection is found, treatment with ACTs may not completely remove the mosquito-infectious gametocyte stages [284]. Consequently, there has been enhanced research on drugs such as primaquine that target gametocytes to try to eliminate infections that are maintaining the disease transmission cycle [285,286]. Because of these factors, the development of sensitive methods to detect infections persisting at low but transmissible levels has become increasingly important.

[4] For submission to *Journal of Clinical Microbiology*. **Krajacich BJ,** Molina-Cruz A, Barillas-Mury C, Foy BD. New molecular methods using mosquito bloodmeals as epidemiological tools to study local *Plasmodium* transmission.

Several highly sensitive molecular methods for *Plasmodium* detection have recently been developed including Quantitative Nucleic Acid Sequence Based Amplification (QT-NASBA) [287], Loop Mediated Isothermal Amplification (LAMP) [192], PCR against multicopy subtelomeric genes [187], and others [288,191,289]. The RNA based methods (QT-NASBA, RT-PCR) allow for stage-specific detection, which helps to discern between asexual parasite stage infections that cause disease but are not infectious, and mature gametocytes that are infectious to mosquitoes and thus maintain transmission. Overall, these methods have increased the detection limit over microscopy by several orders of magnitude. QT-NASBA against *Pfs*25, a gene specific to mature stage V gametocytes, can detect 20-100 gametocytes/mL [95]. RT-LAMP against *Pfs*16, a gene specific to sexually committed gametocytes, can be detected to concentrations of 2 parasites/mL [192]. Subtelomeric PCR can detect 30-150 parasites/mL [187]. Some of the increase in sensitivity of these methods results from the collection of larger volumes of blood (50-2000 µL), which increases the chances of detection [290]. However, with this increase in sampling volume, it is important to consider the amount of blood taken by the mosquito vector, and what most closely approximates a mosquito's bite. The volume of blood taken by a mosquito in each bloodmeal is roughly 2 µL, with some volume of frass (waste) expelled, making the total volume of blood sampled at most 5 µL [291,292]. With these low volumes of blood taken, the chances of infection are limited by the chances of both a male and female gametocyte being ingested [281,96]. Thus these new molecular methods may not accurately reflect true "infectivity" of varying concentrations of parasites.

Furthermore, it has been established that there is significant heterogeneity in mosquito biting patterns across populations, and gametocyte densities have been found to vary considerably across time and space [293,203,294]. Additionally, there is variability in the

infectivity of a bite to mosquitoes even from feeds on same host [295]. Due to this, assays that can account for this variability, and remove assumptive biases of mosquito infectivity solely based on gametocyte density are important to understanding transmission dynamics. Towards this goal, the purpose of this study has been to combine xenodiagnostic approaches with a new, sensitive molecular assay showing transmission and development of *Plasmodium falciparum* parasites in mosquitoes. For accurate quantification of successful "human-to-mosquito" transmission, we tested mosquito bloodmeals with multiplex Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) on intronic *P. falciparum* genes previously unused for detection assays, coupled with droplet digital PCR (ddPCR) for accurate quantification in small sampling volumes. We also demonstrate that this sampling methodology can be utilized with existing QT-NASBA approaches. Through the RT-PCR and ddPCR approaches, we can detect and quantify transcripts of ookinete stage parasites in *An. gambiae* bloodmeals ingested up to 2 days after ingestion of gametocytes. Additionally, as a pilot, proof-of-principle experiment, we investigated a set of samples (human blood spots and mosquito blood meal spots) collected from Liberia during the wet season to look for infection events.

Methods:

Mosquito rearing and Plasmodium culture:

Anopheles gambiae G3 strain mosquitoes were reared at 27°C and 80% humidity under a 12:12 light:dark cycle, and were provided 10% sucrose solution *ad libitum* as previously described [296]. Mosquitoes were infected with mature stage IV/V *Plasmodium falciparum* NF54 strain gametocytes through a membrane feeder at 37°C for 30 minutes. Blood for feedings was from Interstate Blood Bank. In the time course experiment, mosquitoes were held for 0, 2, 6, 12, 24, and 48 hours; at each time point 20 mosquitoes were killed and their bloodmeals

expressed onto FTA cards (see "Blood preservation and RNA extraction" section below). An additional 20 mosquitoes were fed an initial infectious bloodmeal, held for 10 days, and fed an uninfectious bloodmeal that was expressed onto cards after feeding. This additional late time point allowed us to analyze if the contribution of oocyst stage transcripts confounds the assay.

Dilution experiments were performed as follows: three groups of 50, 3-5 day old *An. gambiae* G3 mosquitoes were fed blood containing gametocytes at concentrations of 10 gametocytes/mL, 100 gametocytes/mL, and 10,000 gametocytes/mL. This represented two groups near the previously reported limit of detection [95,189], and one over the ~5,000 gametocyte/mL microscopy limit [95]. These mosquitoes were held for 12 hours, after which their bloodmeals were expressed onto FTA cards (see below for methodology). Ten additional 5 µL spots were made of the input blood from the feeder to see if there was a difference in detection ability after ingestion.

Blood preservation and RNA extraction:

Bloodmeals from mosquito abdomens were expressed onto Whatman FTA cards (WB 120205, GE Life Sciences, Boston, MA) for storage as described previously [292], and stored at -80°C until extraction. For RNA extraction, 3 mm punches were taken from the dried bloodmeal spots using a Harris Uni-core Punch and rocked in 70 µL of RNA Rapid Extraction Solution (Life Technologies, Grand Island, NY) for 20 minutes at room temperature. After shaking, 50 µL of the reagent was processed with the Mag-Bind® Viral DNA/RNA 96 Kit (Omega Bio-tek, Norcross, GA) according to manufacturer's instructions on the 96-well format KingFisher Flex Magnetic Particle Processor (Thermo Fisher Scientific, Waltham, MA) with a 25 µL elution volume of water.

QT-NASBA:

QT-NASBA against the sexual, gametocyte-specific transcript *Pfs25* is the current gold standard for sensitively detecting infectious blood taken from humans by finger prick or venous puncture [287]. Here, we tested this assay against both human blood spots and mosquito blood meal spots from *An. gambiae* that ingested the same human blood. For the assay, 1 µL of RNA product was used in a QT-NASBA reaction according to manufacturer instructions (LifeSciences, St. Petersburg, Florida) at a final KCl concentration of 80 mM in a 10 µL reaction volume. Primer (Integrated DNA Technologies, Coralville, IA), and molecular beacon (Sigma-Aldrich, St Louis, MO) sequences specific for *Pfs*25 were used as described elsewhere (Table 5.1) [95], with final concentrations of 290 nM for primers and 145 nM for probe [297]. Reactions were heated at 65°C for 2 minutes, cooled to 41°C for 10 minutes, and then further held isothermically at 41°C for 91.5 minutes, during the first 1.5 minutes of which the 1.4 µL of enzyme mixture was added. Fluorescence readings were taken on a CFX96 Touch Real-Time PCR Detection system (Bio-Rad Laboratories, Hercules, CA) once per minute during the final 90 minutes of the reaction. Positives and Time-To-Positivity (TTP) were calculated as being higher than 20 standard deviations above the mean of 3 negative controls, or at which time point they reached this value, respectively [298].

Sensitivity and specificity of QT-NASBA and RT-PCR were calculated using bloodmeals from mosquitoes with four dilutions of gametocytes (246,000, 10,000, 100, and 10 gametocytes/mL) at 12 hours post ingestion. Specificity was calculated using RNA extractions from mosquito uninfected bloodmeals 12 hours post ingestion, control uninfected blood spotted onto FTA cards, water-only no template control samples, and blank punches of FTA card material (13-15 samples).

Table 5.1: Primer and probe sequences used for each of the molecular assays. Underlined sequence in the first QT-NASBA primer denotes the T7 promoter sequences used. "N/A" denotes primers created for this project.

Method	Primer Name	Gene ID	Sequence (5'-3')	Reference
ddPCR	ddTrxL1F	PF3D7_0919300	AAGCGGAGAAAAGAAGAACG	N/A
	dd2TrxL1R	" "	CCGGAAATAATATAAGATCCATGTCA	
	dd2-6044F	PF3D7_0604400	AATATGTAAATTTGAATACAAACGTCAAG	
	dd2-6044R	" "	TCTAAGGAATTAATTTTCCCTTCTATGT	
One Step RT-PCR	RT/N-HRPIII-764/554F	PF3D7_1372200	TGTTAGATAACAATAACTCCGAA	N/A
	RT-HRPIII-764R	" "	GTGTAAGTGATGCGTAGTGG	
	RT-TrxL1-492F	PF3D7_0919300	CCATAAACTACTCTGACATGGATCTT	
	RT-TrxL1-492R	" "	GTTGGGATTCTCTTCCGTCA	
	RT-6044-471F	PF3D7_0604400	ATGTTACACGGCGAGCTTTT	
	RT-6044-471R	" "	GGAATTAATTTTCCCTTCTATGTTTG	
Nested PCR	RT/N-HRPIII-764/554F	PF3D7_1372200	TGTTAGATAACAATAACTCCGAA	N/A
	N-HRPIII-554R	" "	GGCATCGTCATGGTGAGAAT	
	N-TrxL1-300F	PF3D7_0919300	TCCCAAATGTAGGGCATTTT	
	N-TrxL1-300R	" "	TTCTTGGGCCTGATCCATAG	
	N-6044-205F	PF3D7_0604400	GGAAAGTTGCATTCCCTTCA	
	N-6044-205R	" "	CGTTTGTATTCAAATTTACATATTCGT	
QT-NASBA	T7+Pfs25-P1	PF3D7_1031000	AATTCTAATACGACTCACTATAGGGAGAAGG CATTTACCGTTACCACAAGTTA	Jones *et al.* 2012 *Malar J*
	Pfs25-P2	" "	GACTGTAAATAAACCATGTGGAGA	
	Pfs25-Beacon	" "	Texas_Red-cgatcg-cccgtttcatacgcttgtaa-cgatcg-DABCYL	

RT-PCR:

To have an assay capable of distinguishing multiple parasite stages on a single sample, a nested, multiplex RT-PCR was developed using three targets. Primers specific to the genes Histidine Rich Protein III (HRPIII, asexual stage parasite biased), Thioredoxin Like Protein-1 (TrxL1, ookinete stage biased) and PF3D7_0604400 (6044, gametocyte stage biased) were created using Primer-BLAST to be exon-exon spanning and are listed in Table 5.1 [299]. Genes were chosen based upon the presence of introns, and which stage had highest transcript abundance determined via RNA transcription profiles generated from the Lopez-Barragan *et al* RNA-seq data set on PlasmoDB [300,301]. Nested multiplex RT-PCR was performed in an initial 12.5 µL reaction volume using the Superscript III One-Step RT-PCR kit (12574-026,

ThermoFisher Scientific, Waltham, MA) with the addition of $MgSO_4$ to a concentration of 2.5mM, and dNTPs to 0.3mM. Thermocycling conditions were as suggested by the manufacturer with initial cDNA synthesis performed at 55.0°C for 30 minutes and a 50.0°C annealing temperature. The nested PCR was performed in a 25 µL reaction using the One*Taq* 2X Master Mix (M0482L, New England Biolabs, Ipswich, MA) with 1.5x concentration of master mix with an annealing temperature of 59.0°C for 60 seconds. This increases the concentration of $MgCl_2$ to 2.7mM and the dNTPs to 0.3 mM to account for the increased reagent demands of a multiplexed assay. Samples were run on a 2% agarose TAE gel with 0.5 µg/mL ethidium bromide. Gel bands for each gene were "semi-quantified" with ImageJ and compared as ratios of TrxL1 to HRPIII and TrxL1 to 6044 [302].

ddPCR:

Droplet Digital PCR was performed by first making cDNA using the M-MLV Reverse Transcriptase (28025013, ThermoFisher Scientific, Waltham, MA) kit with 2µL of RNA per gene target (TrxL1 and 6044) in a 5 µL total reaction volume according to manufacturer's instructions. Primer concentration for the cDNA reaction was 0.5 µM for each gene, and are listed in Table 5.1. Each 5 µL cDNA reaction was added to 20 µL QX200 ddPCR EvaGreen Supermix reactions (#1864034, Bio-Rad, Hercules, CA), droplets were generated (QX200 Droplet Generator #1864002), and the reactions were cycled according to manufacturer's instructions at 59.0°C annealing temperature. Primers were at 100 nM and 150 nM final concentration in the ddPCR reaction for TrxL1 and 6044, respectively. Droplets were read on the QX200 Droplet Reader (1864003, Bio-Rad), and quantified using QuantaSoft Software (Bio-Rad). Absolute quantification ratios of TrxL1 to 6044 were generated from matched samples.

Field samples:

A set of samples collected in the village of Lepelo, Liberia (Lofa County) in 2015 were collected by Joseph Fauver and Dr. James Weger under CSU IRB protocol 15-5896H (principal investigator BD Foy), and used for a preliminary proof-of-principle test of the multiplex RT-PCR. This sample set contained 7 human finger stick blood samples from the inhabitants of one house, and 34 mosquito blood meal spots from blood fed mosquitoes collected in that house over 4 sampling days. All samples were preserved on Whatman FTA cards as described above. QT-NASBA and RT-PCR were performed on the 7 human dried blood spots, and RT-PCR was performed on 24 of the mosquito dried blood meal spots.

Results:

QT-NASBA on mosquito bloodmeals:

QT-NASBA for *Pfs*25 was found to be an amenable approach to detection of gametocytes in recently ingested mosquito bloodmeals. *Pfs*25 transcripts were detected by this method in 100% of 0 hour, 2 hour, 12 hour and 24 hour samples at 246,000 gametocyte/mL dilution. The time effect was tested here because it is a key variable in the practical analysis of blood fed *An. gambiae,* as they are captured from houses at dawn after having blood fed the night prior and subsequently processed later that day or longer. There was 90.0% detection at 6 hours, 80.0% detection at 48 hours, and 55.6% detection of 10 day oocyst samples (Table 5.2).

Table 5.2: Time course positivity for QT-NASBA with *Pfs25*, and all RT-PCR genes targeted. "Any" under RT-PCR constitutes a positive of either *HRPIII*, *TrxL1*, or *6044* on an individual sample. QT-NASBA performed in separate experiments over two days, RT-PCR assays performed in separate experiments over three days.

	QT-NASBA	RT-PCR			
	Pfs25	*HRPIII*	*TrxL1*	*6044*	Any
0 hour	9/9	10/10	10/10	10/10	10/10
2 hour	9/9	9/9	9/9	9/9	9/9
6 hour	9/10	6/10	9/10	10/10	10/10
12 hour	10/10	4/10	10/10	8/10	10/10
24 hour	9/9	7/10	10/10	8/10	10/10
48 hour	7/10	2/10	9/10	1/10	10/10
10 day	5/9	2/8	3/8	0/8	4/8

The mean Time to Positive (TTP) did not vary significantly for groups via a one-way Analysis of Variance (ANOVA) test with Tukey's multiple comparisons correction at $P < 0.05$ (Figure 5.1).

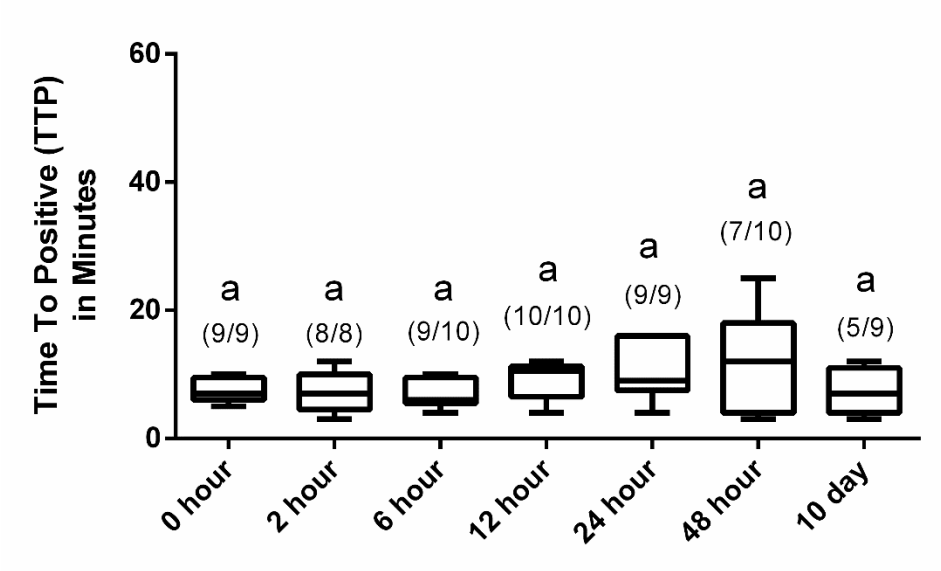

Figure 5.1: Time to Positive (TTP) for Time Course Experiments box (25-75% confidence interval) and whisker (5-95% confidence interval) plots determined by QT-NASBA. Time points 0-48 hour, and 10 day post feed are listed on the x-axis. The same letter above a column indicates that means did not differ significantly by ANOVA with Tukey's multiple comparisons adjustment. Numbers above box and whisker indicate number of positive of those sampled at that time point.

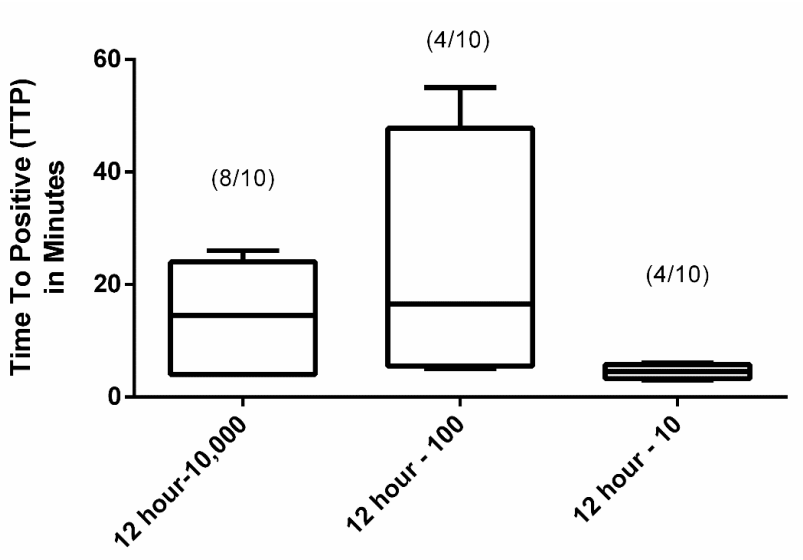

Figure 5.2: Box (25-75% confidence interval) and whisker (5-95% confidence interval) TTP plot for three dilutions of 10,000, 100, and 10 gametocytes/mL at 12 hours post ingestion (x-axis) by QT-NASBA. Numbers above box and whisker indicate number of positive samples of total at that dilution.

Dilution samples showed detection of 80.0%, 40.0%, and 40.0% of 10,000, 100, and 10 gametocyte/mL dilutions, respectively (Figure 5.2). The mean time to positive did not significantly differ between groups analyzed.

Semi-quantitative RT-PCR:

Semi-quantitative RT-PCR showed an association of time point and the ratio of both TrxL1:HRPIII (ookinete:asexual stage) and TrxL1:6044 (ookinete:gametocyte stage). Predominantly, the ratio of TrxL1 to both HRPIII and 6044 increased after 12 hours. This ratio was statistically different for TrxL1:6044 between time points 0, 2, and 6 hours compared to both 24 and 48 hour time points ($P = 0.0012$, <0.0001 for 0 hour compared to 24 and 48 hour; $P = 0.0029$, <0.0001 for 2 hour; $P = 0.0034$, <0.0001 for 6 hour). The mean ratio for 12 to 48 hours also differed significantly ($P < 0.0001$) (Figure 5.3).

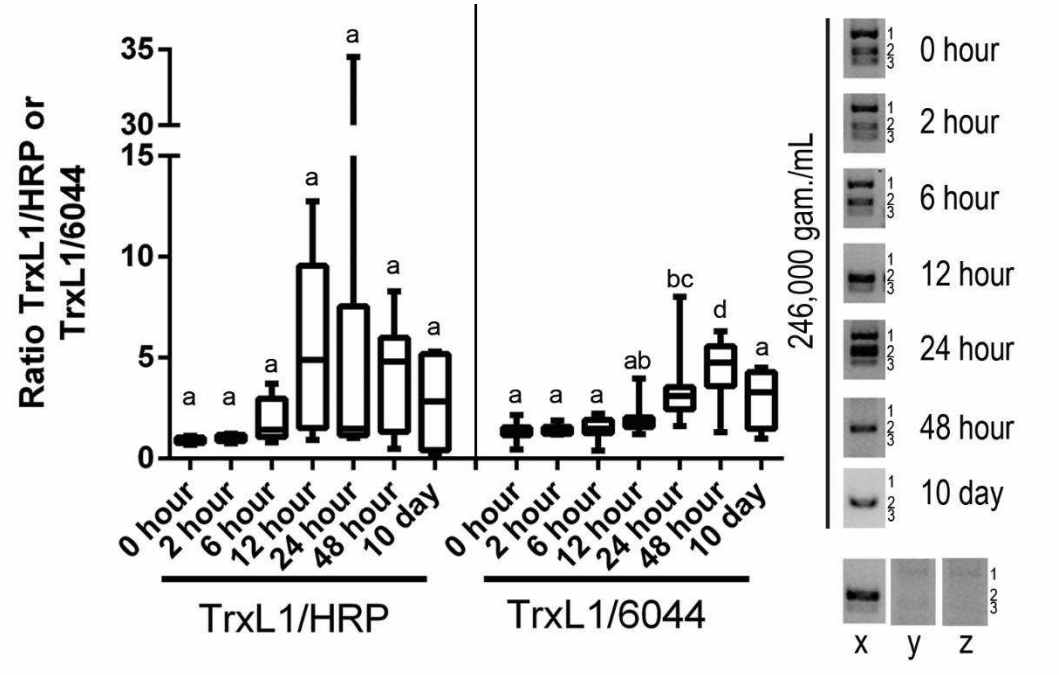

Figure 5.3: Example RT-PCR products from the TrxL1/6044 multiplex, and ratio of TrxL1:HRPIII and TrxL1:6044 for time course experiments. Differing letters above the graphs indicate the means ratio is significantly different ($P < 0.5$) via ANOVA with Tukey's Multiple Comparison's correction. The amplicons from the gel images from top to bottom are HRPIII (Band 1: 554 bp), TrxL1 (Band 2: 300 bp), and 6044 (Band 3: 205 bp). Gel products marked "x", "y", and "z" are 10,000 gametocytes/mL at 12 hours, 100 gam./mL at 12 hours, and 10 gam./mL at 12 hours, respectively.

Qualitatively, differences are seen between representative examples for each time point. Specifically, there is decreasing relative intensity of the 6044 amplicon compared to TrxL1 beginning at 6 hours post ingestion (Figure 5.3, right side). Additionally, these differences can also be seen in the 10,000 gametocyte/mL samples at 12 hours (Figure 5.3, panel X), though this visual distinction is lost at 100 (Panel Y), and 10 gam./mL (Panel Z).

ddPCR:

Quantitative droplet digital PCR showed an increase in the ratio of TrxL1 and 6044 at 12 and 24 hour time points post ingestion of an infectious bloodmeal, with the ratio changing from roughly 1:1 at time points 0, 2, and 6 to 175:1 at 12 hours and 459:1 at 24 hours (Figure 5.4).

This was significant for 0, 2, 6 hour, and 10 day time points vs. the mean ratio at 24 hours (One-way ANOVA, $P < 0.05$).

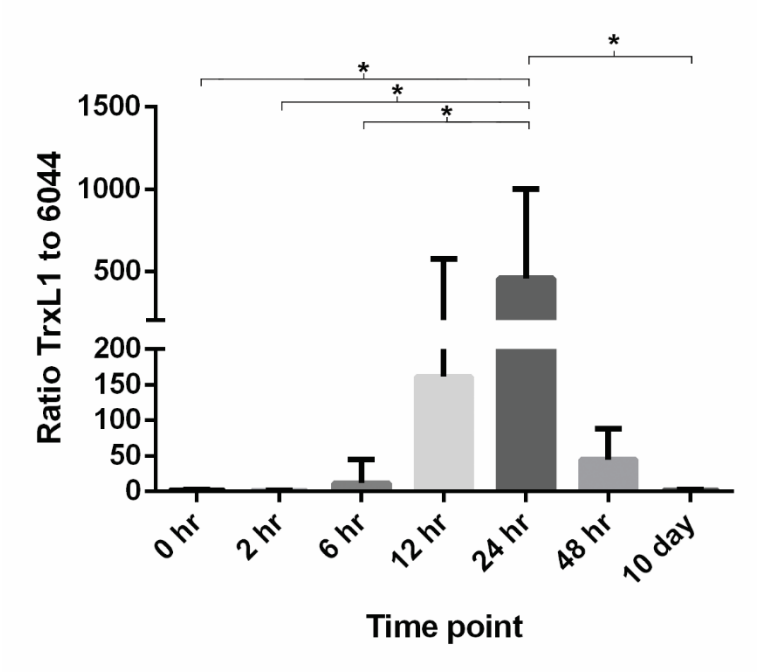

Figure 5.4: Ratio of TrxL1:6044 with droplet digital PCR. Significance via Tukey's multiple comparisons test.

Quantitative ddPCR and semi-quantitative RT-PCR were weakly correlated for the ratios of TrxL1 to 6044 between samples (Figure 5.5, Spearman r=0.3707, P=0.0144). As semi-quantification of gel band intensity is a course measure of quantification, the weak strength of this correlation was not unexpected.

Sensitivity and specificity:

QT-NASBA and RT-PCR sensitivity and specificity for detecting *P. falciparum* transcripts in *An. gambiae* dried bloodmeal spots at 12 hours post ingestion by *An. gambiae* are listed below (Table 5.3). Both sensitivity and specificity were comparable between methods, with sensitivity at 10 gametocytes/mL being higher with QT-NASBA relative to RT-PCR (40.0% to 23.1%) and specificity being slightly higher with RT-PCR (92.9% to 86.7%).

Table 5.3: Sensitivity and specificity of the QT-NASBA and RT-PCR assays at varying dilutions 12 hours post-ingestion.

	QT-NASBA	RT-PCR			
	Pfs25	*HRPIII*	*TrxL1*	*6044*	Any
Sensitivity 246,000[a]	100.00%	33.33%	100.00%	88.89%	100.00%
Sensitivity 10,000[a]	80.00%	14.29%	100.00%	14.29%	100.00%
Sensitivity 100[a]	40.00%	15.38%	30.77%	7.69%	38.46%
Sensitivity 10[a]	40.00%	15.38%	7.69%	15.38%	23.08%
Specificity	86.67%	100.00%	92.86%	100.00%	92.85%

[a] Gametocytes/mL of blood in feeder

Field samples:

Of the 7 human finger blood spot samples collected from Lepelo, one was weakly positive by QT-NASBA (TTP 59 minutes), and none were positive via RT-PCR (Figure 5.6). Of the mosquito blood meal spot samples, 4/25 were positive for TrxL1 only, 1/25 was positive for HRPIII only, and 1/25 was positive for TrxL1 and 6044 (Ratio TrxL1:6044 = 1.45 semi-quantitatively).

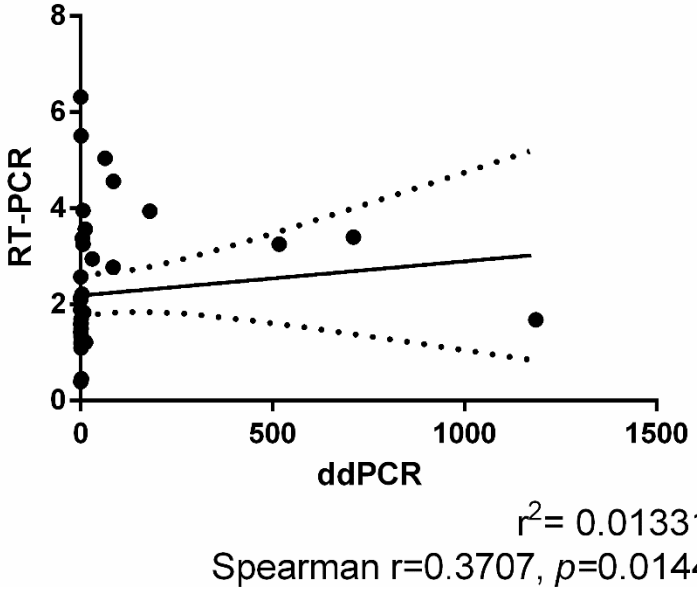

Figure 5.5: Correlation of Semi-quantitative RT-PCR vs. droplet digital PCR across all time points in time course experiment. Dotted lines represent 5-95% confidence intervals.

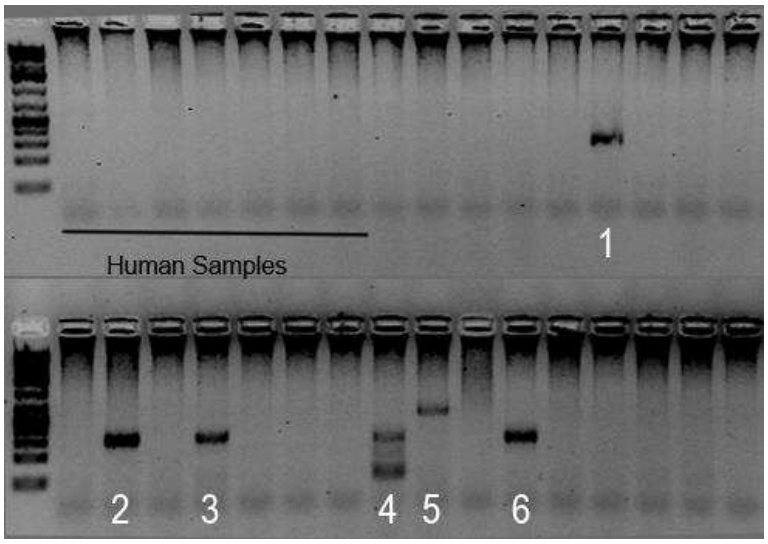

Figure 5.6: RT-PCR screening of human finger blood spot (marked) and mosquito blood meal spot samples (positive samples numbered 1-6). Samples 1, 2, 3, and 6 are positive for the TrxL1 band only (size: 300bp), sample 5 is positive for the HRPIII band only (size: 554bp), and sample 4 is positive for TrxL1 and 6044 bands (300bp and 205bp, respectively). The bottom band in sample 4 appears to be a primer dimer (~100bp).

Discussion:

Sensitive tools that can accurately determine infectivity of humans to mosquitoes are critical to a greater understanding the *Plasmodium* disease cycle, and how the transmission cycle is persisting in the midst of control interventions. Existing tests such as QT-NASBA that have achieved low limits of detection screen individuals broadly with finger blood-sticks to determine the infectious reservoir present in an area [199]. This approach, while thorough, can be quite labor intensive, and can miss individuals who are absent or are highly mobile [93]. Additionally, these broad sampling approaches do not account for the heterogeneity present in mosquito biting. It has been shown recently that blood type, pregnancy, chemical cues, alcohol consumption, body size, proper use of protective measures, and lifestyle affect how humans are bitten [293,303,135,304–307]. This biting heterogeneity is coupled with variability in the parasite's infectiousness at different densities [308], and variability in infectiousness in individual bites on

the same person [295]. To avoid many of these biases, here we present a novel molecular method for detection of low level parasitemias/gametocytemias utilizing naturally biting mosquitoes, and demonstrate how this method compares to existing molecular approaches.

In laboratory studies, we found the sensitivity of this technique correlates well with previously published limits of detection for *Pfs25* QT-NASBA on human blood finger spots (10-100 gametocytes/mL), as well as *Pfs25* QT-NASBA performed on mosquito blood meals (Table 5.3) [189]. This low limit of detection is critical as 14-40% of all mosquito infections may be caused by submicroscopic parasite carriers [281]. Additionally, the prevalence of these submicroscopic infections is higher in adults, a group less likely to be sampled by classic RDT/microscopy measures for any infections [281,178,309]. We also demonstrated this approach with field-caught mosquito samples, and found that it can detect new infections of mosquitoes (Figure 5.6, sample 4), existing or successful infections (Figure 5.6, samples 1-3, 6), and may also detect asexual-only infections that are disease-causing but non-infectious (Figure 5.6, sample 5). This would indicate that with a single, non-invasive sample we can make distinctions of parasite stage across the *Plasmodium* lifecycle. Additionally, by utilizing wild mosquitoes that bite naturally in this approach, we do not have to make assumptions of biting pressure on individuals, or how infectious the bloodmeal is to the mosquito, and we are able to sample in means to most accurately reflect natural transmission.

As has been postulated by Lin *et al.*, the search for the "most-sensitive" assay may be somewhat premature if the assay has poor relation to the true infectivity of that gametocyte density [96]. At low gametocyte densities there is a small probability of a mosquito ingesting two blood cells containing gametocytes that differentiate into a male and a female gamete that successfully meet in the midgut to form a zygote (Figure 5.7), limiting infectivity.

(A)

$$P(detection) = 1 - \left(1 - \frac{i}{t}\right)^{b}$$

(B)

$$P(\text{infection}) = 1 - P(no\ female) - P(no\ male) + P(no\ female\ and\ no\ male)$$

(C)

Concentration gametocytes/mL	Probability of parasite ingestion	Probability of male & female
246,000	100.00%	100.00%
10,000	100.00%	96.59%
100	10.68%	0.25%
10	1.13%	0.00%

Figure 5.7: Theoretical equations denoting (A) the chance of detection (a single parasite of either sex) in mosquito bloodmeal assuming detection sensitivity of 1 parasite per blood meal, and (B) infection (at least one parasite of each sex) in a mosquito bloodmeal. i = number of red blood cells infected (10-246,000 gametocytes/mL), t= total red blood cells per mL (5,000,000,000)[310], and b= total red blood cells per blood meal in mosquito (5,650,000) [311]. Gametocyte sex ratio (male:female) is 3:7 in these equations as previously reported [295,312,313]. Equations assume heterogenous mixing of parasites within blood. (C) Theoretical probability of parasite ingestion and probability of ingestion of a male and female parasite at varying dilutions utilizing equations A and B. Equations generated with assistance from Alex Offerdahl and Xin Chen.

However, xenodiagnostic approaches have shown that these low parasite (gametocyte) densities can and do infect *Anopheles* mosquitoes [314], and in the past may have thwarted control efforts [95,315,73]. A model by Churcher *et al.* showed that mosquito infection is low, but consistent, at low parasite densities up to 100 gametocytes/µL in field settings, at which point success in infection increases with density [308]. This may point to the ability of the parasite to foster their own ingestion by *Anopheles* vectors, possibly by preferential sequestration of gametocytes in capillaries [316]. Additionally, it was shown recently that there are volatile cues produced via the parasite itself to simulate plant terpenes, increasing attractiveness [101].

By demonstrating that parasites have developed into the next stage in this assay, we avoid assumptions of infectivity based solely on gametocyte molecular markers such as *Pfs230* or *Pfs25* that appear to be specific to either male or female gametocytes, respectively [198]. We

also avoid having to hold mosquitoes through the extrinsic incubation period, looking to detect later stage parasites such as oocysts or sporozoites via dissection or molecular assay as is done in purely xenodiagnostic methodology [317,254,318]. By holding mosquitoes artificially with *ad libitum* access to sugar/water, the daily probability of survivorship is also increased artificially, and likely biases results [319].

Classically, limited work has been done with RT-PCR for detection of stage-specific *Plasmodium* parasites due to a limited number of intronic genes, and the necessity of DNase treatment to remove genomic DNA which limits sensitivity [320]. However, with the use of databases such as PlasmoDB (http://www.plasmodb.org), it has become easier to search for intronic genes exhibiting desired transcript expression profiles [301]. Difficulty comes when trying to find transcripts only present in ookinete stage parasites, as many of these transcripts occur but are sequestered in earlier parasite stages, and are under repression by AP2-O or other transcriptional regulators [321,322]. However, by comparing transcript levels between intronic genes more highly expressed in ookinete vs gametocyte stages we can discriminate ookinete stage parasites without DNase treatment, and this remedies the need to find an 'ookinete only' transcript that does not appear to exist. The ratio we developed is also amplified by the degradation of asexual or gametocyte parasite RNA markers (HRPIII and 6044) in the mosquito midgut as those transcripts are not actively being produced by the parasite [300]. This limits the risks of DNA/RNA from dead parasites from 'contaminating' the sample, giving a false positive of a new infection in the mosquito that will never occur [290]. Through showing development of the parasite in the mosquito, our assay circumvents both of these issues, and can more clearly demonstrate true infection.

In conclusion, this approach allows for a view of a largely understudied 'human-to-mosquito' transmission event, with naturally biting wild mosquitoes, acquiring wild parasites. This represents an improvement over current xenodiagnostic and membrane feeding approaches that use wild-caught or laboratory-reared mosquitoes in standardized membrane feeding assays [323,324]. It is able to show successful early-stage 'human-to-mosquito' transmission of *Plasmodium* parasites through transcripts currently unused for detection, including *TrxL1,* an almost completely unstudied, intronic, ookinete marker. Finally, it is amenable to field samples, and with the use of preservative cards, samples can be taken in limited resource settings for study of local transmission.

Chapter 6: Conclusions and future directions

The malaria climate has changed significantly in the last fifty years, and promises to continue doing so for the next fifty years [2,44,45]. As the world works to control malarial disease, novel techniques will be necessary to address the new challenges that come as elimination becomes more realistic [47]. Recently, the Roll Back Malaria Initiative of the World Health Organization has set the goal of reducing malaria mortality and incidence by greater than 40 percent within five years, and by 90 percent within fifteen years [325]. Throughout this dissertation, I have worked to develop new tools and techniques that can help to meet these goals or evaluate new measures working towards them. Our lab has been evaluating ivermectin mass drug administration as an integrative malaria control tool, and we hope that the techniques developed here will also have broader, real world applicability to a range of control measures and epidemiological studies in the field. These tools will be important as we remember the past failures of malaria control, and try to manage the ways in which the parasite and the mosquito persist under new selective pressures. The major findings of this dissertation, and how these tools can be utilized towards these goals in the future are described below.

Tools for the collection of outdoor-biting vectors:

The work presented in Chapters 2 and 3 of this dissertation described the generation and testing of an active, human-baited tent trap that greatly reduced exposure of the collector to biting mosquitoes. The tent caught fewer mosquitoes than human landing catch (HLC), but may more accurately reflect true biting pressure as it is likely that HLC is overestimating this pressure due to its unnatural nature (i.e. sitting still through the night waiting for vectors to bite). Additionally, we found that HLC catch numbers increased over trials, indicating a skill-based component to the technique which can further the collection bias. Importantly, by using the tent

trap the risk to the collector was essentially removed, the ease of sampling was increased, and known exophagic biting species were collected in similar ratios to those caught by outdoor HLC. This work represents an important step to improving host-seeking mosquito collection methodology, and improves the efficiency, safety, vector quality, and comfort of the collector over many alternative tent designs.

The future of this specific tent design could represent a standardized collection platform that is able to catch a range of mosquito species, with a demonstrated high capture efficiency for *Culex* spp. and *An. gambiae* [120,121]. Due to the proprietary nature of the tent design and limited manufacturing ability, the widespread adoption of this tent may be limited, but it could excel in specialized roles. We hope that this study will work to push development of new tent designs that may be more accessible for individuals sampling around the world, and that the need for performing HLC can be removed.

The use of Near Infrared Spectroscopy for age-grading wild mosquitoes:

Chapter 4 of this dissertation described testing of NIRS technology for the age grading of mosquito populations. Due to the limitations in current approaches, even basic knowledge of mosquito population structure remains relatively unknown. The best studies that have shown the age structure of *Anopheles* spp. to date have utilized Polovodova's dissection technique. While this work showed the relative age-distribution of mosquitoes via their post-reproductive states (Figure 6.1), the difficulties in performing this approach have limited its use.

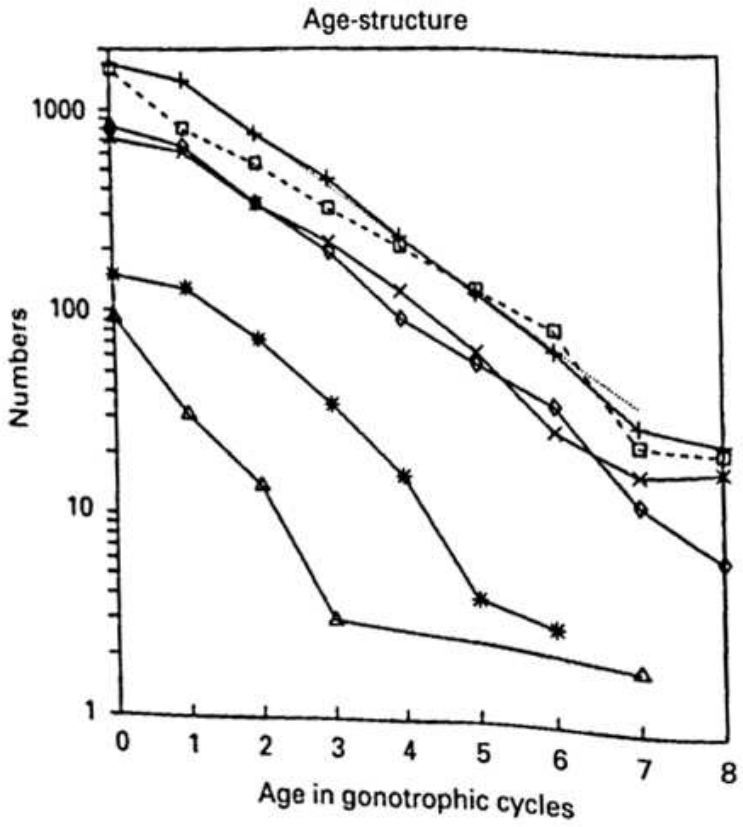

Figure 6.1: The physiological age of female *An. gambiae* caught over a 2-year period in the Muheza, Tanzania area. Data from human biting (-*-), indoor-resting (-×-), and light-trap catches separately (-◊-) and pooled as a total (-+-). Females from a pit trap (-Δ-) and a previous study (-□-) are also shown. Adapted from Lines *et al.* 1991 [326].

A major goal of this study was to apply the recently developed NIRS technique to rapidly determine the age structure of wild *An. gambiae* [163], and to see how the mass drug administration of ivermectin potentially changes this structure. This work started in Senegal in 2012, attempting to use calibration models generated from *An. gambiae* G3-strain mosquitoes that had been in colony for many generations at CSU to predict scans from 1200 wild-caught *An. gambiae*. Using the CSU-G3 calibration, wild-mosquito age predictions were uninterpretable, ranging from -30 to +30 days. This indicated that the chemical background seen as these inbred colony mosquitoes age is too different from the wild-caught mosquitoes. To try to remedy this, we spent two rainy seasons in Burkina Faso collecting wild-caught larvae and rearing them in

water from where they were collected until emergence, and holding them in outdoor insectaries until set ages. Additionally we were provided a new strain of *An. gambiae* that had been recently colonized from the field in Burkina Faso for use in our insectary at CSU. Calibrations from combinations of wild-caught larvae and wild-caught larvae combined with the recently colonized "CSU-IRSS" mosquito strain had the best success in predicting differences in wild-caught mosquitoes whose age had been externally validated by ovary dissection and the presence of *Plasmodium falciparum* sporozoites in their salivary glands. We were able to show significant differences between the mean ages of both young (nulliparous) and middle-to-old age (parous) mosquitoes, and between young (nulliparous) and old (sporozoite positive) mosquitoes. Prediction with independently caught wild-larvae reared to set ages was poor, however these sample sizes were limited. There did appear to be a population shift post ivermectin-MDA that mimics the previously reported shift analyzed by parity analysis [7], though knowing if this shift is representative will require future study.

We see the difficulties of this approach being the large amount of up-front calibration development necessary to create models that accurately represent the study site of interest. However, after this period, the speed of scanning and age-grading individual mosquitoes (a few seconds each) promises to vastly increase the study of mosquitoes in sample sizes that are far more reflective of their population size, while removing the need for burdensome dissection approaches that under sample these populations. A subsequent benefit to this methodology is that after initial cost of the machine, scanning and data processing is essentially reagent-less, and can be performed in areas with minimal infrastructure.

Molecular methods for the detection of 'human-to-mosquito' transmission events:

Chapter 5 of this dissertation described the development of a novel molecular assay for the detection of *Plasmodium* spp. parasites in mosquito bloodmeals. This work was performed to address the need for sensitive ways to detect limited and spatio-temporally heterogenous human-to-mosquito transmission events. Many individuals in endemic communities are often asymptomatic, but can still maintain the disease transmission cycle [281]. Our new methodology analyzed blood fed mosquitoes rather than directly sampling the human population. We found that this sampling technique was amenable to use with the established quantitative nucleic acid sequence based amplification (QT-NASBA) approach [189], and with multiplexed reverse transcriptase polymerase chain reaction (RT-PCR) with two genes currently unused for detection. With both methods we achieved high sensitivity, and through the RT-PCR assay we were able to determine whether parasites began to develop in the mosquito. This is unique as it removes the need to artificially hold mosquitoes for end-point measures of infection which introduces many biases. Additionally, this RT-PCR approach contained a gene specific for asexual, disease-causing parasites, which allows for detection of individuals or clusters of people who were not infectious to mosquitoes, but still had active infections. This allows determination from one wild, naturally blood fed mosquito sample: 1) if the mosquito was capable of transmitting *Plasmodium* spp. parasites to the person it bit (by detection of sporozoites in the salivary glands), 2) if the person it bit had disease causing parasites ("asexual stage"), 3) if the person it bit was infectious to the mosquito ("gametocytes"), and 4) if the mosquito became infected by the bite ("ookinete development").

This work has exciting possibilities for use as a less biased measure of parasite transmission. We believe it holds promise for use in local scale epidemiological studies as a

method to track how parasites are moving through a population. It can easily be combined with standard sporozoite detection assays to see parasite transmission at both ends of the disease cycle (i.e. from mosquitoes to humans and from humans to mosquitoes). Additionally, there may be a role for this approach in looking at the anti-sporogonic effects of ivermectin [214]. As crossing of the midgut is the time of highest stress for the parasite post ingestion [327,328], this is likely the point at which ivermectin may disrupt successful invasion due to disruption of midgut physiology [214]. In absence of successful midgut traversal, it is likely that the TrxL1 transcript would decrease as does HRPIII and 6044 as the parasites are degraded, which may be seen with the assay. This would also be applicable to alternative transmission blocking vaccines or other strategies [329,330]. This work was initially planned with our current ivermectin trials, though due to RNA degradation in our samples was unable to be performed. Future work will also determine how well this method correlates with end-point infectivity (tested by oocyst dissection), and how indicative the transcript ratios are to ookinete formation (tested with the use of heat-treated gametocytes that fail to develop into ookinetes upon ingestion).

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Appendix I: Search for ookinete specific *Plasmodium* spp. markers.

In the development of the approach presented in Chapter 5 for the detection of *Plasmodium* transcripts, we screened many gene candidates to find one that was induced only in the ookinete (the first major developmental stage in the mosquito). The purpose of this was to have an indicator not only of the presence of parasites in the mosquito that were previously in human blood, but of actual sexual-stage fertilization and development to indicate a successful early infection. Initial screens used the transcriptomics data set generated by López-Barragán *et al.* [300]. This data set is searchable on PlasmoDB (http://www.plasmodb.org, [301]), with the ability to compare the fold change in transcript expression between stage V gametocytes (the final, mosquito-infectious parasite stage) and ookinete. Initial gene candidates were identified by the highest reads per kilobase million (RPKM). These were chitinase (CHT1, PF3D7_1252200), and circumsporozoite- and TRAP-related protein (CTRP, PF3D7_0315200).

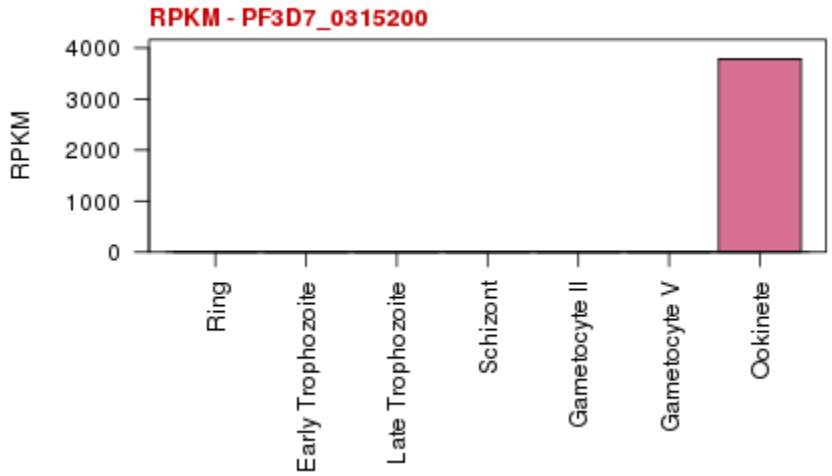

Figure 1A.1: Transcript abundance of circumsporozoite and TRAP-related protein (CTRP) based on parasite stage, figure generated by PlasmoDB with the López-Barragán dataset [300,301].

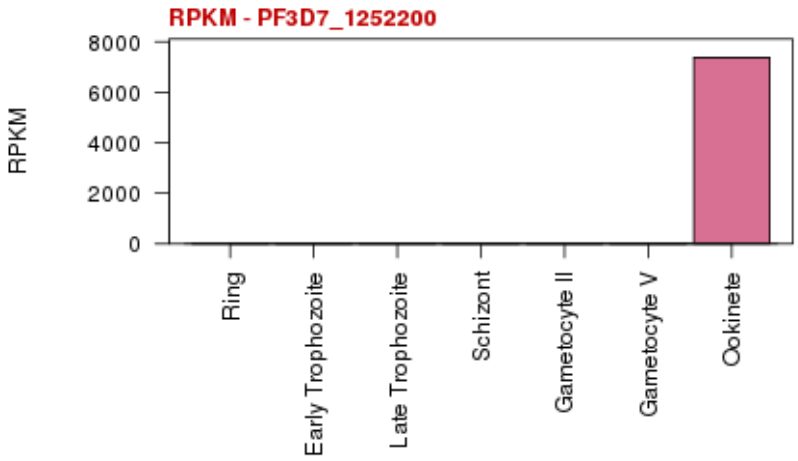

Figure 1A.2: Transcript abundance of chitinase (CHT1) based on parasite stage, figure generated by PlasmoDB with the López-Barragán dataset [300,301].

These genes have both been shown to be important to early infection events as described in Chapter 1 [25,26,331], and were first attempted to be detected by quantitative nucleic sequence based amplification (QT-NASBA). This approach, summarized below (Figure 1A.4), amplifies solely from single-stranded RNA product, and thus does not require DNAse treatment for removal of genomic DNA. Initial trials had poor success (Figure 1A.3), showing only weak amplification with CHT1 in 'feeder' blood collected prior to being in the mosquito. After primer and amplicon optimization following the guide by Deiman *et al.* [332], CTRP and WARP (von Willebrand factor A domain-related protein, PF3D7_0801300), a different gene suspected of being "ookinete-specific" based on transcript abundance, were able to be amplified (Figure 1A.5). While QT-NASBA for WARP was initially positive due to its seeming presence only post-ingestion, it was later found to be related to sensitivity, not to transcript presence (Figure 1A.6).

Due to the cost of development of the fluorescent probes for QT-NASBA, we switched after this finding to screening genes with reverse-transcriptase polymerase chain reaction (RT-

PCR). In total, we screened 25 different intronic gene targets, shown in tables 1A.1 and 1A.2 below. All of the genes screened either had detectable presence in feeder blood samples (prior to ingestion), issues with the PCR (multiband, poor/no amplification), or presence in oocyst samples. Later, we found literature describing AP2-O mediated repression of a variety of transcripts. It is likely that the transcripts are present in low, but detectable levels in sexual human stages, but simply under repression. However, it was through these experiments that the idea of comparing ratios, rather than just presence/absence came to mind, and this approach was ultimately what was utilized for the presented work. There still may be a role for the detection of genes such as chitinase, WARP, and CTRP with QT-NASBA as these may have differing sensitivity/specificity to gametocyte sex than does *Pfs*25, but this is still unexplored.

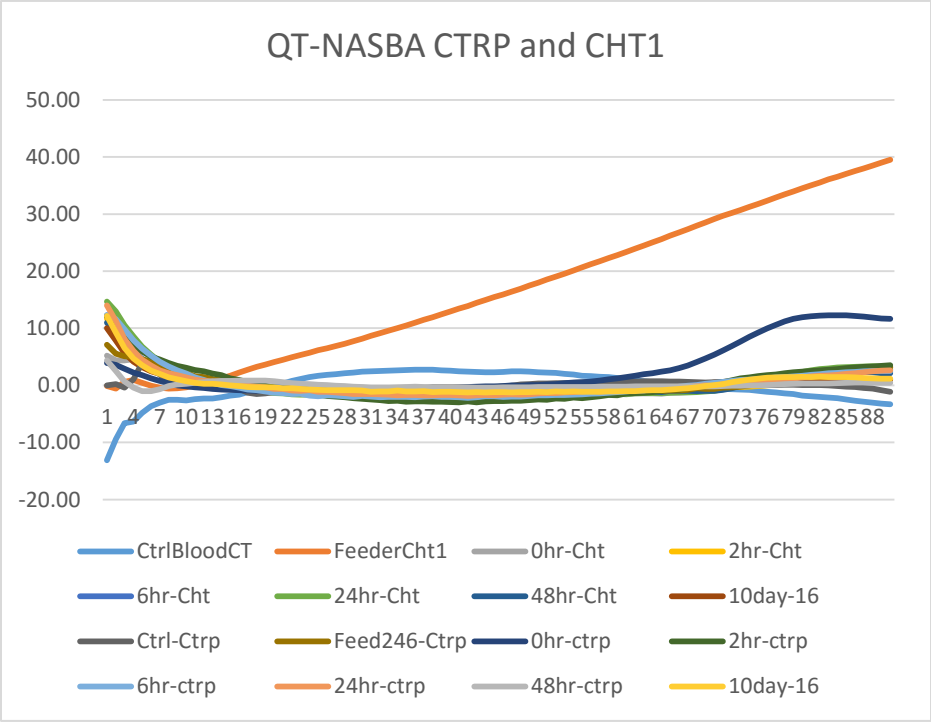

Figure 1A.3: Trial of CHT1 and CTRP amplification with QT-NASBA. Y-axis denotes relative fluorescence units (RFU), and the x-axis denotes time in minutes.

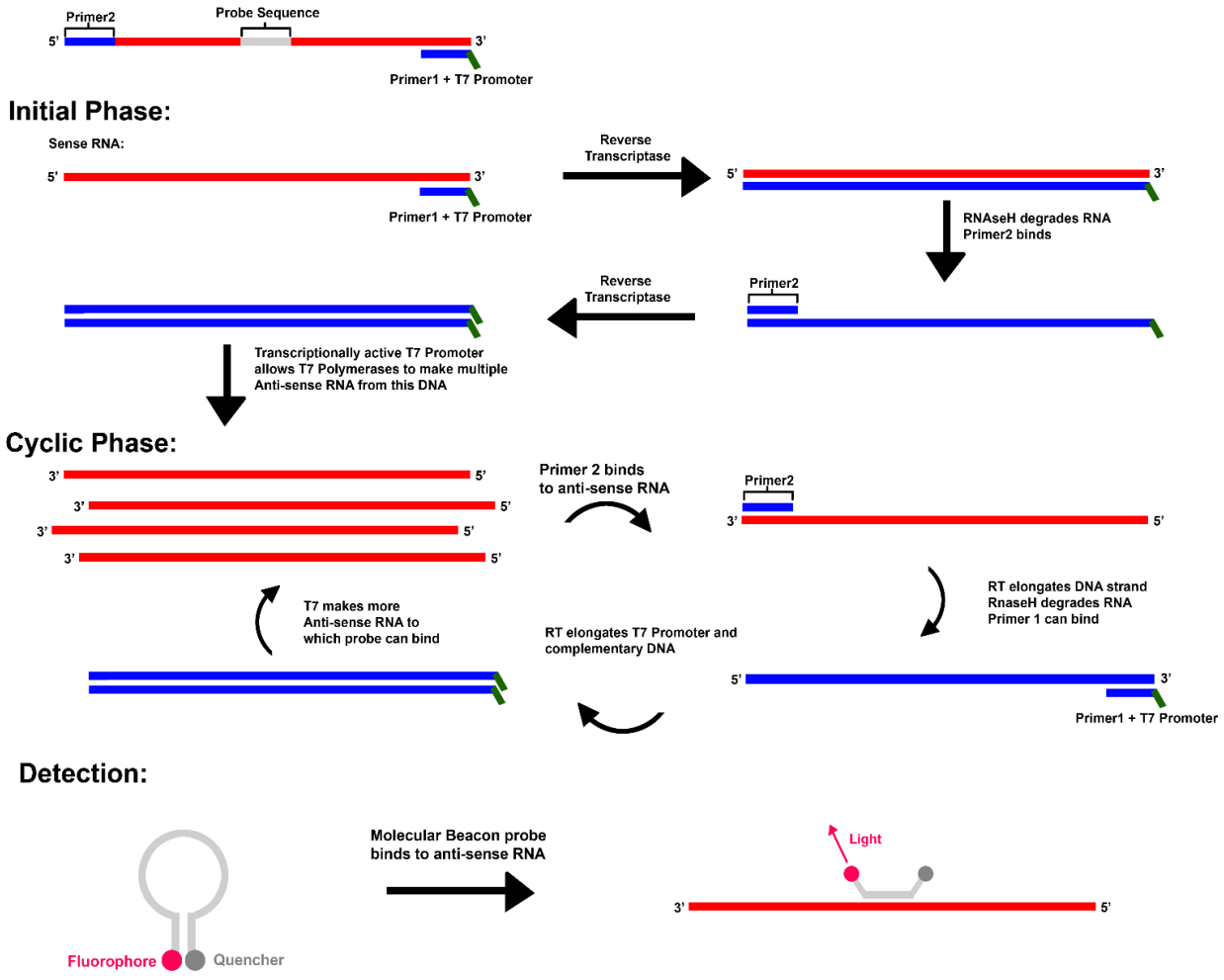

Figure 1A.4: Summary of RNA amplification by QT-NASBA. Briefly, target transcript (shown in red) is bound by a primer containing a 5' T7-promoter sequence and a sequence of 15-25 nucleotides specific to the gene of interest. A complementary DNA strand (shown in blue) is generated by Avian Myeloblastosis virus reverse transcriptase (RT), after which RNaseH (an enzyme that only degrades RNA in RNA-RNA or RNA-DNA hybrids [333]) degrades the RNA strand. This allows Primer 2, a second gene specific primer ~150 nucleotides from primer1, to bind. RT elongates from this primer to create a double stranded DNA amplicon with a transcriptionally active T7 promoter sequence. From this, T7 polymerase produces multiple single-strand anti-sense RNA copies of the amplified region, to which Primer 2 can bind and be elongated. This produces sense DNA for Primer 1 to bind and produce more amplicons with transcriptionally active T7 promoter regions for amplification. This all happens isothermally at 41°C over 90 minutes, and can be performed without a thermocycler [334]. Detection of these products is through a molecular beacon that binds to the anti-sense RNA copies, emitting fluorescence. This can be detected in real time on a standard thermocycler.

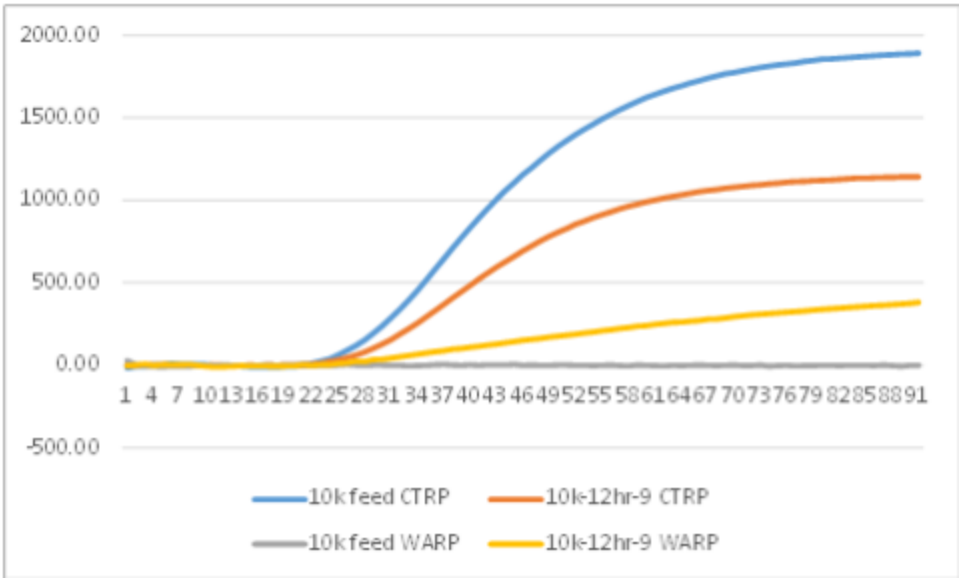

Figure 1A.5: Amplification via QT-NASBA of CTRP and von Willebrand factor A domain-related protein (WARP) from *Plasmodium* parasites pre/post mosquito ingestion. Y-axis denotes relative fluorescence units (RFU), and the x-axis denotes time in minutes.

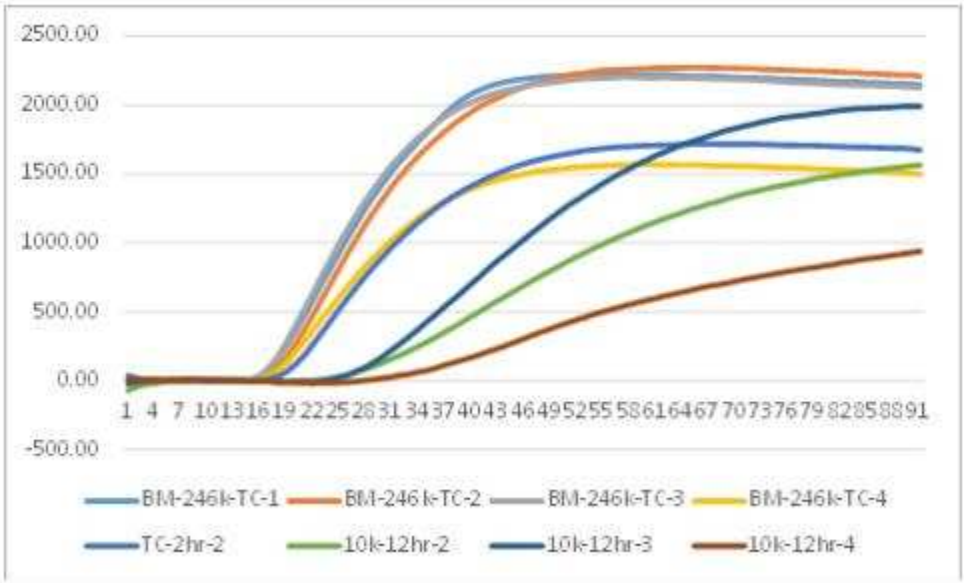

Figure 1A.6: WARP QT-NASBA from blood with 246,000 *P. falciparum* gametocytes/mL (BM-246k-TC-1 to 4), the same blood 2 hours post ingestion by an *An. gambiae* mosquito (TC-2hr-2), and three 10,000 gam./mL samples 12 hours post ingestion. Y-axis denotes relative fluorescence units (RFU), and the x-axis denotes time in minutes.

Table 1A.1: Detection of various genes by RT-PCR from blood containing varying dilutions of *P. falciparum* parasites prior to ingestion by mosquito.

	Dilution			
Gene	10 feeder	100 feeder	10k feeder	246k feeder
TrxL1	2/2	4/7	4/7	2/2
6044	2/2	4/7	4/7	2/2
PfCDPK3			0/2	1/2
Pf74			1/1	
Sera8			1/3	1/2
DEADbox			1/1	
IIb			0/1	
2080			1/1	
178			faint	
215			1/1	
Slarp				1/1
TSP				1/1
Spect				1/1
Pfl0995c				1/1
MAL13p1				1/1
PFL1095c				1/1
PFE480c				1/1
HRPIII				1/1
Hsp101				0/1
0940c				multiband
MAC				wrongsize
Kinesinlike				1/1
TREP				1/1
MyoA				multiband
MAEBL				1/1

Table 1A.2: Detection of various genes by RT-PCR from blood containing *P. falciparum* parasites (246,000 gametocytes/mL) after ingestion by *An. gambiae* at various time points.

	Time point						
Gene	0 Hr	2 Hr	6 Hr	12 Hr	24 Hr	48 Hr	10 day
TrxL1	6/6	7/7	7/7	4/4	7/7	5/5	5/14
6044	6/6	5/5	7/7	0/4	0/7	2/5	0/8
PfCDPK3	6/7	3/3	1/1	0/1	1/1	1/1	6/10
Pf74	4/4						2/4
Sera8	1/4	4/5					0/5
DEADbox	1/1						2/3
IIb	1/1?	1/1?	1/1?				1/1?
2080	1/1	1/1	1/1				1/1
178	1/1	1/1	1/1				1/1
215	1/1	1/1	1/1				0/1
Slarp							1/1?
TSP							0/1
Spect							1/1
Pfl0995c							1/1
MAL13p1							0/1
PFL1095c							1/1
PFE480c							1/1
HRPIII							0/1
Hsp101	1/1	2/2	1/1	1/1	1/1	1/1	1/2
0940c							0/1
MAC							0/1
Kinesinlike							1/1
TREP							1/1
MyoA							1/1
MAEBL							1/1

Table 1A.3: PlasmoDB gene ID's and gene names for all genes evaluated via RT-PCR and/or QT-NASBA methodology.

Gene:	PF3D7 ID:	Full name
TrxL1	PF3D7_0919300	thioredoxin-like protein 1, putative (TrxL1)
"6044"	PF3D7_0604400	conserved Plasmodium protein, unknown function
PLP1	PF3D7_0408700	
Tubulin	PF3D7_1008700	Tubulin beta chain
Hsp20	PF3D7_0816500	Heat Shock Protein 20
CDPK3	PF3D7_0310100	Calcium Dependent Protein Kinase - 3
"Pf74"	PF3D7_1407700	conserved Plasmodium protein, unknown function
Sera8	PF3D7_0207300	Serine repeat antigen 8
deadbox	PF3D7_1331100	DEAD box helicase, putative
"IIb"	PF3D7_0508400	transcription factor IIb, putative
"2080c"	PF3D7_1243300	conserved Plasmodium protein, unknown function
"178"	PF3D7_1018400	conserved Plasmodium protein, unknown function
SLARP	PF3D7_1147000	sporozoite asparagine-rich protein (SLARP)
TSP1, TRSP	PF3D7_0104000	thrombospondin-related sporozoite protein (TRSP)
SPECT1	PF3D7_1342500	sporozoite protein essential for cell traversal (SPECT1)
Pfl0995c	PF3D7_1220700	conserved Plasmodium protein, unknown function
MAL13P1.154	PF3D7_1327100	conserved Plasmodium protein, unknown function
PFL1095c	PF3D7_1222800	conserved Plasmodium protein, unknown function
PFE0480c	PF3D7_0509700	conserved Plasmodium protein, unknown function
HRPIII	PF3D7_1372200	histidine-rich protein III (HRPIII)
Hsp101	PF3D7_1116800	heat shock protein 101 (HSP101)
PSOP13 / 0940c	PF3D7_0518800	secreted ookinete protein, putative (PSOP13)
PLP1 / MAC	PF3D7_0408700	sporozoite micronemal protein essential for cell traversal (PLP1)
kinesin	PF3D7_1211000	kinesin-7, putative
TREP	PF3D7_1442600	TRAP-like protein (TREP)
myoA	PF3D7_1342600	myosin A (myoA)
MAEBL	PF3D7_1147800.1	merozoite adhesive erythrocytic binding protein (MAEBL)
CHT1	PF3D7_1252200	Chitinase (cht1)
CTRP	PF3D7_0315200	circumsporozoite- and TRAP-related protein (CTRP)
WARP	PF3D7_0801300	on Willebrand factor A domain-related protein (WARP)
SOAP	PF3D7_1404300	secreted ookinete adhesive protein, putative (SOAP)
"Pf62"	PF3D7_0620000	conserved Plasmodium protein, unknown function
Cap380	PF3D7_0320400	oocyst capsule protein (Cap380)
"4088"	PF3D7_0408800	conserved Plasmodium protein, unknown function

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